Exploring the role of Leucine Rich Repeat Kinase 2 within the innate immune system

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September 2017

This dissertation is submitted for the degree of Doctor of Philosophy
Title: Exploring the role of Leucine Rich Repeat Kinase 2 within the innate immune system
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Summary:

Leucine rich repeat kinase 2 (LRRK2) is a 286 kDa protein expressed in a variety of tissues and cell types, including neuronal tissue and innate immune cells. Mutations in LRRK2 have been linked to inflammatory diseases, most notably Crohn’s disease and Parkinson’s disease. Further to this, LRRK2 expression is induced by innate immune stimuli, and can be phosphorylated by Myd88 directed TLR signalling. Accordingly, a range of experiments and experimental approaches were taken, each designed to assess the role of LRRK2 in innate immunity.

An initial focus was placed upon the LRR domain of LRRK2. LRRs play an important role as ligand binding domains of many innate immune receptors, including the TLR and NLR families. An attempt was made to express and purify the LRR domain of LRRK2 in order to build upon results from earlier work that suggested nucleic acid binding activity of the domain through binding of heparin. Attempts to replicate initial work using a construct termed ‘LRR6’ were unsuccessful, most likely due to batch variation associated with baculoviral expression of protein using insect cell lines. Therefore alternative constructs were designed and successfully tested using insect cell expression. These constructs systematically incorporate VLR capping structures that have recently been utilised for the purification and expression of other LRR containing receptors, including drosophila Toll.

Functional experiments were performed using macrophages from WT and LRRK2 knockout mice. Many phenotypes and interactions have been described for LRRK2 in a neuronal or in vitro context; therefore experiments in macrophages were specifically designed to investigate these phenotypes and interactions in an innate immune context. LRRK2 interacts with a range of small GTPase proteins called Rabs, which coordinate and carry out vesicular trafficking, including that of innate immune receptors. Further interactions have been shown with clathrin-mediated endocytic machinery and phagocytic machinery; including cytoskeletal components actin and tubulin. Accordingly, the role of LRRK2 in the expression, membrane localisation, and ligand-induced endocytosis of the innate immune receptors such as TLR4 were assayed. TLR4 plays an important role in immune responses to alpha-synuclein, an immunogenic protein aggregate that accumulates as part of Parkinson’s disease pathology, making it a particularly interesting target for this assay. No effect was shown for LRRK2 on TLR4 expression or receptor mediated endocytosis, so attention was focused upon LRRK2 cytoskeletal interactions. An unclear role of LRRK2 has been described in phagocytosis. Application of LRRK2 KO macrophages in a series of systematic phagocytosis assays was used to demonstrate and clarify that there is no role of LRRK2 in the phagocytosis of simple beads, opsonised material, or complex bacterial targets expressing a range of immunogenic molecules such as LPS.

A genome wide approach was applied to further investigate the role of LRRK2 in TLR4 mediated signalling, as well as NOD2 mediated signalling. Comparison of LPS responses between WT and LRRK2 KO genotype macrophages identified a role of LRRK2 in modulating transcription of a range of chemokines and chemokine receptors. This indicates a specific role of LRRK2 in regulating chemotaxis in LPS stimulated cells. Knockout of LRRK2 resulted in a complete reversal of the regulation of the expression of EPAC1, a cAMP inducible protein working in parallel with a previously described LRRK2 interacting protein PKA. EPAC1 acts, at least in part, via Ca\(^{2+}\) signalling. Modulation of signalling through pathways such as Ca\(^{2+}\), Wnt and cAMP appear as a theme in results described in this transcriptomic experiment. A parallel metabolomic approach allowed analysis of ceramide levels in resting and innate immune stimulated macrophages. Ceramides are lipid molecules able to activate the NLRP3 inflammasome, as well as modulate alpha-synuclein pathology via ceramide metabolomic products. In contrast to results described in neuronal tissue, LRRK2 has no effect on ceramide levels in resting macrophages, however stimulation of NOD2 via MDP resulted in a dramatic LRRK2 specific increase in ceramide levels. Together, these results indicate a role of LRRK2 in activated innate immune cells. A differential effect of LRRK2 is described under different innate immune stimuli; with a range of transcriptional effects described upon LPS stimulation, compared to a metabolomic effect on ceramide levels following MDP stimulation.
Declaration

• This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

• It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

• It does not exceed the prescribed word limit for the relevant Degree Committee.
Dedicated to my parents

- who thought I should be a lawyer.
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Acronym List

4E-BP  Eukaryotic initiation factor 4E (eIF4E)-binding protein
6-OHDA  6-Hydroxydopamine
Abtb2  Ankyrin repeat and BTB/POZ domain containing protein-2
ADP  Adenosine triphosphate
ALR  AIM2-like receptor
ALS  Amyotrophic lateral sclerosis
ARP2/3  Actin-related protein-2/3
aSyn Oligo  Alpha-synuclein oligomers
Atf3  Activating transcription factor 3
BAC  Bacterial artificial chromosome
bp  Base pairs
BPOZ-2  Ankyrin-rich BTB/POZ domain containing protein-2
BSA  Bovine serum albumin
CAMK2  Calcium-calmodulin-dependent protein kinase II
cAMP  Cyclic adenosine mono-phosphate
CARD  Caspase recruitment domain
CCL  Chemokine (C-C motif) ligand
CLR  C-type lectin receptor
CMA  Chaperone mediated autophagy
COR  C-terminal of Roc
Csrnp1  Cysteine And Serine Rich Nuclear Protein 1
DAMP  Danger associated molecular pattern
Disc1  Disrupted in schizophrenia 1
DMEM  Dulbecco's modified eagle medium
DMSO  Dimethyl sulfoxide
DSS  Dextran sodium sulphate
EBSS  Earle's Balanced Salt Solution
eIF4E  Eukaryotic translation initiation factor 4E
ELISA  Enzyme-linked immunosorbent assay
EPAC1  Exchange factor directly activated by cAMP 1
ERK5  Extracellular signal-regulated kinase 5
ERM  Ezrin Radixin Moesin
ESI  Electrospray ionisation
F-actin  Filamentous actin
FCS  Foetal calf serum
FRET  Fluorescence resonance energy transfer
GABA  Gamma-Aminobutyric acid
GEF  Guanine nucleotide exchange factor
GFP  Green fluorescent protein
GSK3  Glycogen synthase kinase 3
GWAS  Genome wide association studies
Hcar2  Hydroxycarboxylic acid receptor 2
HDAC-2  Histone deactetylase 2
HPLC  High-performance liquid chromatography
HSC70  Heat shock chaperone 70
HSP90  Heat-shock protein 90
Ifn-γ  Interferon gamma
IKK  IkappaB Kinase
IL  Interleukin
iPSC  Induced pluripotent stem cell
IRF3  Interferon regulatory factor 3
KO  Knockout
L-DOPA  Levodopa
L2in1  LRRK2-IN-1
LB  Luria broth
LIC  Ligation independent cloning
LPS  Lipopolysaccharide
LRP6  Lipoprotein receptor-related protein 6
LRR  Leucine rich repeat
LRRK2  Leucine Rich Repeat Kinase 2
LTA  Lipoteichoic acid
M-CSF  Macrophage colony-stimulating factor
MAL  MyD88-adapter-like
MAPK  Mitogen-activated-protein kinase
MAVS  Mitochondrial antiviral signalling
MBP  Maltose binding protein
MD2  Myeloid differentiation factor 2
MDP  Muramyl dipeptide
MIP-1  Macrophage inflammatory protein 1
MLK  Mixed-Lineage Kinase
MOI  Molarity of infection
MPTP  1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS  Multiple sclerosis
MXD1  MAX Dimerization Protein 1
Myd88  Myeloid differentiation primary response gene 88
NAC  Non-amyloid-β component of AD amyloid plaques
NDAT  Nuclear factor of activated T-cells
NF-κB  Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR  NOD-like receptor
NOD  Nucleotide-binding oligomeration domain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NRON</td>
<td>ncRNA repressor of the nuclear factor of activated T cells</td>
</tr>
<tr>
<td>padj</td>
<td>Adjusted p-value</td>
</tr>
<tr>
<td>PAK</td>
<td>p-21 activated kinase</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>pBMDM</td>
<td>Primary bone marrow derived macrophage</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline tween</td>
</tr>
<tr>
<td>PcP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN induced putative kinase 1</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PLS-DA</td>
<td>Partial least squares discriminant analysis</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion protein</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>qRT-PCT</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>QToF</td>
<td>Quadrupole Time of Flight</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation endproducts</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>Rapgef3</td>
<td>Rap guanine nucleotide exchange factor 3</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RIP2</td>
<td>Receptor-interacting serine/threonine-protein kinase 2</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptor</td>
</tr>
<tr>
<td>Roc</td>
<td>Ras of complex proteins</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S2</td>
<td>Schneider 2</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sf9</td>
<td>Spodoptera frugiperda</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope labelling of amino acids in cell culture</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOCS-3</td>
<td>Suppressor of cytokine signalling 3</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1R</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMED7</td>
<td>Transmembrane emp24 domain-containing protein 7</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor protein inducing IFNβ</td>
</tr>
<tr>
<td>TRIM25</td>
<td>Tripartite motif-containing protein 25</td>
</tr>
<tr>
<td>VLR</td>
<td>Variable lymphocyte receptor</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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1. Introduction

1.1 Immunology and inflammation

1.1.1 Innate and adaptive immunity

The immune system plays an important role in maintaining health. This role is extremely broad in its scope due to the sheer diversity of threats that an organism will face during its life. Two distinct branches of the immune system exist which reflect this diversity; these are the innate and the adaptive immune responses. The adaptive immune response requires, as the name suggests, adaptation of pre-existing genes to overcome a pathogen or threat. This is a process that takes an extended length of time to occur. Innate immunity forms a complementary branch of the immune system, which informs adaptive immune responses of threats through a degree of cross-talk (Janeway, 1989; Iwasaki and Medzhitov, 2015), while directly countering threats through inflammatory processes.

The innate immune system is key to immunity due to the immediate nature of its action. The term ‘innate’ is used as germline-encoded receptors available to an organism without any adaptation or genetic recombination are utilised. This facilitates rapid immunological responses, allowing threats from pathogens to be countered quickly, often negating the need to develop an adaptive response altogether (Kumar et al., 2011). Innate immune responses are often pro-inflammatory in nature, relying on the secretion of inflammatory cytokines (Dinarello, 2000). Cytokines such as ‘tumour necrosis factor-alpha’ (TNFα) act on cell surface receptors to induce protective processes exemplified by apoptosis (Rath and Aggarwal, 1999), while others such as interleukin-8 (IL-8) can act to recruit further innate immune cells, and thus amplify the pro-inflammatory response (Baggiolini and Clark-Lewis, 1992).

1.1.2 Pattern recognition receptors program the inflammatory response

In the case of infection, innate immune responses are triggered by specifically recognised molecules termed ‘pathogen associated molecular patterns’ (PAMPs). Receptors have evolved which are tailored to the detection of PAMP molecules; these are termed pattern recognition receptors (PRRs) (Kumar et al., 2011). The most well-established and studied group of these PRRs in mammalian species are the ‘Toll-like receptors’ (TLRs). The
number of TLRs varies between species, with the human genome encoding ten members of the TLR family (Barreiro et al., 2009). The TLR family are type-I transmembrane receptors, each with a ‘leucine rich repeat’ (LRR) ligand binding ectodomain and a transmembrane ‘Toll/IL-1R’ (TIR) domain (Gay and Gangloff, 2007). The diversity of TLRs reflects the range of PAMPs expressed by pathogens. TLRs 1, 2, 4, 5, and 6 are expressed as membrane receptors, exposed to the extracellular environment (Gay et al., 2014). Diversity of ligand recognition by some TLRs such as TLRs 1, 2 and 6 is extended by the ability and functional requirement for the formation of heterodimers upon ligand binding. The classical ligands for these TLRs are bacterial associated PAMPS, ranging from acylated lipopeptides to bacterial flagellin and lipopolysaccharide (LPS). TLR4 is a particularly interesting receptor, and the focus of a great deal of study. Unlike other TLRs, TLR4 is functionally cycled between the cell surface and acidified endosomes, with consequences for down-stream innate immune signalling and inflammatory output (Kagan et al., 2008). TLR4 recognises bacterial LPS as a heterodimer complexed with ‘myeloid differentiation factor 2’ (MD2) (Chow et al., 1999; Kim et al., 2007). LPS is a highly modified lipid found on the outer membrane of Gram-negative bacteria that is not found in mammalian cells under healthy conditions (Bryant et al., 2010). Therefore, detection of LPS by cells expressing TLR4 allows an immune response tailored to intracellular Gram-negative bacteria such as Salmonella if signalling from the endosomal system, or extracellular Gram-negative bacteria such as Escherichia coli if signalling from the cell surface. TLRs 3, 7, 8 and 9 exist as pre-formed dimers in the endosomal system where they are tailored to the detection of viral infections through recognition and binding of nucleic acids (Gay et al, 2014). Other pattern recognition receptors are also encoded in the mammalian genome including the ‘nucleotide-binding oligomerisation domain’ (NOD) pattern recognition receptors; members of the ‘NOD-like receptor’ (NLR) family. These cytosolic receptors contain LRRs, a nucleotide binding domain, and caspase recruitment domains (CARDs) (Franchi et al., 2009; Boyle et al., 2013). NOD1 and NOD2 recognise bacterial peptidoglycan during infection, and perhaps also from the gut microbiota – tolerance of which is essential as part of gut homeostasis (Philpott et al., 2014). Pattern recognition receptors can therefore be found at the plasma membrane, at endocytic vesicles, and in the cell cytoplasm; providing a wide coverage of immune surveillance (fig 1).

While the immune system is classically associated with the cellular response to infection as discussed above, the innate immune systems role is more nuanced, with a continual role in
surveillance not just for infection, but also for signs of cellular damage. Harmful processes often result in cell death. This can occur through mechanical injury or through disruption of cellular homeostasis. In either case, activation of innate immunity is achieved by ‘danger associated molecular patterns’ (DAMPs) (Midwood and Piccinini, 2010). These are molecules that, like PAMPs, are able to activate innate immune responses. However DAMPs are not derived from an external source such as a pathogen, but are instead released from cellular environments where they would not normally be exposed to immune-surveillance and PRR detection. A good example of this is ‘adenosine triphosphate’ (ATP), a molecule that would normally act as an energy source inside a cell, but in the external environment acts as a signal to innate immune cells that a disruptive process is occurring (Bours et al., 2006; Seiffert et al., 2006).

**Figure 1: Immune surveillance occurs at all major cellular compartments.** Various innate immune receptors are expressed at the cell surface and at endocytic vesicles. This arrangement of receptors ensures that immune responses can be orchestrated against a range of threats: from viruses, to both intracellular and extracellular bacterial pathogens. Illustrated are a range of TLRs located within the plasma membrane both at the cell surface and endocytic vesicles, while NLRs such as NOD2 can detect PAMPs and DAMPs within the cell cytoplasm.
As discussed, innate immune responses are activated by stimuli termed PAMPs and DAMPs, depending on the source of the activating molecule. The specific combination of these molecules and receptors bring about corresponding inflammatory signalling that can dictate the type of response being generated. Some innate immune responses are geared more towards repair than inflammation, creating a polarisation paradigm where cells such as macrophages can be activated towards a pro-inflammatory (M1) response, or an anti-inflammatory, pro-repair response (M2) (Italiani and Boraschi, 2014). While macrophage polarisation is a helpful concept when applied broadly, the diversity of response that can be employed by a macrophage means the M1/M2 paradigm should only be a guide, and not a phenotypical definition (Martinez and Gordon, 2014). Importantly, while activation of innate immunity is activated with a great degree of molecular-specificity, the inflammatory response is inherently non-specific and can lead to pathology if unregulated (Chaudhry et al., 2013). An example of this can be seen with TLR4 recognition of LPS. In the case of a huge dose of LPS from an uncontrolled infection, sepsis can occur where the inflammatory response causes pathological harm. As such, it is the dampening of inflammatory responses that is the aim of novel therapies for sepsis (Wittebole et al., 2010).

1.2 Immunology in the brain

1.2.1 Neuroinflammation and immune privilege

The nervous system consists of the peripheral nervous system, and the central nervous system. Within the central nervous system, the brain has long been considered an immune-privileged site due to the presence of the blood brain barrier. This barrier severely impairs the ability of circulating immune cells and stimuli in the periphery to access brain tissues and induce inflammatory responses (Ballabh et al., 2004).

The central reason for maintaining immune privilege of the brain is the delicate nature of neuronal cells. Neurons, and the synapses formed between them, are generated during development and further shaped throughout life. The intricate networks generated this way encode information in ways we are only beginning to understand. Neuronal cells themselves, unlike the vast majority of other cells, typically do not undergo cellular division in order to maintain their populations (Bond et al., 2015). Neuronal cells and their synaptic contacts are not only precious, but they are also extremely delicate. Neurones have an
extreme morphology and highly specialised structures and function. Maintaining the
elongated morphology and meeting energetic demands placed on neurones results in
significant cellular stress (Cavanagh, 1984). The blood brain barrier is the major reason for
the immune privileged nature of the brain; effecting reduced exposure to toxins from the
periphery, as well as inflammatory mediators such as cytokines, and infiltration of
inflammatory cells (Ballabh et al., 2004). The result of this immune privilege is not absolute
isolation of the brain, but protection from stresses that would otherwise prove deleterious
to neuronal maintenance. Immune privilege is also maintained by the action of resident
immune cells present within the brain, namely the microglia as well as other glial cells such
as astrocytes (Ousman and Kubes, 2012) – thus a degree of immunological protection is
possible without the input of non-neuronal cells.

1.2.2 Blood brain barrier

The blood brain barrier is a multi-layered structure centred around endothelial cells
connected by tight-junctions which limit the ability of solutes and circulating cells to transfer
from blood to the brain, while still maintaining brain oxygenation through efficient transfer
of oxygen. Barrier function is enhanced by several further layers, including a thick glycocalyx
that acts as a buffer between blood vessels and endothelial cells (Abbott and Friedman,
2012). Pericytes can be found wrapped around endothelial cells, where they are able to
respond to extracellular signals to regulate aspects of blood brain barrier function through
pericyte-endothelial cell signal transduction (Sweeney et al., 2016). A basement membrane
of extra-cellular matrix is enhanced by the glia limitans between the central nervous system
and the blood brain barrier. The glia limitans forms the outer-most layer of neuronal tissue
associated with the blood brain barrier, consisting of astrocytic-end-feet which provide
support to the blood brain barrier, and promote the formation of tight junctions during
development (Abbott, 2002). These structures together form a unit that is remarkably tight
when compared to the leaky vasculature found in peripheral regions of the body (fig 2).
Similar structures complement the blood brain barrier to separate the cerebrospinal fluid,
neuronal tissue, and the blood (Liddelow, 2015). The blood brain barrier functions as both a
physical barrier and a molecular barrier. While the physical properties of the barrier
discussed above can prevent movement of molecules and infiltration of cells through and
between epithelial cells, structures such as the glycocalyx are also thought to prevent
physical interaction of molecules such as toxins in the blood from interacting with receptors on the vasculature side of the barrier (Lipowsky, 2012), thus providing a molecular barrier between the brain and the periphery. Molecular barrier function is served on a more basic level through regulated expression of transporters and signalling molecules on the blood brain barrier surface, thus regulating the interactions between central nervous system and the periphery, as well as specifically tailoring the central nervous system cellular environment.

Figure 2: The blood brain barrier. Multiple layers of cells constitute the blood brain barrier neurovascular unit: including endothelial cells linked by tight junctions, pericytes, and astroglia. These are supported by secreted glycoproteins of the glycocalyx layer (facing the vasculature), and the extracellular matrix of the basement membrane (facing the brain parenchyma). Tight junctions between endothelial cells and the glycocalyx create an obstacle again the infiltration of peripheral immune cells from the circulatory system, while still allowing the diffusion and active transport of other molecules. Pericytes and astrocytic end feet facilitate communication of extracellular signals to endothelial cells and provide trophic support. Adapted from Sweeney et al., 2016.
While the blood brain barrier provides a remarkable layer of separation between the periphery and the brain from an immunological perspective, limited infiltration of peripheral immune cells does occur, and is part of healthy homeostasis of the brain; this is often upregulated during disease (Ransohoff et al., 2003; Ousman and Kubes, 2012). Therefore, while the brain can be considered immune privileged, it is far from immunologically isolated. Communication between the central nervous system and the periphery is essential for regulating homeostatic conditions such as body temperature via the autonomous nervous system. Small regions of interface between the blood and the brain where the tightly controlled blood brain barrier are lacking make communication possible. At these regions, vascularised interfaces consisting of highly fenestrated capillaries are found between the circulatory system and neuronal parenchyma; these are termed the circumventricular organs (Ganong, 2000). The choroid plexus and leptomeninges also present softer barriers that permit small molecule diffusion (Rivest, 2009). Such leaky regions of the neuronal interface can permit toxins such as LPS, from outside the central nervous system, to be detected by immune cells in the brain (Nadeau and Rivest, 2000).

1.2.3 Microglia

Microglia are often considered to be tissue resident macrophages of the brain, and are present in the brain at much higher numbers than infiltrating immune cells under normal conditions (Vilhardt, 2005). There is a significant amount of overlap between the known functions of microglia and bone marrow derived macrophages, with primary roles of both cell types including immune surveillance, phagocytosis of pathogenic material, and transduction of innate immune signals from PRRs (Ousman and Kubes, 2012). Both cell types display encountered antigens on MHC molecules, release appropriate cytokines to promote inflammation, and recruit further inflammatory cells. On top of this, both microglia and bone marrow derived macrophages play a role in ‘cleaning-up’ after pathological processes through phagocytosis of cell debris, and the release of growth factors to promote repair and cell survival. Historically, the similarities between tissue resident macrophages such as the microglia, and bone marrow derived macrophages led to a lack of appreciation of the differences between these phagocytic cell types. More recently however, a growing acceptance of the specialised nature of microglia has been established (Prinz and Priller, 2014). In vivo fate mapping studies in mice have demonstrated that microglia are derived
from the embryonic yolk sac from embryonic day 7, and are found to colonise the brain via
the blood stream by embryonic day 9, confirming earlier reports (Alliot et al., 1991; Alliot et
al., 1999; Ginhoux et al., 2010). In the adult brain, the blood brain barrier provides a layer of
isolation to microglia, which were found to self-renew from within the brain rather than rely
on infiltration of macrophages from the blood stream to maintain phagocytic cell numbers
(Ajami et al., 2007; Askew et al., 2017). Thus, while microglia and macrophages are
remarkably similar, they are developmentally distinct, and differences in cellular function
reflect this.

Microglia carry out many specialist tasks in the brain during development and
homeostasis, and play a more conventional role during disease (Wolf et al., 2017). In the
developing brain, microglia promote neurogenesis as well as outgrowth of neurons (Walton
et al., 2006). Under homeostatic conditions, microglia maintain a ramified morphology
characterised by long processes extending away from the cell body (Glenn et al., 1992). This
morphology assists in forming transient physical contacts with neuronal axons, dendritic
spines, and the synapses between neurons. This permits efficient provision of trophic
support to healthy and developing neurons, as well as phagocytosis of apoptotic neurons
both in neurogenesis and disease (Sierra et al., 2010; Nayak et al., 2014). Phagocytosis of
apoptotic neurons is an important process towards maintaining homeostasis, and avoiding
unwanted and damaging activation of pro-inflammatory responses to cell death within the
delicate neuronal environment of the brain. Phagocytosis of neurons by microglia may also
occur as part of a process known as synaptic pruning, a developmental process involved in
learning and plasticity; this is an example of a specialised function of microglia (Geier et al.,
2012). Such pruning may be mediated by immunological signalling pathways such as the
expression of complement component 3 in microglial exosomes (Bahrini et al., 2015),
however it has been suggested that the relationship between microglia and synaptic
pruning may be one of association rather than a direct mechanism (Perry and O’Connor,
2010). Disruption of innate immune related proteins such as the complement or fractalkine
receptors were found to inhibit this process, and led to abnormalities in neuronal
development (Hoshiko et al., 2012; Bialas and Stevens, 2013; Hong et al., 2016). Recent
research has also highlighted how microglia may not simply react to apoptotic extracellular
exposure of ‘eat-me’ signals such as phosphatidylcholine, but may actually drive
phagocytosis of viable neurons or react to reversible (non-apoptotic) exposure of
phagocytosis signals, through a process known as phagoptosis (Brown and Neher, 2014). Destruction of neurons in this manner may act as a pathogenic mechanism, through the destruction of otherwise viable neurons as an aberrant response to neuronal stress (Brown and Neher, 2012).

Our understanding of how the immune system can influence the nervous system is still in its infancy, with microglia and inflammation able to modulate neuronal development as discussed, but also able to modulate behaviour more directly. An interesting example can be seen in the role of innate immunity in the compulsive grooming phenotype of Hoxb8 loss of function mutant mice. In this study, wild type (WT) bone marrow transplantation in conjunction with whole body irradiation of mice expressing mutant Hoxb8 was able to reverse compulsive behaviour, thus a neuronal-driven behavioural disorder can be mechanistically accounted for by perturbation of peripheral immune cells (Chen et al., 2010). More recently, neuroinflammation has been strongly associated with behavioural and psychiatric disorders such as depression (Blank and Prinz, 2013; Miller and Raison, 2016). These revelations suggest we may be able to better understand and treat psychiatric disorders by modulating immunity rather than overcoming challenges associated with treating neuronal circuitry directly. Overall these new areas of research highlight the significant and previously underappreciated interconnectivity of the immune system and the nervous system.

The extended processes of ramified microglia allow efficient sampling not just of neurons and their synapses, but also of the extracellular environment in the brain (Nimmerjahn et al., 2005) (fig 3). This is important for immunosurveillance, and the more classical immunological roles of microglia. As mentioned previously, immunosurveillance revolves around detection of PAMPs and DAMPs, which can signal to the immune system that homeostasis has been disrupted and an inflammatory response may be required. Detection of such molecules is achieved by the expression of the full range of the TLR family of PRRs by human microglia (Jack et al., 2005), which as resident macrophages of the brain, are the primary cell type involved in this process. In the context of neurodegenerative disease, microglial release of inflammatory cytokines is also the primary driver of neuroinflammation (Vilhardt, 2005). Activation of microglia causes a dramatic change in morphology from ramified to amoeboid, where processes are retracted towards the central
cell body (fig 3). Activated, amoeboid microglia display an enhanced pro-inflammatory and phagocytic phenotype compared to the ramified microglia which are involved in homeostatic processes (Kreutzberg, 1996; Lull and Block, 2010). While activated microglia are pro-inflammatory in nature, it is generally considered that inflammatory responses are dampened as compared to bone marrow macrophages from the periphery (Gautier et al., 2012; Perry and Teeling, 2013). This is a further example of the immune privileged nature of the brain, where an overabundance of inflammation can be pathological to delicate neuronal networks in a way that would not be experienced outside of the brain.

Figure 3: Microglial morphology is highly dynamic, and varies with innate immune activation state.

The cellular and inflammatory properties of microglia change with their state of activation: with resting ramified microglia less proliferative and less inflammatory than fully activated amoeboid microglia.
The ‘immunologically dampened’ nature of microglia in comparison to peripheral macrophages is comparable to the divergence of many different tissue resident macrophages from canonical macrophage function and immunological reactivity to suit a particular environment and its associated functions (Davies et al., 2013). Other such examples include adipose tissue macrophages involved in the development of conditions such as type 2 diabetes, and cardiac resident macrophages that facilitate electrical conduction in the heart (Boutens and Stienstra, 2016; Hulsmans et al., 2017). While macrophages clearly vary in activity and function by localisation within the body, so to do microglia within different neuronal subsections. This has been highlighted in work by McColl and colleagues who identified brain region specific transcriptional identities that vary independently with age (Grabert et al., 2016). The use of such next-generation transcriptional methodology suggests that regulation of immunological responses varies not just between peripheral immune cells, and those situated within the brain, but also between brain regions; affected by factors such as where immunological function is being carried out, and where microglia are recruited from within the brain. Further implications may exist in the immunological contribution known to be made by microglia towards region-specific neurodegenerative effects observed with ageing, and changes in microglial transcriptional profiles during on-going neurodegenerative diseases (Vincenti et al., 2016).

1.2.4 Astrocytes and glial scarring

Microglia comprise one cell type within a larger category of cells known as the glia. Astrocytes are another major glial cell type with a role in the innate immune response, and have been discussed in the context of the blood brain barrier. The glia collectively have been shown to provide support to neuronal cells through provision of growth factors and nutrients (Volterra and Meldolesi, 2005). In particular, astrocytes regulate the cellular environment of the nervous system, and recycle valuable molecules such as neurotransmitters that may leak during synaptic transmission (Chaturvedi et al., 2014). Astrocytes form close contacts with neuronal cell bodies and the synapses between neurons; with a single astrocyte able to contact around 100,000 synapses forming ‘tripartite’ synapses (Halassa et al., 2007; Chung et al., 2015).

Secreted factors by astrocytes promote the formation of neuronal synapses, with cultures grown in the absence of astrocytes displaying fewer synapses than mixed astrocyte
and neuronal co-cultures (Diniz et al., 2012; Korn et al., 2012; Farhy-Tselnicker et al., 2017). Furthermore, astrocytes play a functional role in the synapse remodelling during the course of development and associated neuronal plasticity, but also following some instances of brain pathology (Kim et al., 2016; Kim et al., 2017). Such remodelling is activity-dependent, suggesting a fundamental sensitivity of astrocytes to neuronal activity at the synapse (Flores and Méndez, 2014). More recently, astrocytes have been suggested to contribute towards the regulation of synaptic signalling directly, in a process known as gliotransmission (Panatier et al., 2011). This has stemmed from the discovery that astrocytes respond to various neurotransmitters or electrical stimulation, and as a consequence are able to secrete neurotransmitters such as glutamate, D-serine, ATP and ‘gamma-Aminobutyric acid’ (GABA); thus demonstrating neuronal like properties (Auld and Robitaille, 2003; Sahlender et al., 2014). There exists an on-going debate as to the significance of the contribution of the astrocyte neurotransmission machinery to established mechanisms of neuronal calcium transient transmission, with some groups suggesting astrocytic calcium transients may be artifactual rather than physiological (Fiacco et al., 2009). The advent of more sensitive calcium detection techniques such as two-photon fluorescence methods have uncovered that astrocyte calcium transients at astrocyte extended processes occur differentially to the easily detected calcium transients at the soma (Kanemaru et al., 2014). The ability to detect astrocyte electrical signalling at a subcellular compartment significantly more proximal to neuronal synapses has helped to improve our understanding of gliotransmission substantially (Bazargani and Attwell, 2016).

Within an immunological context, astrocytes complement microglia in the innate immune response, and provide a powerful role in the regulation of responses in a way that is made possible by unique and specific cellular distribution. Human astrocytes express some, but not all TLRs. Human astroglial expression of TLR3, and lower levels of TLR1, 4, 5, and 9 have been reported (Jack et al., 2005), while there are also reports of the expression of TLR2 in mice (Bowman et al., 2003). This suggests that astrocytes are able to detect a range of innate immune ligands to complement the microglial response. Unlike microglia, which are highly motile and dynamic, astrocytes form barriers both at the blood brain barrier, but also at the meninges, and around aggregates of inflammatory leukocytes (Sofroniew, 2015a). Astrocytes can act at multiple levels: to directly induce inflammation by the production of inflammatory cytokines, to attract peripheral immune cells to the central
nervous system through the action of chemokines such as ‘chemokine (C-C motif) ligand 2’ (CCL2), to control diapedesis of recruited cells via controlled regulation of astrocytic end-feet at the glia limitans, and by regulating the micro-environment to which recruited lymphocytes are exposed (Sofroniew, 2015a). In the context of the blood brain barrier, the ‘astrocyte functional barrier’ exists between the neural parenchyma and the parenchymal basement membranes, acting as a pool to which recruited immune cells collect before astrocyte-regulated entry into the brain parenchyma. Regulation of this microenvironment through controlled release of pro-inflammatory and/or anti-inflammatory cytokines and membrane receptors can influence the activity of recruited cells, and may therefore play a role in the control, and eventual resolution of an inflammatory response. This has implications in disease and the protection of neurons from excessive or chronic inflammation and resulting neuronal damage (Owens et al., 2008).

Another functional role of astrocytes is in the generation of glial scars. Such structures have long been considered entirely detrimental to the brain, however this view is now being overturned, and an appreciation of their beneficial attributes is developing. Astrogliosis is interesting as it is a process that can be activated upon insult to the central nervous system; with direct immunological consequences (Sofroniew, 2015b). Astrogliosis is a heterogeneous process with a range of severities: from simple morphological changes in individual astrocytes, to rapid proliferation of astrocytes and secretion of various extracellular matrix components, which results in a continuous plasma membrane forming between cells, and the generation of a ‘glial scar’. This web of cells, to which other cells types such as microglia also contribute, can effectively act as a functional barrier with similarities to the previously discussed blood brain barrier (Sofroniew, 2015b). The beneficial or damaging nature of a glial scar may be very much context dependent, defined by considerations such as the nature of the initial insult to the brain and the required response to manage (Adams and Gallo, 2017). For instance, glial scars have classically been considered to be inhibitory to axonal regrowth and myelination by inhibiting axonal outgrowth and oligodendrocyte access to the neuronal lesion (Fawcett and Asher, 1999). However in some contexts, the astrocytic scar may also inhibit the infiltration of pro-inflammatory macrophages and microglia, and therefore reduce the inflammatory environment of the wound, thus reducing neuronal stress and promoting neuronal repair (Wanner et al., 2013). Subsequently it has also been shown that astrocytes in scars express
molecules which promote axon repair, and that inhibiting scary formation limited this repair process (Anderson et al., 2016). Similarly, like microglia, using next generation sequencing technology a growing appreciation is developing of the diversity of astrocyte activity across different regions of the central nervous system, and during ageing (Farmer and Murai, 2017; Lin et al., 2017). As such, it should be expected that glial scars may differ in their functional activity when forming in different brain regions of the brain or at different times across the lifespan of an individual, as such heterogeneity should be expected in the effect of glial scar formation of neuronal regeneration.

The growing appreciation of astrocyte diversity has further implications upon neurobiology, with effects not just on glial scarring but also previously mentioned astrocytic roles including electrochemical signalling at the synapse, synaptic development and innate immune responses. These considerations may contribute towards future astrocyte (or indeed microglial) targeted disease therapies by shifting cells of a detrimental gene expression profile, to that of a beneficial profile, potentially by mimicking environmental cues of another brain region (Anderson et al., 2016; Adams and Gallo, 2017).

1.2.5 Neuroinflammatory disease

A relationship between innate immunity and the nervous system is clear from the diverse roles of glia in immunity, but also in maintaining neuronal function under homeostatic conditions. The interconnectivity of the innate immune system and the nervous system is strengthened by the expression of TLRs on neuronal cells themselves. Neuronal TLR expression is variable during development, and appears to play a specialised role in neurogenesis and development (Kaul et al., 2012). A prime example of the specialised nature of TLR expression in neurones can be found in TLR7. Cellular localisation and function of TLR7 are variable between different types of neurone at different regions of the brain. Cortical and hippocampal neurons display endosomal localisation of TLR7, with activation bringing about neurodegeneration (Lehmann et al., 2012). In comparison, sensory neurons of the dorsal root ganglia display an unusual membrane localisation of TLR7, with activation of the receptor eliciting a pain response by coupling to the ion channel TRPA1 (Park et al., 2014).
TLR3 represents another interesting example of a classical innate immune receptor serving a neurological role in a neuronal context, as viral infections at foetal or neonatal stages has been linked to the development of autism and schizophrenia (Knuesel et al., 2014; MacDowell et al., 2017). TLR3 is the innate immune receptor for double stranded RNA (Gay et al., 2014). Activation of neuronally expressed TLR3 has been shown to cause down-regulation of several psychiatric disorder associated genes including ‘Disrupted in schizophrenia 1’ (Disc1), leading to various developmental deficits including defective neuronal arborisation and increased density of neuronal spines (Chen et al., 2017).

While inflammatory processes must exist, as elsewhere in the body, to resolve infections and mop up immunogenic molecules; they also play a role in neurological processes including plasticity, development, and behaviour. As such, a wide range of diseases of the nervous system can be linked to when neuroinflammation goes wrong. These include, but are not limited to: neurological disorders such as depression, and more classical inflammatory conditions such as meningitis, autoimmune encephalitis, multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (World Health Organization (WHO), 2006; Dendrou et al., 2016).
1.3 Parkinson’s disease

1.3.1 Introduction to Parkinson’s disease

Parkinson’s disease is a neurodegenerative disease characterised by the selective destruction of a specific subset of neurons responsible for the generation of dopamine and fine control of motor function (Orr et al., 2002). These dopaminergic neurons are located at the substantia nigra pars compacta, a region of the midbrain that is specifically affected in Parkinson's sufferers at early stages of the disease (Naoi and Maruyama, 1999) (fig 4). Classical symptoms of Parkinson’s disease relate directly to the loss of the dopaminergic neural circuitry; and include tremor at rest, loss of gait control, and bradykinesia. Parkinson’s disease is the most prevalent neurodegenerative movement disorder, affecting 1% of the population over 60, increasing to 5% of the population over 85 years of age (de Lau and Breteler, 2006). With an ageing population, an improved understanding of the mechanisms leading to the onset of Parkinson’s pathology and/or the molecular pathways involved in neuronal degradation are of increasing importance in order to develop the effective long-term therapeutic strategies that are currently lacking (Toulouse and Sullivan, 2008).

Parkinson’s disease is a progressive condition, with pathology and symptoms progressing with time. At later stages of pathology, neurodegeneration spreads to regions surrounding the substantia nigra, including higher-order sensory areas of the cortex. The uniform progression of the disease with little variation between individuals is reflected in the progressive development of pathology at a cellular level; this has become known as ‘Braak staging’ (Braak et al., 2003). Symptoms resulting from neurological damage lead to devastating changes to the quality of life of individuals, affecting not just movement, but also mental acuity and behaviour (Fahn, 2003; Wolters et al., 2008). As a chronic and progressive degenerative disease, once classical movement related symptoms are prevalent and Parkinson’s disease is diagnosed, there is no known way to halt or reverse progression of pathology. This is another example of the delicacy and irreplaceability of neuronal networks within the brain.
Figure 4: Schematic of dopaminergic neuronal pathways within the basal ganglia. The nigrostriatal pathway consists of dopaminergic neurons between the substantia nigra and the striatum. This pathway is part of a larger neuronal network known as the basal ganglia. While loss of dopaminergic neurons within the substantia nigra results in loss of motor control and Parkinson’s disease, other regions of the basal ganglia such as the cortex are more closely associated with behaviour and may be affected at later stages of Parkinson’s disease progression. Adapted from an image originally created by the National Institute of Health. VTA = ventral tegmental area.
1.3.2 Parkinson's disease treatments

Treatment of Parkinson’s disease has not progressed significantly since the first use of levodopa (L-DOPA) in the 1960s. L-DOPA is able to cross the blood brain barrier where it is metabolised to dopamine, and is able to attenuate the effect of the loss of dopamine from the degeneration of dopaminergic neurons that occurs as part of Parkinson’s pathology (Contin and Martinelli, 2010). Unfortunately, L-DOPA acts as a treatment of symptoms and not pathology, therefore as pathology progresses, the effectiveness of the drug decreases significantly; and symptoms progress beyond motor control and dopamine-related pathology. After 4-6 years of L-DOPA therapy, 40% of patients begin to experience L-DOPA induced motor fluctuations and dyskinesia (Nutt, 2008).

Another form of treatment developed is deep brain stimulation, which can be applied to initially alleviate disease burden and restore quality of life. But like levodopa, deep brain stimulation only acts to treat symptoms and not disease pathology; resulting in decreased efficacy over time (Toulouse and Sullivan, 2008). Application of deep brain stimulation also requires brain surgery, meaning therapeutic intervention is severe and carries risks both during and after the procedure through complications such as infection. Therefore, deep brain stimulation is most often not considered beneficial for older patients who are most commonly affected by Parkinson’s disease. In spite of this, deep brain stimulation is considered a more effective method of treating motor disability and improving quality of life than L-DOPA; so remains an appealing route for treatment of fitter Parkinson’s patients (Weaver et al., 2009). Patient response to deep brain stimulation has been positively correlated to L-DOPA response, and has been shown to reduce the requirement for parallel L-DOPA treatments in ‘long-term’ clinical follow-up case studies (Kleiner-Fisman et al., 2003).

While Parkinson’s disease pathology is mostly considered and diagnosed as a movement disorder, it is believed that some non-motor symptoms may manifest before pathology has progressed to affect the substantia nigra. Such symptoms have been referred to as ‘pre-diagnostic’ markers for Parkinson’s disease, and are thought to be less homogenous than the later pathology of Braak staging (Zis et al., 2015; Noyce et al., 2016). Much research emphasis is placed on the use of pre-diagnostic symptoms as early markers for Parkinson’s disease to allow early-intervention and preservation of dopaminergic
neurons. Symptoms such as anosmia and dizziness are examples of these markers, and are thought to precede clinical Parkinson’s disease by four years (Ross et al., 2008; Noyce et al., 2017). As a progressive disease, early diagnosis could help enhance currently applied treatments by allowing intervention with lower doses of levodopa before disease severity has worsened. Recent research using germ-free mice has even suggested that Parkinson’s pathology may in fact not originate in the brain, but may spread from the gut via short chain fatty acid signalling from the gut microbiota (Sampson et al., 2016). If substantiated, such a finding not only changes out understanding of the pathobiology of Parkinson’s disease, but also raises the possibility of preventative medical interventions, or treatments to sever the spread of disease before reaching the brain.

1.3.3 Alpha-synuclein in Parkinson’s disease

The hallmark of Parkinson’s pathobiology is the accumulation of toxic aggregates of misfolded proteins known as Lewy bodies within cells; these classify Parkinson’s disease as a proteinopathy. Lewy bodies consist primarily of the archetypal Parkinson’s associated protein alpha-synuclein, decorated with an array of other proteins including ubiquitin, and tau (Spillantini et al., 1998; Giráldez-Pérez et al., 2014). The specific morphology of Lewy bodies can vary in different brain regions, and may reflect different stages in Lewy body development, but the core components of Lewy bodies remain the same. Lewy body formation is seeded through toxic misfolding of alpha-synuclein. The role of non-pathogenic alpha-synuclein during health is not well established, however a function has been suggested in the regulation of neurotransmitter release and recycling at neuronal synapses, with alpha-synuclein overexpression inhibiting neurotransmitter release (Nemani et al., 2010). Mutations in alpha-synuclein which make the protein more prone to aggregate, or gene multiplication events, have been shown to significantly increase the risk of developing Parkinson’s disease, showing a clear mechanistic link between alpha-synuclein and the development of the disease (Polymeropoulos et al., 1997; Singleton, 2003; Kay et al., 2008).

A prion-like spreading of toxic alpha-synuclein has been proposed, where the formation of the toxic β-amyloid species of alpha-synuclein is capable of seeding the further misfolding of functional forms of alpha-synuclein in a prion-like manner (Olanow and Brundin, 2013). This phenomenon was first observed in Parkinson’s patients who developed Lewy body pathology in transplanted neurons which had previously been healthy,
suggesting host-to-graft disease propagation (Kordower et al., 2008; Li et al., 2008). Aggregation of β-fibrils revolves around the formation of stacks of extremely thermostable hydrophobic β-sheets, which eventually grow into large hydrophobic structures. This stacking is brought about by a hydrophobic central region of alpha-synuclein termed the ‘non-amyloid-β component of AD amyloid plaques’ (NAC) domain (Giasson et al., 2001). Under normal conditions, large hydrophobic surfaces are inherently internalised and contribute a substantial energetic component of protein folding. The presence of such a large hydrophobic structure within cells therefore has potential to disrupt cellular functions at even the most fundamental level. The nature of the spread of alpha-synuclein between cells is a matter of current research, with suggestions that seeds of toxic alpha-synuclein aggregates could be transferred between cells by normal cellular exocytosis and endocytosis events, released from cells through toxic alpha-synuclein mediated cell death, or ectopically released from cells through processes mediated by the biophysical properties of alpha-synuclein aggregates themselves (Lashuel et al., 2013).

Alpha-synuclein is clearly intricately associated with Parkinson’s disease pathology, with increasing concentration and spread of toxic aggregates positively correlated with disease severity. As such, targeting the accumulation and aggregation of alpha-synuclein has been a priority for the development of novel treatments for the disease, as well as attempts to remove aggregated alpha-synuclein (Chu and Kordower, 2007). Unfortunately no attempts so far have successfully halted disease progression, raising the possibility that other important processes are being overlooked that work either in conjunction with, or as well as, alpha-synuclein aggregation in the pathobiology of Parkinson’s disease. An example of such thinking can be found in the therapeutic inhibition of the unfolded protein response in misfolded prion protein (PrP) diseased mice. PrP is a prion protein with some similarities to the misfolded proteins in other neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease. Researchers found that inhibition of the unfolded protein response was able to ameliorate neurodegenerative processes in PrP mice by restoring normal protein translation (Moreno et al., 2012). Most interestingly, recovery and prevention of disease in these mice was achieved after protein aggregation had already occurred, meaning PrP aggregates were tolerated in these mice (Moreno et al., 2013). This would be analogous to alpha-synuclein aggregation occurring as part of Parkinson’s disease, but not leading to neuronal pathology due to treatment of pathological mechanisms other than
alpha-synuclein aggregation itself. Neuroinflammation is an example of such a pathological process.

1.3.4 Parkinson’s disease is a neuroinflammatory condition

Advances in our understanding of neurodegenerative disease have highlighted an important role of the innate immune system in disease pathology (Long-Smith et al., 2009). This involvement stems from peripheral immune cell activation, as well as the notable action of microglia, the brains resident macrophages (Collins et al., 2012). Indeed, microglial cells in particular have been demonstrated to have a close relationship to Parkinson’s disease. For instance, post-mortem biopsies from Parkinson’s disease sufferers have shown large numbers of activated microglia at the substantia nigra (McGeer et al., 1988), and these microglia exhibit the amoeboid morphology associated with activation by innate immune stimuli and inflammatory cytokine secretion (Lull and Block, 2010). In tissue culture experiments, conditioned media from activated microglia has been demonstrated to be toxic to dopaminergic neurons, which suggests susceptibility of these physiologically relevant cells to innate immune processes. Accordingly, elevated levels of inflammatory cytokines have been observed in the blood and cerebrospinal fluid of patients suffering from Parkinson’s disease (Dobbs et al., 1999; Scalzo et al., 2010; Lindqvist et al., 2012). In vitro cultures of mixed neurons and glia also facilitate investigation into the role of specific molecules on dopaminergic neuronal survival. For example, neuronal degeneration was shown to be preceded by activation of microglia upon innate immune stimulation of such cultures, and the extent of degeneration was proportional to the level of microglial activation as measured by inflammatory mediator release (Gao et al., 2002).

Alpha-synuclein has been shown by several groups to be an activator of the innate immune system. Alpha-synuclein acts as a DAMP and contributes to a strong immune response through detection by TLR2 (Kim et al., 2014). Activation of microglial TLR2 by oligomeric alpha-synuclein caused characteristic proliferation of microglia, as well as changes in microglial morphology from ramified to amoeboid, with corresponding production and release of inflammatory mediators IL-1β and nitric oxide. More recently, it has been shown that alpha-synuclein can be detected and signal through a TLR1/2 heterodimer (Daniele et al., 2015). TLR4 has been shown to mediate phagocytosis of alpha-synuclein and modulate an array of downstream pro-inflammatory responses such as
cytokine release and ‘reactive oxygen species’ (ROS) production in microglia (Fellner et al., 2013). As a protein aggregate, alpha-synuclein takes on crystalline characteristics and is able to activate the NLRP3 inflammasome (Codolo et al., 2013). The NLRP3 inflammasome acts to cleave pro-IL-1β and pro-IL-18 into their mature forms by activating caspase-1 processing (Martinon et al., 2002). NLRP3 is highly promiscuous, and has been associated with a plethora of activators, including crystalline substances such as uric acid, silica, aluminium hydroxide, and protein aggregates including amyloid-beta and alpha-synuclein (Codolo et al., 2013; Heneka et al., 2013; Hari et al., 2014). Given the promiscuity of NLRP3, some believe activation may not occur directly through interaction with these molecules, but via induction of secondary messengers such as ROS or K+ (Abderrazak et al., 2015). NLRP3 is expressed in microglia but not astrocytes (Gustin et al., 2015), suggesting the microglia and macrophage lineages play an important and direct role in Parkinson’s associated neuroinflammation. Indeed, cultured TLR4 knockout (KO) astrocytes have been shown a suppressed pro-inflammatory response to alpha-synuclein (Fellner et al., 2013; Rannikko et al., 2015).

The link provided by alpha-synuclein between Parkinson’s as a protein misfolding disease and as a neuroinflammatory condition has not gone un-noticed by researchers. The two processes are inter-linked and exacerbate each other through a process termed ‘reactive microgliosis’ (Lull and Block, 2010) (fig 5). Alpha-synuclein oligomers cause stress to cells and result in cell death. Dead neurons then release alpha-synuclein aggregates that are able to activate inflammatory processes in microglia and other innate immune cells of the brain. Activated innate immune cells release inflammatory cytokines, recruiting further microglia to the affected regions of the brain and causing damage to the sensitive dopaminergic neurons located there. Death of dopaminergic neurons exacerbates Parkinson’s symptoms and causes the release of fresh alpha-synuclein aggregates (Block et al., 2007). Understanding the interplay between inflammation and alpha-synuclein misfolding is important as a ‘chicken-or-the-egg’ scenario currently exists between the two; does alpha-synuclein misfolding initiate inflammatory processes, or do underlying inflammatory processes initiate alpha-synuclein misfolding? Alpha-synuclein, and Lewy body pathology levels have long been used as a diagnostic indicator of Parkinson’s disease, and as a marker for disease progression in post-mortem biopsy (Gibb and Lees, 1988); however therapeutic strategies targeting alpha synuclein accumulation have not so far been
successfully applied. Efforts are being made to use inflammatory profiling as a marker for the progression of Parkinson’s disease, or to predict future prognosis of patients by classifying sufferers by inflammatory profile (Chen et al., 2008; Brockmann et al., 2017). Specific targeting of inflammatory processes involved in early stages of disease, or in later stage reactive microgliosis, could have potential in preventing, treating, or attenuating Parkinson’s disease pathology and its progression.

Figure 5: Reactive microgliosis. A ‘vicious cycle’ of neurodegeneration and microglial activation can exist within the diseased brain. Activation of microglia causes inflammatory stress to neurons leading to neurodegeneration. Resulting release of debris and toxic molecules from degenerating neurons causes further activation of microglia. As such, reactive microgliosis is a cycle of immune activation leading to neuronal damage. Intervention to control this process may provide a viable method of controlling immunopathology of Parkinson’s disease. Adapted from Block et al., 2009.
1.3.5 Genetic advances have linked LRRK2 to Parkinson's disease

Genetic advances have taught us much about Parkinson’s disease and neurodegenerative conditions more generally. It has long been appreciated that Parkinson’s can be inherited in specific families. Familial cases of Parkinson’s disease account for 10 % of cases, with the remaining 90 % of cases described as sporadic in western populations. Genes encoding alpha-synuclein (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004), parkin (Kitada et al., 1998), DJ-1 (Bonifati et al., 2003), ‘PTEN induced putative kinase 1’ (PINK1) (Valente et al., 2004) and ‘leucine rich repeat kinase 2’ (LRRK2) (Paisán-Ruiz et al., 2004; Zimprich et al., 2004), have been linked to Parkinson’s disease this way. Interestingly, clinical differences were observed between most cases of genetic Parkinson’s disease when opposed to sporadic cases. Namely, it was noted that genetic cases appeared to be predominantly early-onset (diagnosed before the age of 50) as compared to sporadic cases, and levels of Lewy body pathology observed varied between genes (Pankratz and Foroud, 2007). It is interesting therefore, that LRRK2 associated cases are considered clinically indistinguishable from the sporadic disease, with late disease onset and classical Lewy body presentation focused around the brainstem (Adams et al., 2005; Ross et al., 2006). This suggests at the possibility that by understanding the role of LRRK2 in the underlying pathobiology of Parkinson’s disease, we may gain an insight into mechanisms that are at play in even sporadic, non-LRRK2 associated Parkinson’s cases.

The development of genome wide association studies (GWAS) has provided a deeper insight of the genetics of Parkinson’s disease, and has revealed that single nucleotide polymorphisms (SNPs) of the PARK8 gene, encoding LRRK2, are associated not just with familiarly inherited Parkinson’s, but also as risk factors for the development of the so-called sporadic disease (Satake et al., 2009; Simón-Sánchez et al., 2009). The most common mutation in the LRRK2 gene results in a mutation from glycine to serine at amino acid 2019 (G2019S). This SNP is the highest known risk factor for the development of Parkinson’s disease, accounting for 5-7 % of autosomal-dominant familial cases (Di Fonzo et al., 2005; Nichols et al., 2005), and 1-2 % of sporadic cases in western populations (Gilks et al., 2005). In specific populations, the rates of G2019S associated Parkinson’s can be significantly higher. In Ashkenazi Jews for instance, the G2019S LRRK2 mutations is present in 30 % of familial cases and 13 % of sporadic cases (Lesage et al., 2006). In North African Arabs, the
mutation is present in 37% of familial cases and 40% of sporadic cases (Lesage et al., 2006). Mutations of the LRRK2 gene increase in disease penetrance with age (Goldwurm et al., 2007).

1.4 Biochemistry of LRRK2

1.4.1 LRRK2 possesses both GTPase and Kinase activity

LRRK2 is a large protein of 286 kDa consisting of a complex and unique arrangement of protein-protein interaction and functional domains. This arrangement consists of N-terminal repeats, including ankyrin repeats, an LRR domain, a Ras of complex proteins (Roc) GTPase, with associated C-terminal of Roc (COR) domain, a Ser/Thr protein kinase, and finally a WD40 domain at the C-terminus of the protein (Mills et al., 2014). The presence of a Roc-COR tandem domain defines LRRK2 as a member of the RocO protein family, a family first detected in the slime mould Dictyostelium discoideum (Marín et al., 2008; Russo et al., 2015). While similarities in LRRK2 exist to RocO proteins in D. discoideum, LRRK2 itself has been shown to have emerged much later in animals, following the protostome-deuterostome split after which point N-terminal repeats were acquired; separating LRRK2 from its paralogues (Marín, 2006). The array of domain types in LRRK2 suggests that the cellular role of LRRK2 is complex and potentially wide-ranging, an assertion that is reflected

Figure 6: LRRK2 domain organisation and associated pathogenic mutations. Pathogenic mutations and associated domains of LRRK2 are indicated. Mutations listed are non-exhaustive; taken from Greggio and Cookson, 2009, and http://omim.org/allelicVariant/609007. Well-characterised mutations are indicated in bold. M2387T is associated with non-Parkinson’s inflammatory disease. Domain boundaries adapted from Corti et al., 2011. ARM = Armadillo repeats, ANK = Ankyrin repeats, LRR = Leucine rich repeats, Roc = Ras of complex proteins, COR = C-terminal of Roc.
in our shallow understanding of LRRK2 biology. Over 80 mutations have been detected across the full range of LRRK2 domains, making LRRK2 the most frequently mutated Parkinson’s associated gene (Nuytemans et al., 2010). Confirmed pathological SNPs for Parkinson’s, Crohn’s Disease, and Leprosy are clustered at the GTPase and kinase functional domains of the protein, which suggests that modulation of enzymatic activity is especially important to LRRK2 associated pathology (Corti et al., 2011) (fig 6). Accordingly, functional domains of LRRK2 have been the most intensely studied, and so are the best understood of LRRK2 domains.

From a structural perspective, the Roc-COR tandem domains of LRRK2 are by far the best understood, having been resolved by X-ray crystallography to reveal a dimeric GTPase. (Deng et al., 2008) (fig 7). The back-to-back dimer is stabilised by domain swapping between separate monomers, resulting in GTPase active sites that are each formed from two gene products. Mutations of an arginine residue at position 1441 of the LRRK2 gene are strongly associated with Parkinson’s disease, and are the most prevalent of Roc domain disease-causing mutations (Nuytemans et al., 2008). This arginine residue constitutes a mutational hotspot within LRRK2, with pathogenic mutations to any of a cysteine, glycine, or histidine residue (R1441C/G/H) reported (Simón-Sánchez et al., 2006; Haugarvoll et al., 2008; Ross et al., 2009). This suggests that the change from arginine may be more important for LRRK2 pathogenicity than which amino acid is encoded by the SNP. Accordingly, the coordination of the LRRK2 Roc domain positions residue 1441 at the interface of the homodimer; with the arginine residue providing stabilising hydrogen bonds and stacking forces. Mutation to smaller, or oppositely charged residues such as the cysteine, glycine or histidine residues associated with Parkinson’s, would therefore act to destabilise dimer formation (Deng et al., 2008). Interestingly, GTPase dimer formation is co-ordinated by complementation, with dimerisation generating completed β-sheets that span the dimer. Each monomer contributes three β-strands to each sheet. Pathogenic mutations within the Roc domain of LRRK2 do not affect the structure of the GTPase active site directly, and yet a consensus is forming that R1441 mutations decrease GTPase activity (Lewis et al., 2007). There is a less clear relationship between the Roc-COR SNPs at position 1441 or 1699, and activity of the kinase domain, with mixed reports as to whether such SNPs increase kinase activity (Smith et al., 2006; Guo et al., 2007), or simply have no effect (Jaleel et al., 2007). It has been suggested that GTP binding activity may have a more significant effect on LRRK2 biology
than GTP hydrolysis (Taymans et al., 2011), with GTP binding acting to stabilise the LRRK2 dimer, as well as acting as a critical requirement for normal kinase activity (Biosa et al., 2013). However not all reports are in agreement, with evidence from LRRK2 purified from murine brain suggesting that GTP binding is not essential for kinase activity (Liu et al., 2010). In comparison, mutations affecting kinase activity appear to have no effect on GTPase activity (West et al., 2007; Biosa et al., 2013).

While LRRK2 is classified as a Roco protein largely due to its domain architecture, the kinase domain of LRRK2 bears highest homology to ‘Mixed-Lineage Kinase’ (MLK) family of serine/threonine kinases (West et al., 2005). The MLKs are themselves part of a larger family of ‘mitogen-activated-protein kinase’ (MAPK) kinase kinases (MAPKKKs) which are strongly associated with inflammatory signalling, as well as neurodegeneration (Gallo and Johnson, 2002; Kaminska, 2005; Kim and Choi, 2010). LRRK2 has been shown to phosphorylate MKK3/6 and MKK4/7, which lead to p38 and JNK signalling respectively (Gloeckner et al., 2009). As mentioned previously, the G2019S SNP is positioned within the kinase domain of LRRK2, specifically, within the activation loop of the domain (fig 8). The kinase activation loop can be phosphorylated resulting in conformation change in an N-terminal regulatory

**Figure 7:** Human LRRK2 Roc dimer crystal structure. The two Roc domains of LRRK2 form a head-to-tail dimer. β-strand complementation between domains forms dimerisation stabilised β-sheets that contribute to GTP binding coordination. Arg1441 of each monomer is labelled. One of two dimerisation sites and GTP molecules labelled. PBD: 2ZEJ
helix initiated by electrostatic repulsion from the negatively charged phosphate modification. This conformational change activates the kinase by increasing accessibility of the ATP binding pocket, permitting phosphoryl transfer to target proteins (Adams, 2003). The G2019S mutation of LRRK2 is the single most studied aspect of LRRK2 biology. This is due to the fact that this mutation is the most frequent LRRK2 SNP, and strongest risk factor for the development of Parkinson’s disease (Goldwurm et al., 2005). Mutation of the activation loop ‘DFG motif’ from glycine to serine has been suggested to promote the active conformation of the LRRK2 kinase domain (Gilsbach et al., 2012). This results in a clear three-fold increase in kinase activity of mutant LRRK2 as compared to wild-type protein (West et al., 2005; MacLeod et al., 2006; Jaleel et al., 2007; Greggio and Cookson, 2009). The clarity and consistency of this result is in contrast to many of the contradictory results reported for other SNPs such as the 1441 mutations previously described within the Roc-COR domains, and even the I2020T mutation just a single amino acid away, next to the ‘DFG motif’ (Gloeckner et al., 2006; Jaleel et al., 2007).

**Figure 8: Kinase domain of LRRK2 homologue Roco4 (from Dictyostelium discoideum).** An ATP binding site is formed between β-sheet rich N-terminal, and α-helix rich C-terminal lobes. Kinase activity is enhanced by phosphorylation of the catalytic loop, which results in conformational change at the regulatory helix. The DFG motif (black) coordinates ATP via Mg$^{2+}$, and may be mutated to DFS in the G2019S mutant, resulting in hydrogen bonding with R1077 of the regulatory helix (Gilsbach et al., 2012). *D. discoideum* residues are labelled, bracketed residues are human LRRK2 equivalent residues. PDB code: 4F0F
1.4.2 Measuring LRRK2 kinase activity

Biochemical evidence of LRRK2 kinase activity suffers from a lack of consistency across different experiments, as there is no known and robust physiological substrate for LRRK2. Instead, analysis of kinase activity relies upon analysis of various sites of auto-phosphorylation, phosphorylation of artificial substrates, or phosphorylation of biological substrates of unknown physiological significance such as myelin basic protein.

Auto-phosphorylation of the LRRK2 dimer was first described in 2007 by autoradiography experiments, using $^{32}$P as a substrate to label phosphorylated LRRK2 (Jaleel et al., 2007; Luzón-Toro et al., 2007). Such experiments demonstrated that LRRK2 had the capacity to phosphorylate itself, and that auto-phosphorylation activity was dependent on an intact C-terminus of the protein (Greggio et al., 2008); but did not give detailed biochemical information as to which residues were phosphorylated. Modern, sensitive techniques such as mass spectroscopy have highlighted over 20 auto-phosphorylation sites across the length of the LRRK2 protein, with a clear substrate preference of threonine residues over serine residues (Kamikawaji et al., 2009; Webber et al., 2011). Auto-phosphorylation sites for LRRK2 have been described as clustering within two regions, perhaps reflecting accessibility to the LRRK2 kinase domain in the full-length LRRK2 dimer. Of interest is that one of these auto-phosphorylation clusters falls within the GTP binding pocket of the Roc domain; perhaps reflecting the cross-regulation observed by some groups between LRRK2 GTPase and kinase domains. The other auto-phosphorylation cluster lies just upstream of the LRR domain, at the N-terminus of the protein (Gloeckner et al., 2010). The most studied LRRK2 auto-phosphorylation site is Ser$^{1292}$, which is now widely used as a measure of LRRK2 kinase activity (Sheng et al., 2012). The use of auto-phosphorylation is especially useful in vivo, where more physical methods are not amenable to experiment design. Further phosphorylation sites have been identified as targets for other kinases, notably ‘protein kinase A’ (PKA) (Muda et al., 2014), and the ‘IkappaB Kinase’ (IKK) family (Dzamko et al., 2012).

One of the early proteins to be identified and validated as a substrate for LRRK2 kinase activity was moesin, of the ERM family of proteins (along with ezrin and radixin) (Jaleel et al., 2007). Moesin acts to bridge the actin cytoskeleton to the plasma membrane, and is phosphorylated at a threonine residue at position 558, responsible for binding
filamentous actin (F-actin) (Arpin et al., 2011). This suggests a potential role of LRRK2 in controlling processes that require dynamics changes in cell shape and associated cytoskeletal structures; such as cell migration or endocytosis. The finding that other members of the ERM protein family are also phosphorylated by LRRK2 has led to the development of a peptide substrate for LRRK2. This peptide, termed ‘LRRKtide’ was designed using mass spectroscopy to identify the LRRK2 phosphorylation sites of the ERM proteins, then applying sequence homology analysis to identify favourable surrounding amino acids (Jaleel et al., 2007). Shortly after, a positional scanning peptide library approach was taken to generate a completely artificial substrate for LRRK2. This library consisted of 20 amino acid long peptides, varying in the amino acid sequence around a central serine or threonine residue. Ultimately, this approach led to the generation of an optimised peptide for phosphorylation by LRRK2 (Nichols et al., 2009). Known as ‘Nictide’, this peptide possesses favourable phosphorylation reaction kinetics as compared to LRRKtide. Both molecules are able to measure LRRK2 kinase activity using radiolabelled $^{32}$P, but neither are physiological substrates of LRRK2.

The final method for LRRK2 kinase activity measurement is through phosphorylation of a protein of interest. This was the method that led to the development of LRRKtide, however the approach was often used with other LRRK2 ligands, such as myelin basic protein. (West et al., 2005). Myelin basic protein in particular was used before the development of artificial substrates. These experiments are suitable for individual studies, but can lead to confusion when comparing the effects of LRRK2 mutations across different studies, as the effect of LRRK2 mutations on kinase activity is likely to be substrate specific. Indeed, there is no lack of potential LRRK2 substrates, as the range and breadth of protein-protein interactions for LRRK2 is breath-taking (Manzoni et al., 2015; Porras et al., 2015). Such a range of LRRK2 interactions reflects the size and complexity of the LRRK2 protein and its multiple protein-protein interaction surfaces.
1.5 Functions of LRRK2 in biology

1.5.1 LRRK2 is an innate immune related protein

As well as auto-phosphorylation, LRRK2 is phosphorylated at a number of sites by other kinases. Particular interest was placed on Ser\textsuperscript{910} and Ser\textsuperscript{935}, at the N-terminus of the predicted LRR domain of LRRK2. Before interest settled on Ser\textsuperscript{1292}, Ser\textsuperscript{910} and Ser\textsuperscript{935} were used as indirect measures of LRRK2 kinase activity as their phosphorylation status correlated positively with chemical inhibition of LRRK2 kinase activity (Zhao et al., 2015). While use of Ser\textsuperscript{910} and Ser\textsuperscript{935} phosphorylation to indicate LRRK2 activity is no longer the most appropriate approach available (Ito et al., 2014), the finding that phosphorylation status can be altered by TLR signalling provides a direct link between LRRK2 physiological function and the innate immune system (Dzamko et al., 2012) (fig 9). The IkappaB kinase family, which is responsible for phosphorylation of these residues, is normally associated with the phosphorylation of IkB proteins that sequester ‘nuclear factor kappa-light-chain-enhancer of activated B cells’ (NF-κB) in the cytoplasm. Phosphorylation and ubiquitination of IkB proteins leads to proteolysis, and subsequent transfer of NF-κB into the nucleus; resulting in altered gene transcription (Karin, 1999). In response to detection of pro-inflammatory stimuli such as that of TLR4 signalling in response to LPS, IkB kinases initiate NF-κB signalling, but also appear to directly phosphorylate LRRK2 (Dzamko et al., 2012). This LRRK2 phosphorylation was shown to be dependent on the TLR adaptor ‘myeloid differentiation primary response gene 88’ (Myd88), an innate immune adaptor molecule which mediates signalling from cell surface TLRs, as well as TLR7, 8, and 9 which signal from the endosomal compartment (Dzamko et al., 2012). This suggests a level of specificity in the phosphorylation of LRRK2 to particular innate immune stimuli, as TLR3 activation or the non-TLR immunogenic ligands Zymosan and Curdlan failed to cause phosphorylation. Later work has shown that Ser\textsuperscript{910} and Ser\textsuperscript{935} can also be phosphorylated by the action of PKA in response to cyclic adenosine mono-phosphate (cAMP) in the cell (Li et al., 2011). cAMP acts as a secondary messenger in many cell signalling contexts. PKA was later shown to further phosphorylate LRRK2 at Ser\textsuperscript{1444} (Muda et al., 2014). Ser\textsuperscript{910} and Ser\textsuperscript{935} phosphorylation is important for the binding of the chaperone protein 14-3-3, with disruption of this interaction causing aberrant LRRK2 localisation into discrete cytoplasmic pools (Dzamko et al., 2010; Nichols et al., 2010), and affecting the secretion of LRRK2 into exosomes (Fraser et
Ser$^{1444}$ phosphorylation was also demonstrated to be important for 14-3-3 binding. Interestingly, decreased Ser$^{1444}$ phosphorylation was described for R1441C/G/H SNPs of LRRK2, resulting in increased LRRK2 kinase activity in this study; this suggests that 14-3-3 binding modulates LRRK2 kinase activity (Muda et al., 2014). Together, these cellular effects of 14-3-3 binding demonstrate a potentially important role of regulated LRRK2 phosphorylation for maintaining homeostasis which may be disrupted by LRRK2 pathogenic mutations (Li et al., 2011; Muda et al., 2014) and can be modulated by TLR mediated innate immune signalling (Dzamko et al., 2012).

**Figure 9: LRRK2 interaction with 14-3-3 is regulated by immune signalling events.** Phosphorylation of LRRK2 at 14-3-3 binding residues is mediated by Myd88 mediated TLR4 signalling via the IκB Kinase family, and PKA. LRRK2 phosphorylation and relevant residues are indicated in red. These interactions demonstrate a direct signalling interaction between innate immune stimuli and LRRK2 with implications upon LRRK2 localisation, secretion and kinase activity. As such downstream LRRK2 activity is regulated at least in part by innate immune signalling.
The expression profile of LRRK2 mRNA and protein suggests a fundamental role of LRRK2 in non-neuronal processes, including innate immunity. LRRK2 mRNA is detected not just in the brain, but also at high levels in the kidney, spleen and lungs of normal mice (Maekawa et al., 2010), and can be detected in soluble extracts of human brain, liver and heart (Miklossy et al., 2006). Notably, expression of LRRK2 in systemic tissues is actually higher than in the brain (Biskup et al., 2006; Maekawa et al., 2010). TLR4 stimulation has been shown to affect LRRK2 localisation, causing translocation to the cell membrane (Schapansky et al., 2014), while during bacterial infection, LRRK2 specifically localises near bacterial membranes (Gardet et al., 2010).

Directly linking LRRK2 to an innate immune function is the fact that LRRK2 expression is enriched in macrophages, B-cells and dendritic cells (Gardet et al., 2010), and innate immune stimuli such as interferon gamma (Ifn-γ) have been reported to stimulate LRRK2 expression; revealing a responsiveness to the activation of innate immune signalling pathways (Thévenet et al., 2011). While Ifn-γ is reproducibly linked with an increase in LRRK2 expression in immune cells, other stimuli such as LPS (Gardet et al., 2010; Hakimi et al., 2011; Moehle et al., 2012) and IL-1β (Hongge et al., 2014) have less well documented effects on LRRK2 expression. LRRK2 mRNA expression has been demonstrated in cultured human astrocytes, microglia and oligodendrocytes (Miklossy et al., 2006). In the peripheral immune system, LRRK2 expression is highly enriched in monocytes and B-cells, with very little expression detected in T cells (Thévenet et al., 2011). In contrast, a more recent study has examined LRRK2 expression in primary peripheral immune cells, and shown LRRK2 to be expressed at comparable levels in B and T-lymphocytes, but that there is greater expression in monocytes. Further to this, this study showed enrichment of LRRK2 expression in Parkinson’s patients as compared to healthy controls (Cook et al., 2017). A monocyte-specific increase in inflammatory cytokine release was detected for Parkinson’s sufferers as compared to healthy controls in this study, correlating inflammatory cytokine release with LRRK2 expression in these cells. Interestingly, these results were recorded independently of LRRK2 genotyping, which suggests an underlying role of LRRK2 in regulating inflammation in idiopathic Parkinson’s disease. This agrees with reports of LRRK2 knockdown attenuating inflammatory responsiveness to LPS in microglial cultures (Kim et al., 2012), and a reported increase in serum and cerebrospinal fluid inflammatory cytokine levels in patients expressing the over-active kinase G2019S mutant (Dzamko et al, 2016). These data would
suggest a simple gain-of-function of LRRK2 leading to pathogenic inflammation as a causative model for Parkinson’s disease, however other reports suggest that LRRK2 over-expression, or G2019S mutant LRRK2 have no bearing on classical inflammatory cytokine release (Moehle et al., 2015). There are also mixed reports of the effect of LRRK2 GTPase R1441G/C mutations. Microglia overexpressing the LRRK2 R1441G mutation displayed enhanced TNFα release in response to LPS stimulation, while another report showed a slight decrease in NF-κB signalling in response to LPS in R1441C expressing microglia (Gillardon et al., 2012; Kim et al., 2012). Differences in reports of inflammatory signalling by mice expressing SNPs at R1441 may be accounted for by the different amino acids encoded by these SNPs. Different mutations may drive pathology by different mechanisms. Similarly, it is not known whether mutations in the Roc domain of LRRK2 would drive pathology by a common mechanism or independently of the G2019S kinase mutation.

1.5.2 LRRK2 is associated with non-neuronal inflammatory disease

At the genetic level, GWAS studies have shown that LRRK2 is not just linked with Parkinson’s disease, but also with Crohn’s disease and leprosy. Genetic links with these diseases demonstrate a non-neuronal, but firmly innate immune component to LRRK2 biology (Greggio et al., 2012). The fact that LRRK2 modulates susceptibility to multiple diseases, with pathology ranging from the gut to the brain, shows a remarkable breadth in function.

Leprosy is a chronic disease brought about through infection by the causative agent Mycobacterium leprae. Skin, eyes and peripheral nerves are affected by the formation of granulomas within macrophages and Schwann cells, leading to impairment of nerve function in the peripheries, skin lesions and potentially blindness (Britton and Lockwood, 2004). LRRK2 was first identified as a risk factor for leprosy in 2009 (Zhang et al., 2009) and later confirmed in 2015, along with four further LRRK2 polymorphisms, strengthening the link between LRRK2 and leprosy (Wang et al., 2014). The capacity of LRRK2 to affect susceptibility to bacterial infection directly links LRRK2 function to the host anti-bacterial response; including immunological inflammatory processes.

Crohn’s disease in particular is an archetypal polygenic inflammatory disease with 71 susceptibility loci (Barrett et al., 2009; Franke et al., 2010). Pathology is thought to stem
from the aberrant interaction of gut microbiota and environmental factors with the host innate immune system. Experimental mouse models of colitis have shown that mice deficient in LRRK2 expression are particularly susceptible to ‘dextran sodium sulphate’ (DSS)-induced colitis (Liu et al., 2011; Liu and Lenardo, 2012).

One mechanism believed to be responsible for the effect of LRRK2 on experimental colitis revolves around regulation of the ‘nuclear factor of activated T-cells (NFAT) and its translocation to the nucleus. NFAT is a transcription factor that regulates gene transcription in immune cells including T-cells and dendritic cells (Müller and Rao, 2010). More recently, a role for NFAT has begun to be defined in macrophages (Elloumi et al., 2012; Zanoni and Granucci, 2012). Under homeostatic conditions, NFAT is highly phosphorylated, and retained in the cytoplasm. In response to inflammatory stimuli, NFAT is able to translocate to the nucleus via dephosphorylation by calcineurin, a Ca\(^{2+}\) responsive phosphatase (Müller and Rao, 2010). LRRK2 was shown to modulate NFAT signalling through retention of the transcription factor in a cytoplasmic complex involving LRRK2, NFAT and a large noncoding RNA: ‘ncRNA repressor of the nuclear factor of activated T cells’ (NRON); this constitutes a non-canonical Ca\(^{2+}\) independent form of NFAT regulation. The M2397 allele of LRRK2 led to decreased inhibition of NFAT signalling, correlating with decreased detection of LRRK2 protein (Liu et al., 2011). M2397 is thought to act independently of direct perturbation of LRRK2 kinase activity (West et al., 2005), and has recently been linked to pathological immune responses in leprosy sufferers (Fava et al., 2016).

Another potential role for LRRK2 in Crohn’s disease was recently discovered in Paneth cells of the gut. Paneth cells sit within the crypts of the gut epithelium, and are responsible for secreting anti-microbial molecules to maintain sterility within the crypt itself, and to control microbial growth in the gut to promote barrier function and maintain microbiota sympathy with the host (Clevers and Bevins, 2013). LRRK2 was found to interact with another Crohn’s disease associated protein: NOD2, as well as the GTPase Rab2 within Paneth cells. The presence of each of these components was shown to be essential for the sorting of lysozyme into dense core vesicles for secretion into the gut lumen. Disruption of the complex led to lysosomal degradation of lysozyme (Zhang et al., 2015). While lysozyme sorting appears to be a very specific function for Paneth cells, the process is initiated by NOD2 mediated detection of ‘muramyl dipeptide’ (MDP), a component of the bacterial
peptidoglycan, which acts on the immune system as a PAMP. Interestingly, LPS was also shown to activate lysozyme sorting by an unknown detection mechanism (Zhang et al., 2015). Later, ‘receptor-interacting serine/threonine-protein kinase 2’ (RIP2), a kinase intimately associated with immunological signalling pathways and NF-κB activation was also found to be essential for lysosomal sorting (Wang et al., 2017), demonstrating a strong link between LRRK2 and innate immune signalling components. A recent report has further linked LRRK2 and NOD2 signalling by suggesting that LRRK2 acts as a positive regulator for RIP2 phosphorylation upon stimulation of NOD2; with consequences on TNFα, IL-6, and IL-1β production in response to LPS and MDP co-stimulation (Yan and Liu, 2017).

1.5.3 LRRK2 kinase inhibition has misled our understanding of LRRK2 function

Despite strong fundamental evidence that LRRK2 plays an important role in inflammation and inflammatory disease, the majority of attention in LRRK2 has been placed on a role in neuronal cells. This is because of LRRK2’s fundamental association with the degeneration of neurones, and status as a leading candidate for pharmaceutical targeting for the treatment of Parkinson’s disease; but also as a consequence of setbacks in the use of LRRK2 kinase inhibitors.

An ideal method for investigating the role of a kinase in disease or cellular processes is the use of small molecule inhibitors. Much was initially made of evidence from the inhibitor ‘LRRK2-IN-1’ (L2in1), a cell permeable competitor of ATP for LRRK2 binding (Deng et al., 2011). In particular, evidence from L2in1 inhibition suggested that LRRK2 kinase activity played a key role in innate immune processes, with results ranging from a change in microglial activation in response to LPS (Moehle et al., 2012), phagocytosis of foreign protein (Marker et al., 2012), macroautophagy (Manzoni et al., 2013), cell migration (Caesar et al., 2013), and innate immune signalling pathways (Luerman et al., 2014). In 2014, a quantitative phosphoproteomic screen was performed using ‘stable isotope labelling of amino acids in cell culture’ (SILAC). This experimental design was able to look at, and compare, protein phosphorylation significantly altered by L2in1 inhibition of endogenous LRRK2, and G2019S LRRK2 transduced cells. While this analysis was performed on the dopaminergic neuronal cell line SH-SY5Y, bioinformatic analysis of differential peptide phosphorylation revealed immunological pathways as top hits. Notably ‘extracellular signal-regulated kinase 5’ (ERK5) signalling’ was one of the top signalling pathways identified. ERK5
is a MAPK protein with a role in innate immune signalling (Luerman et al., 2014). While these results coincided with many previous studies at the time, it was noted that L2in1 was originally derived from XMD8-92, an inhibitor of ERK5 (Yang et al., 2010; Deng et al., 2011). Further analysis was performed, this time in an immunological context by using astroglial cells cultures from mice. It was noted that both L2in1 and XMD8-92 inhibited LPS-stimulated cytokine production, and that L2in1 had a stronger effect on LRRK2 kinase activity than XMD8-92, as measured using LRRK2 S910 phosphorylation as a read-out of LRRK2 kinase activity. This suggested that L2in1 displays improved targeting to LRRK2 compared to XMD8-92, and that both inhibitors show similar immunological effects in tissue culture. However, L2in1 was then applied to and compared between WT and LRRK2 KO astrocytes. This control experiment revealed inhibition of TNFα and CXCL10 production in response to LPS stimulation of LRRK2 KO astrocytes, indicating that many immunological effects ascribed to inhibition of LRRK2 kinase activity may in fact be caused by significant off-target effects of L2in1 on other kinases; such as ERK5 (Luerman et al., 2014). In light of this, evidence of LRRK2 function using L2in1 should be treated with caution, and considered only in parallel with structurally unrelated inhibitors or genetic methods of LRRK2 manipulation.

1.5.4 Modulation of protein degradation and translation by LRRK2

Other kinase inhibitors have subsequently been developed for LRRK2, and have indicated that LRRK2 kinase activity is important for maintaining LRRK2 protein stability by balancing proteolytic degradation.

Inhibition of LRRK2 kinase activity by any of a panel of six different LRRK2 inhibitors led to increased proteosomal degradation of LRRK2 in vitro. In a separate experiment, decreased LRRK2 protein levels were observed in the brain, kidney and lungs of mice following LRRK2 kinase inhibition, but not in the cortex of the brain, or in primary astrocyte cultures (Lobbestael et al., 2016). Similar observations were made upon treatment with LRRK2 inhibitors in human cultured peripheral blood mononuclear cells, or primary cells cultured from patients suffering from Parkinson’s disease (Perera et al., 2016). Dephosphorylation of LRRK2 has been shown to result in hyper-ubiquitination, leading to proteosomal degradation (Zhao et al., 2015); possibly as a consequence of loss of 14-3-3 chaperone binding (Dzamko et al., 2010). Interestingly, loss of 14-3-3 binding by LRRK2 kinase inhibition was also described as causing LRRK2 to accumulate in protein aggregates.
A similar observation was made of LRRK2 over-expressing mice, where the activity of the ubiquitin-proteasome system was impaired; leading to accumulation of proteins and aggregate formation (Lichtenberg et al., 2011).

Accumulation of LRRK2 has also been reported in a process associated with autophagy; the other major protein degradation pathway. LRRK2 stability was affected by the expression of kinase-dead mutants of LRRK2. Mice expressing this mutant displayed markers of autophagic disease in the kidneys, while LRRK2 KO mice showed similar pathology in the kidneys and lungs (Herzig et al., 2011; Tong et al., 2012). LRRK2 has been linked to two major forms of autophagy; macroautophagy and ‘chaperone mediated autophagy’ (CMA). Autophagy is particularly interesting in the context of neurodegenerative disease, as macromolecular protein aggregates are a hallmark of neurodegenerative pathology, and defective degradation of such aggregates would lead to toxic accumulation and cellular stress (Vogiatzi et al., 2008; Nixon, 2013). Interestingly, in neurotoxic models of Parkinson’s disease, over-induction of autophagy is observed (Zhu et al., 2007), however the clinical significance of this is open to question and will be discussed in a later focus on animal models of Parkinson’s disease. Macroautophagy is the most common and well-known form of autophagy, where material is sequestered within a double-membrane compartment, which matures into an autophagosome. Degradation of cargo occurs following lysosome-autophagosome fusion (Feng et al., 2014). Homeostasis of LRRK2 macroautophagy has been suggested to be regulated via direct LRRK2 phosphorylation of the macroautophagy machinery (Park et al., 2016). Indeed, involvement of LRRK2 in the modulation of macroautophagy as a process extends to substrates other than LRRK2 itself. Therefore LRRK2 associated pathology may result not just from dis-homeostasis of the LRRK2 protein, but also of other substrates (Gómez-Suaga et al., 2012). The importance of LRRK2 affecting macroautophagy is highlighted by the fact that recruitment of LRRK2 to autophagosome membranes occurs following TLR4 stimulation, suggesting a link between LRRK2 in innate immunity and autophagy (Schapansky et al., 2014). LRRK2 has been linked not just with macroautophagy, but also with CMA. CMA is characterised by chaperone mediated unfolding and transfer of targeted cytosolic proteins directly across the lysosomal membrane, without the formation of new membrane bound compartments (Kaushik et al., 2011). WT LRRK2 is efficiently degraded by CMA, while G2019S mutant LRRK2 is not; with G2019S LRRK2 depending to a greater extent on macroautophagy for proteolysis (Orenstein
et al., 2013). This is interesting in itself as it suggests the balance between these degradative pathways may play a role in disease. However, LRRK2 binding to lysosomes was also enhanced in the presence of other CMA substrates, thus displaying the opposite degradative dynamics to all other known CMA substrates. LRRK2 at the lysosome interfered with organisation of the CMA machinery; therefore LRRK2 interferes not just with its own degradation, but also with the degradation of other CMA substrates (Orenstein et al., 2013).

While the proteasome and autophagy regulate protein homeostasis through proteolysis, LRRK2 also interacts with, and modulates the protein synthesis machinery. Much of this work was performed using the drosophila homologue to LRRK2, dLRRK. In particular, ‘eukaryotic initiation factor 4E (eIF4E)-binding protein’ (4E-BP) has been suggested as a substrate for dLRRK (Imai et al., 2008). This phosphorylation event was also observed by human LRRK2. 4E-BP acts as a negative regulator to protein translation by sequestering the eukaryotic translation initiation factor ‘eukaryotic translation initiation factor 4E’ (eIF4E) from the ribosome (Richter and Sonenberg, 2005). Hyperphosphorylation of 4E-BP, mediated by dLRRK, is proposed to cause release of 4E-BP from eIF4E. Release of eIF4E then permits formation of the protein translation initiation complex, involving eIF4E and a target mRNA molecule for translation. dLRRK knockout conferred increased susceptibility to oxidative stress, and resultant dopaminergic neuronal degeneration in drosophila (Imai et al., 2008). A separate report has shown LRRK2 directly phosphorylates the small ribosomal subunit protein S15, with increased phosphorylation detected for LRRK2 kinase mutants such as G2019S. LRRK2 G2019S toxicity in neuronal cultures as measured by DNA fragmentation was seen to be rescued by mutating the phosphorylation site of the S15 subunit, suggesting a direct link between the LRRK2/S15 interaction and Parkinson’s disease pathology (Martin et al., 2014).

LRRK2 has also been suggested to regulate protein translation, not just through interaction with cellular translation machinery, but also by modulating microRNA-mediated translational repression (Gehrke et al., 2010). MicroRNAs are able to exert targeted translational regulation of specific genes through base-pair complementarity guided binding between miRNA molecules and target mRNAs. The mechanism by which miRNA action occurs is not fully understood, with regulation now thought to function at the level of mRNA degradation, as well as through translational repression by the RNA-induced silencing
complex (Huntzinger and Izaurralde, 2011). This interaction of LRRK2 with the miRNA machinery is intriguing, as miRNAs have previously been linked to neurodegenerative disease. Knockout of miRNA pathways is neurotoxic, and specific miRNA molecules have been linked to the transcriptional regulation of proteins such as alpha-synuclein and amyloid-beta; which accumulate during Parkinson’s disease, and Alzheimer’s disease respectively (Eacker et al., 2009). LRRK2 was shown to interact with and antagonise the action of Argonaut, one of the main components of the RNA-induced silencing complex (Gehrke et al., 2010). Translational profiling in drosophila showed that this interaction regulated the miRNAs let-7 and miR-184, which in turn are transcriptional repressors of E2F1 and DP. Therefore, LRRK2 acts to antagonize the inhibition of translation of the E2F1/DP transcription factor complex. Mutant forms of LRRK2, including the over active kinase mutant G2019S disinhibited E2F1/DP further. This suggests that the role for LRRK2 in modulating miRNA activity may be a pathogenic mechanism for Parkinson’s disease. The relevance of this role for LRRK2 is highlighted by a previous report of over-expression of E2F1 in post-mortem neuronal biopsies of Parkinson’s sufferers, and a protective effect of E2F1 knockout from dopaminergic neuronal degeneration in a neurotoxic mouse model of Parkinson’s disease (Höglinger et al., 2007). It is also intriguing that LRRK2 should play a role in let-7 mediated mRNA regulation, with mammalian let-7b having previously been associated with activation of the innate immune receptor TLR7, leading to neurodegeneration (Lehmann et al., 2012). The mammalian let-7 family has also been associated with suppression of innate immune responses including transcription of IL-10, IL-6, and TLR4 (Androulidaki et al., 2009; Schulte et al., 2011). Other notable interactions between LRRK2 and miRNAs include the small microRNA NRON, previously discussed for its association with LRRK2 in the modulation of NFAT signalling implicated in Crohn’s disease pathology (Liu et al., 2011); and a reported role of miR-712 in transcriptional regulation of LRRK2 itself (Talari et al., 2017).
1.5.5 LRRK2 has a distinct role in regulating neurone specific processes, morphology and function

While work discussed in this thesis focuses on the role of LRRK2 in innate immune cells, the majority of the attention of LRRK2 research has been placed on the role of LRRK2 in a neuronal context. This is owing to the fact that the majority of groups studying LRRK2 are interested in Parkinson’s disease, and generally come from a neuroscience background where a strong emphasis is placed on neuronal cells; little attention is paid to peripheral circulating immune cells. On top of this, interest that existed in the role of LRRK2 in innate immune pathways has been set back by the revelation that many effects ascribed to LRRK2 were in fact a result of off-target effects of the L2in1 LRRK2 kinase inhibitor (Luerman et al., 2014).

Neuronal cells are most noticeable for their extreme morphology and functional specialisation of transducing and rapidly transmitting cellular signals in the form of electrochemical currents. The detection and propagation of such currents is achieved through a high density of voltage-gated ion channels that populate the axonal membrane of neurons. These voltage-gated channels are closed under the negatively charged conditions of the resting cell, however upon detection of a positive membrane potential are opened allowing the rapid influx of Na⁺ or Ca²⁺ ions (Bean, 2007). As such, these channels allow the efficient propagation of an initial positive action potential along the axon. Specific morphology of neurons can be extremely diverse. For instance, signals from the peripheral nervous system need to travel much longer distances along the spinal cord than signals in relay neurones, which must only travel short distances within the brain. Therefore, while all neurons possess an axon which gives neuronal cells their characteristic extended morphology, the axon of a sensory neurone can be as long as over a meter in order to span the length of the spinal cord. Therefore, neurons are highly specialised not just for their general role in signal propagation, but also for their specific role within the nervous system (Marmigère and Ernfors, 2007).

Neurons within the central nervous system are highly networked, with multiple contacts made between cells. These contacts are made via synapses, often between the axon terminal of one cell, and spines localised to the highly branched structure of the dendrites of the next (Hering and Sheng, 2001). Synapses are enclosed spaces through
which electrical impulses are transduced via diffusion of a chemical intermediates known as neurotransmitters (Li and Sheng, 2003). Acetylcholine was the first neurotransmitter to be identified, and is found at neuromuscular junctions. Secretion of acetylcholine by motor neurons at the neuromuscular junction acts to transduce a neuronal electrical signal from the nervous system to a mechanical responses by muscle (Nishimaru et al., 2005). Between neurons, synapses can be broadly classified as excitatory or inhibitory in nature. The balance of the input signals at these synapses controls whether an ‘excitatory post synaptic potential’ is generated to continue downstream electrical signalling. One of the defining features as to the excitatory or inhibitory nature of a synapse is the neurotransmitter secreted at that synapse. Indeed, a vast array of neurotransmitters have been identified, and can be broadly classified as excitatory or inhibitory. For instance, the amino acid Glutamate is a classic neurotransmitter secreted at excitatory synapses, while GABA is classically found at inhibitory synapses (Mihic and Harris, 1997; Meldrum, 2000). Recent evidence however shows that individual synapses may actually secrete multiple neurotransmitters, suggesting that our understanding of neurotransmission may in fact still be in its infancy (Tritsch et al., 2016). Dopaminergic neurons of the substantia nigra play a specialised role in synthesising dopamine. Dopamine can be considered to be either excitatory or inhibitory depending on the receptor repertoire found at the synapse to which it is secreted, as well as neurotransmitters with which it may be co-secreted (Bucher and Wightman, 2015).

The nigrostriatal system is part of a larger neuronal network known as the basal ganglia, which is responsible for voluntary movement, as well as procedural learning, routine behaviours, cognition and emotion (Nelson and Kreitzer, 2014). It is the degeneration of dopaminergic neurons within this pathway that leads to tremors and other motor control symptoms associated with Parkinson’s disease. No dopamine can be supplied to the dorsal striatum of the basal ganglia neuronal circuits without these cells (fig 4). The input of the nigrostriatal pathway acts as part of a complex integration of basal ganglia signals at the dorsal striatum. Indeed the striatum forms connections to various regions of the brain including the globus pallidus and substantia nigra pars reticula; and receives input not just from the substantia nigra pars compacta, but also the cortex, thalamus and ventral tegmental area (Nelson and Kreitzer, 2014). Connections to the striatum are spatially organised, with dopaminergic neurons from the substantia nigra pars compacta projecting
to the dorsal and lateral regions associated with sensorimotor function, while the more medial and ventral regions of the striatum are associated more with cognitive and behavioural functions.

It is thought that the extremely specialised nature of dopaminergic neurons, even compared to surrounding neurons, makes them vulnerable to degenerative processes. More specifically, dopaminergic neurons possess extended and highly branched axons with low levels of myelination, meaning conducting electrical impulses is hugely energetically demanding (fig 10). To express this quantitatively, the rat substantia nigra pars compacta contains approximately 12,000 dopaminergic neurons, with each dopaminergic neurone contributing between 100,000 and 250,000 synapses to neurons of the striatum (Matsuda et al., 2009; Bolam and Pissadaki, 2012). To put this in perspective, the neuronal type contributing the next greatest number of synapses in the basal ganglia contributes only around 5,000 synapses. Each synapse requires active transport of molecules all the way from the cell body, as well as maintenance of the cytoskeleton, maintenance of membrane potential, and the generation of action potentials (Bolam and Pissadaki, 2012). The fact that dopaminergic neurons lack an electrically insulating myelin sheath exacerbates the energetic demands of propagating action potentials, as without myelin, saltatory conduction is not possible. On top of such morphological issues, dopaminergic neurons face a considerable inherent metabolic burden of dopamine synthesis (Wang and Michaelis, 2010). Pathogenic mutants of LRRK2 have been shown to modulate many of the facets that render dopaminergic neurons sensitive to stress, and it is theorised that this may lead to dopaminergic neuronal degeneration.

At a cellular level, some of the most consistently reported phenotypes of LRRK2 pathogenic mutations have been reported in the modulation of neuronal morphology. In particular, there are reports of defects in neurite branching, both during neuronal development and neuronal ageing. Neurites are a general term for projections from a neuronal cell body, including axons and dendrites. Issues have been reported in the development of neurons using induced pluripotent stem cells, where dopaminergic neuronal differentiation from skin fibroblasts was shown to be less efficient for G2019S LRRK2 mutation bearing neurons, compared to WT LRRK2 controls (Borgs et al., 2016). Deficiencies in differentiation have previously been reported in human neural-stem-cell
populations (Liu et al., 2012). While the physical reprogramming process of cells through induced pluripotency is somewhat artificial, it is interesting that G2019S dopaminergic neurons possessed a hyper-branched phenotype at early stages in this process, as compared to WT dopaminergic neurons (Borgs et al., 2016). Indeed the increased complexity of axonal arborisation observed in freshly developed G2019S LRRK2 expressing dopaminergic neurons is in contrast to the decreased branching complexity observed in aged induced pluripotency derived G2019S LRRK2 dopaminergic neurons (Sánchez-Danés et al., 2012). This suggests a differential role of LRRK2 and its pathogenic mutations both in neuronal development, and

**Figure 10: Dopaminergic neurons are exposed to oxidative stress.** Intrinsic and extrinsic pressures faced by dopaminergic neurons lead to a vulnerability to oxidative stress. Specifically, dopaminergic neurons must produce large quantities of dopamine in an environment of low glutathione, an antioxidant normally associated with protection from oxidative stress. On top of this, dopaminergic neurons must transmit electrical impulses along an unmyelinated axon to an extensively arbourised axonal terminal, requiring significant energy input. Such intrinsic sources of oxidative stress are said to place dopaminergic neurones ‘on the edge’, and are therefore highly vulnerable to degeneration. Finally dopaminergic neurons are exposed to external oxidative stresses from sources such as environmental toxins and inflammation that have been associated with the development of Parkinson’s disease. It has been speculated that such sources of stress may push vulnerable dopaminergic neurons ‘over the edge’.
in neuronal ageing where factors such as oxidative stress and autophagy are thought to play a strong part. This latter reports correspond with findings made using primary midbrain cultures of LRRK2 G2019S transgenic mice (Ramonet et al., 2011). A bi-phasic effect of LRRK2 on neurite branching mirrors findings by one group in the autophagy field, suggesting the cellular function of LRRK2 may be affected by age (Tong et al., 2012); with increased autophagy thought to correlate with increased neurite branching (Sánchez-Danés et al., 2012). Early observations of the effect of LRRK2 on neurite branching were made in vitro by transfection of LRRK2 mutant constructs, or knockdown of LRRK2. These cultures revealed not just effects on neurite branching, but also on neurite length; with G2019S LRRK2 causing a decrease in neurite length, and decreased branch complexity (MacLeod et al., 2006). These findings have subsequently been replicated in transgenic rodent models that have also revealed that fewer dendritic spines develop in G2019S LRRK2 transgenic animals (Winner et al., 2011; Häbig et al., 2013). As discussed previously, dopaminergic neurones display such extreme branching under homeostatic conditions that energetic demands makes these cells extremely vulnerable to stress (Bolam and Pissadaki, 2012; Pacelli et al., 2015). Perturbation of branch density and axonal length by pathogenic LRRK2 mutations could therefore be reasonably predicted to push these cells over-the-edge, leading to neurodegeneration (Pissadaki and Bolam, 2013).

Dopaminergic neurons are wholly responsible for the generation and supply of dopamine to the striatum. Dopamine is stable in synaptic vesicles, however in excess amounts, cytoplasmic dopamine has been shown to be neurotoxic to cells through formation of ROS and reactive quinones (Miyazaki and Asanuma, 2008). In whole rat brain lysate, LRRK2 is expressed in microsomal and synaptic vesicle-enriched cytosolic fractions, as well as on the mitochondrial outer membrane. Immunohistochemical analysis has shown this expression to be prevalent on neuronal vesicular structures, and mitochondria of the cell body, dendrites and axons (Biskup et al., 2006). It is of note that the underlying vulnerability of dopaminergic neurons is thought to revolve, at least in part, around the action of oxidative stress (Pacelli et al., 2015); so it is interesting that LRRK2 should localise to mitochondrial membranes, and therefore associate with the cellular machinery most intimately linked to the energetic demands of the cell. While interaction of LRRK2 with the mitochondria is not specific to the neuronal cell type, combination of mitochondrial
localisation with the vulnerability to oxidative stress of dopaminergic neurons is particularly interesting.

Mitochondria are implicated in Parkinson’s disease pathology not just due to their role in buffering of oxidative species, but also calcium. As previously discussed, calcium ions flux contributes to action potential propagation in neurons, and so buffering of calcium carries functional significance to this process. It is therefore of note that the G2019S and R1441C mutations of LRRK2 have been shown to result in upregulated mitochondrial expression of the up-regulation of the ‘mitochondrial calcium uniporter’ and ‘mitochondrial calcium uptake 1’ proteins, with a resulting increase in depolarisation-induced mitochondrial calcium uptake (Verma et al., 2017). In comparison, another group has shown a decrease in the calcium buffering capacity of neurons expressing the same LRRK2 mutants compared to wild type (Cherra et al., 2013). It is possible that as well as an effect on neuronal toxicity, calcium dis-homeostasis brought about by mutant LRRK2 may be responsible for enhanced current responses upon stimulation of G2019S or R1441C LRRK2 expressing neurons (Plowey et al., 2014). A further role of LRRK2 in signal transduction has been reported through an interaction with the voltage-gated Ca\textsuperscript{2+} channel complex (Ca\textsubscript{V2.1}). Such Ca\textsuperscript{2+} channels are expressed at the membrane of presynaptic terminal of many neurons, with a role in the regulation of neurotransmitter release and resulting postsynaptic signal transduction. Indeed LRRK2 co-expression was shown to increase cellular Ca\textsuperscript{2+} current densities compared to cells expressing Ca\textsubscript{V2.1} alone (Bedford et al., 2016). While LRRK2 may affect electrophysiology of neurons through the indirect mechanisms such as the buffering of calcium discussed here, there is also a body of evidence suggesting a more direct role in processes such as vesicular trafficking of neurotransmitters for synaptic secretion (Cirnaru et al., 2014; Penney et al., 2016), and endocytosis of synaptic vesicles (Arranz et al., 2015); as well as surface levels of dopamine receptors (Migheli et al., 2013).
1.6 Animal models of Parkinson’s disease

Much of the evidence discussed has been generated using animal models of Parkinson’s disease. Rodent models in particular are hugely important to our ability to mimic conditions that may lead to Parkinson’s disease, or genetic backgrounds that may lead to predisposition towards disease. This said, it is also important that these models are used and interpreted with an understanding of their relative strengths and weaknesses.

1.6.1 Chemically induced neurodegeneration

Chemically induced models of Parkinson’s disease pathology are generally an excellent way of mimicking the degeneration of dopaminergic neurons, and generating animals with movement and behavioural deficits akin to that of Parkinson’s sufferers (fig 11). Crucially, the mimicking of pathophysiological loss of dopaminergic neurons in these models allows the testing of disease modifying drugs and therapeutics on animals without an intact dopaminergic-striatal system. By these means, an assessment of drug efficacy and amelioration of disease symptoms can be made (Bové et al., 2005). A strong limitation of such models however, is the artificial nature of the mechanism of dopaminergic destruction. Most chemical models of Parkinson’s disease are completely artificial in their method of action on dopaminergic neurons, and so tell us little of the pathophysiological processes underlying the disease, or the cause of the destruction of these dopamine-secreting neurons. This process was initially termed ‘chemical denervation’ following the discovery of the effect of 6-Hydroxydopamine (6-OHDA) on neuronal cells in the 1970s (Jonsson, 1980).

6-OHDA is a hydroxylated analogue of dopamine, which is able to target dopaminergic and noradrenergic neurons via an affinity for the dopamine and noradrenergic transporters (Ungerstedt, 1968). Once present in the cytosol, 6-OHDA is rapidly oxidised causing the release of a large amount of ROS, which results in cell death through oxidative stress. The size of the lesions resulting from 6-OHDA injection, as well as the timescale required for damage to occur varies depending on the site of injection. These range from between 12 hours, and 3 days for injection into the substantia nigra (Jeon et al., 1995), to 3 weeks for injection to the striatum (Sauer and Oertel, 1994). 6-OHDA needs to be injected past the blood brain barrier, and into specific brain regions in order to avoid off-target death of non-dopaminergic neurons. As a result, 6-OHDA is largely restricted to use in
rats rather than mice, and has largely been surpassed by other chemical models of Parkinson’s disease. While 6-OHDA mimics the degeneration of dopaminergic neurons, no Lewy pathology is observed in 6-OHDA models of disease (Tieu, 2011).

The most commonly used chemical model of Parkinson’s disease in mice utilises 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The link between MPTP and Parkinsonism was made in 1983, following accidental self-administration of MPTP by intravenous drug users who presented with symptoms similar to severe Parkinson’s disease (Davis et al., 1979). Patients were responsive to L-DOPA treatment, demonstrating similarity in pathology between MPTP damage and naturally occurring Parkinson’s disease. More recently, an MPTP exposed patient has been successfully treated with deep brain stimulation. This demonstrates that the similarity in pathology of humans exposed to MPTP to Parkinson’s disease goes beyond simple damage to dopaminergic neurons, and into the overall electrophysiology of the brain. This in turn suggests great translatability of the MPTP model to Parkinson’s patients (Christine et al., 2009). MPTP is largely employed in mice and non-human primates. Non-human primates have been shown to be more sensitive to MPTP administration than mice, however MPTP none-the-less causes specific degeneration of dopaminergic neurons in the substantia nigra in mice. Interestingly, rats have been shown to be less sensitive to MPTP, limiting their suitability as a model for MPTP induced Parkinson’s disease (Giovanni et al., 1994). MPTP, unlike 6-OHDA, is systemically active, and can pass through the blood brain barrier due to its lipophilic nature. This makes MPTP easier to use, requiring much less expertise in animal surgery through simple intraperitoneal injection. As a result, MPTP administration is less prone to human error, and is thus more reproducible than 6-OHDA Parkinson’s models (Tieu, 2011). MPTP itself is specifically metabolised in neuronal astrocytes to its toxic form of MPP+. MPP+ is released from astrocytes (Cui et al., 2009), and taken up by the dopamine transporter of dopaminergic neurons where toxicity is achieved through inhibition of mitochondrial complex I, resulting in oxidative stress (Mizuno et al., 1987). While the pathology of MPTP treated mice closely resembles Parkinson’s disease in terms of the loss of dopaminergic neurones and responsiveness to therapy, like 6-OHDA treatment, Lewy pathology is incomplete. Low dose MPTP exposure over 30 days is required to recreate Lewy pathology (Fornai et al., 2005).
Environmental toxins are thought to play a role in the pathophysiology of Parkinson’s disease, and so are also employed in the lab to model the disease. The common environmental contaminants Paraquat and Rotenone are employed in this way. Paraquat is a pesticide with a similar molecular structure to MPP⁺. However, Paraquat can pass the blood-brain-barrier, and the mechanism of Paraquat toxicity appears to be independent of mitochondrial complex I inhibition (Richardson et al., 2005). Neurodegeneration observed following Paraquat exposure is also seen to be inconsistent compared to better-characterised neurotoxins such as MPTP. This makes the Paraquat model somewhat interesting to investigate the role of environmental toxins on the brain, but not necessarily to model Parkinson’s disease pathology itself (Tieu, 2011). Rotenone is a pesticide employed in bodies of water. Rotenone readily passes the blood brain barrier and acts through inhibition of mitochondrial complex I (Betarbet et al., 2000). Interest in Rotenone as a model of Parkinson’s disease peaked with the discovery that although the molecule exhibits no specificity of action to dopaminergic neurons, and inhibition of the electron transport chain was seen across the brain, prolonged exposure to Rotenone at a low dose specifically caused dopaminergic neuronal degeneration. This suggests that dopaminergic neurons are much more vulnerable to oxidative stress than surrounding neurons (Betarbet et al., 2000). Correspondingly, unlike most other chemically induced Parkinson’s models at the time, Rotenone appeared to induce Lewy body pathology following prolonged low dose exposure. However, irreproducibility and variability in reports of such Parkinson’s disease like features has led to marginalisation of Rotenone exposure as a model of disease (Tieu, 2011). Environmental toxins can be considered more meaningful than entirely synthetic molecules such as MPTP or 6-OHDA in terms of relevance to lifetime exposure to neurotoxins, but provide less consistent models of degeneration.

Some of the most appreciable evidence for a role of the innate immune system in the pathology of Parkinson’s comes from an LPS model of the disease. As previously discussed, LPS is a very widely used molecule in immunological studies and is the well-known ligand of TLR4 (Chow et al., 1999). LPS can be administered directly to the substantia nigra, where irreversible degeneration of dopaminergic neurons of the substantia nigra pars compacta is observed a week after injection (Castaño et al., 2002; Iravani et al., 2005). Of note is the fact that non-dopaminergic neurons of the nigrostriatal system, as well as proximal dopaminergic neurons not associated with the nigrostriatal pathway, remain
unaffected by direct LPS injection. Therefore LPS injection and the resulting inflammatory insult demonstrates remarkable sensitivity and specificity to the dopaminergic circuitry associated with Parkinson’s disease. Unlike molecules such as MPTP, this specificity is not derived through targeting of dopaminergic neurone specific transporters, but as with Rotenone exposure, reflects an underlying vulnerability of dopaminergic neurons which may be relevant to pathogenesis. LPS experiments also allow the coordination of immunological events in the brain, with activation of microglia and up-regulation of inflammatory cytokine release occurring within hours of LPS injection, while astrocyte activation occurs only a week after injection. This suggests a secondary role of astrogliosis in observed neurodegeneration (Herrera et al., 2000; Liu and Bing, 2011). In comparison to a single LPS injection, prolonged infusion of a lower dose of LPS to the substantia nigra over a two-week period successfully delayed the onset of innate immune activation and resulting Parkinson’s-like pathology (Gao et al., 2002). Activation of microglia in the substantia nigra occurred over the first two weeks of LPS exposure. Dopaminergic neuronal degeneration was then observed by four weeks, becoming significant by six weeks, and reaching 70% after ten weeks. Degeneration was again found to be specific to the substantia nigra as opposed to proximal regions, and to dopaminergic neurons as opposed to GABAergic or other neurons within the substantia nigra. A subsequent study demonstrated that the same pattern of Parkinson’s disease-like microglial activation, followed by neurodegeneration over ten months was observed when LPS or TNFα were administered systemically in mice via intraperitoneal injection (Qin et al., 2007). Activation and neuronal degeneration was not observed in mice lacking the TNFα receptors: TNFR1 and TNFR2. This suggests systemic innate immune activation plays a role in the development of neurodegeneration over a prolonged time period, and that progressive dopaminergic neurodegeneration continues even following resolution of the inflammatory cytokine response from the periphery (Qin et al., 2007). The fact that innate immune activation can be so closely linked to neurodegeneration by the LPS model is an indication that inflammation may be more than just a consequence of neuronal damage, and could play a causative role in disease pathology over time. The ability of innate immune receptors to transduce environmental signals to cellular events could, in this way, contribute towards the environmental component thought to underlie the onset of Parkinson’s, in the same way that environmental contaminants are thought to. In humans, a laboratory worker accidentally exposed to Salmonella derived LPS developed many
symptoms of Parkinson’s disease including bradykinesia, rigidity and tremor at rest, as well as other neurological problems resulting from damage to the substantia nigra as well as the cerebral cortex (Niehaus and Lange, 2003). Human Parkinsonism has been further linked to immune activation through the role of neurotrophic viral infection, and in particular, infection by the human influenza virus. Individual cases of viral infection leading to neuropathology and death have been reported, as well as increased incidence of Parkinson’s disease following pandemic flu, such as experienced in 1918 (Jang et al., 2009). Unlike some of the previously described neurotoxins, the use of LPS has not been identified purely due to its ability to specifically destroy dopaminergic neurons. Rather, neurodegeneration can be observed as a consequence of the natural process of inflammation.

Overall, LPS is an interesting molecule for the study of the potential immunological mechanisms by which Parkinson’s may develop in the idiopathic disease state. The LPS system is also notably more consistent in its neurological effects than the environmental contaminants, and benefits from a well studied and defined mechanism of action. Chemically induced Parkinsonism in rodent models by molecules such as 6-OHDA and MPTP are the best-known way to trial new therapeutics for capacity to ameliorate the physical symptoms of Parkinson’s disease, or overcome neurodegenerative processes specifically resulting from oxidative stress. The use of environmental toxins and inflammatory stimuli such as LPS can be used to model the role of more feasibly encountered environmental contaminants and the role of the innate immune system in the development of Parkinson’s disease. Notably, LPS is only one of many described ligands for TLR4 (Bryant et al., 2015). Other activators of TLR4 include endogenous DAMPs which may also be able to lead to an over activation of innate immunity which could lead to the development of neuropathology with age. Molecules capable of causing Parkinson’s through innate immune activation or oxidative stress can also be combined with genetic models of Parkinson’s disease to investigate how genetic effects modulate processes that lead to Parkinson’s symptoms.
Figure 11: Characteristics of chemically induced Parkinson’s disease mouse models. a: Chemical structures of neurotoxins. b: Simplified mechanisms of neurotoxin uptake and neurotoxicity. Specificity of some neurotoxins such as MPTP and Rotenone is brought about by targeting dopamine transporters found on dopaminergic neurons, while LPS and 6-OHDA act more generally. c: Level of specificity of toxin introduction required for targeted dopaminergic neurodegeneration. In particular, the lack of specificity of 6-OHDA as a neurotoxin requires specific injection to the substantia nigra to act as a model of Parkinson’s disease. LPS structure taken from Dong et al., 2014.
1.6.2 Genetic models

Models exist for the study of alpha-synuclein in mouse brains. Mice expressing mutant forms of alpha-synuclein: A30P, A53T and E46K, which cause familial Parkinson’s disease in humans, have been unsuccessful in replicating the pathology of the disease in mice. Such mouse models are not uniform, with different promoters and forms of alpha-synuclein expressed, leading to different cellular effects. The most commonly used promoters include Prion and tyrosine hydroxylase promoters, which generated mice with limited dopaminergic neuronal loss, and only moderate to mild motor phenotypes and microglial activation; without clear alpha-synuclein aggregation in the substantia nigra (Matsuoka et al., 2001; Giasson et al., 2002; Thiruchelvam et al., 2004; Su et al., 2009). These results can be interpreted as an indication that overt alpha-synuclein aggregation is not necessary for the onset of limited immunopathology, or that these mice model human Parkinson’s disease poorly. It seems the progressive pathology associated with dopaminergic degeneration is poorly replicated by genetic mutations in a rodent context. Numerous models bearing mutations to recessive Parkinson’s disease associated genes have also failed to replicate the disease (Blesa and Przedborski, 2014). Indeed, a triple knockout mouse lacking the mitochondrial-associated genes: Parkin, DJ-1, and PINK1 lacks any kind of neuronal cell pathology (Kitada et al., 2009). Genetic knockout of any Parkinson’s associated gene has failed to lead to neurodegeneration. In contrast to this, overexpression of alpha-synuclein through SNCA gene multiplication in mice (Janezic et al., 2013), or viral delivery of the SNCA gene in rats, does lead to alpha-synuclein associated pathology, and progressive dopaminergic degeneration (Decressac et al., 2012). While it is mechanistically interesting that an over-abundance of alpha-synuclein can lead to pathology in rodents, it should not be overlooked that such overexpression of alpha-synuclein is rarely observed in humans. Alpha-synuclein duplications or triplications are the cause of Parkinson’s disease in only individual cases (Singleton, 2003; Ibáñez et al., 2004).

Multiple LRRK2 based animal models of Parkinson’s disease have been developed, and multiple lines of evidence pertaining to LRRK2 function at a molecular level have been referenced previously. Here, LRRK2 mouse models will be considered at an organismal level. As with other genetic Parkinson’s disease models, LRRK2 KO mice demonstrate no overt signs of nigrostriatal pathology (Tong et al., 2010; Hinkle et al., 2012). However, some
behavioural deficits are observed compared to WT mice. There are mixed reports of accumulation of alpha-synuclein and Tau in LRRK2 KO mice. Interestingly, histology shows consistent pathology of the kidney (Tong et al., 2010; Hinkle et al., 2012; Tong et al., 2012), and some reports of lung pathology (Herzig et al., 2011). Kidney pathology consistently corresponds with changes in autophagy, however these reports are mixed as to whether autophagy is enhanced or suppressed, with variation evident with age.

Knock-in mice for G2019S or R1441C mutant LRRK2 show no obvious signs of dopaminergic neurodegeneration or alpha-synuclein related pathology (Tong et al., 2009; Herzig et al., 2011); with no pathology reported in the lung or kidneys of these mice. Mice overexpressing G2019S LRRK2 experience some dopaminergic neuronal loss (Ramonet et al., 2011; Chen et al., 2012), and this loss was not experienced to the same extent when overexpressing WT LRRK2. Mice overexpressing R1441C mutant LRRK2 showed no obvious signs of neurodegeneration in one study (Tsika et al., 2014), but mild dopaminergic neurodegeneration in another; alpha-synuclein pathology was not observed in this model (Weng et al., 2016). Another model using bacterial artificial chromosome (BAC) transgene expression achieved 5-10 fold overexpression of R1441G LRRK2, leading to dopaminergic neurodegeneration and motor defects (Li et al., 2009). G2019S and WT LRRK2 overexpression in BAC transgenic mice both led to abnormalities in dopamine transmission, but no dopaminergic neurodegeneration (Melrose et al., 2010). It should be noted that LRRK2 is endogenously expressed in the motor cortex and striatum of both mice and rats, however expression is only found in dopaminergic neurons of the substantia nigra pars compacta in mice, not rats (West et al., 2014).

Evidence from LRRK2 transgenic mice corresponds with observations made using other genetic rodent models of Parkinson’s, in that genetic risk factors associated with human Parkinson’s disease are not directly translatable to mice. Overexpression of LRRK2 or other Parkinson’s disease associated genes do appear to cause pathology, but these systems lack the physiologically relevant conditions which transgenic animals should mimic when studying disease pathogenesis. There are many reasons why rodent models of disease are limited, not least that in human disease there is a clear but ill-defined interaction between genetic and environmental factors that cannot be truly replicated in the lab. An environmental component of Parkinson’s disease is thought to contribute to the incomplete
penetrance of most genetic risk factors of the disease (Horowitz and Greenamyre, 2010). Another factor at play, which an animal model cannot easily replicate, is that of ageing. Ageing is a huge factor in Parkinson’s disease pathology, with disease prevalence quintupling between the ages of 60 and 85, to 5% of the population (de Lau and Breteler, 2006). In spite of this, the effects of ageing at the molecular level are not truly understood. The difference in the age of disease onset associated with SNPs in different Parkinson’s disease genes also hints that age-related processes are a strong modifier of underlying pathology. It is generally accepted that inflammatory processes become less tightly regulated with age, a process colloquially known as ‘inflamming’ (Franceschi and Campisi, 2014). Increased basal levels of inflammation could very well play a role in Parkinson’s disease pathology, however whether rodents and humans age similarly enough to mimic the contribution of ageing to Parkinson’s pathology in humans is unknown. Other age related deficits in processes such as protein degradation by the proteasome also become evident with age, and may play a role in the clearance of toxic alpha-synuclein aggregates (Collier et al., 2011).

While evidence from whole animal models needs to be carefully considered due to their inherent limitations in modelling such a complicated age related human disease, genetic models of Parkinson’s disease in particular allow specific aspects of biology to be studied closely for effects of individual genes on molecular and cellular processes.

1.7 Summary

LRRK2 is a complex protein with involvement in a range of cellular processes. A genetic association between LRRK2 and the archetypal inflammatory and immune related conditions of Crohn’s disease and leprosy demonstrate the importance of understanding the role of LRRK2 in immune cells. Much of the research attention focused on LRRK2 comes from a neuronal context in the study of Parkinson’s disease. It is now well established that Parkinson’s, and neurodegenerative diseases more generally, involve a strong inflammatory component. Therefore, while LRRK2 can be directly associated with neurodegeneration via a role in neuronal cells themselves, understanding the role of LRRK2 in an inflammatory or neuroinflammatory context may shine a light on the role of inflammation in Parkinson’s disease.
1.8 Hypothesis and aims

The interaction between the innate immune and nervous systems is increasingly appreciated as fundamental both in health and disease. In particular, a role of the innate immune system in neurodegenerative conditions such as Parkinson’s disease is now clear. Given that LRRK2 is associated with inflammatory disease such as Crohn’s disease and leprosy as well as Parkinson’s disease, is expressed in innate immune cells such as macrophages and microglia, is upregulated upon innate immune stimuli, and is phosphorylated and modulated upon Myd88 associated activation such as by TLR4 signalling; I hypothesise that LRRK2 plays a role in immune cell processes that may be relevant to Parkinson’s disease.

The role of LRRK2 in innate immunity is investigated at three levels, biophysical, cellular, and genome-wide; each with a specific aim towards investigating this hypothesis.

Aims

Biophysical investigation of LRRK2 is focused upon the LRR domain of LRRK2. Early evidence suggested a nucleic acid binding function of this domain; a function associated with similar LRR domains of innate immune proteins such as TLR3. Work described here aims to express the LRR domain of LRRK2 in order to investigate potential ligand binding activity of the LRR domain, which if confirmed would provide a direct ligand recognition function of LRRK2 in innate immunity. A range of approaches and optimisation of LRR domain expression are described towards this aim.

Cellular investigation of LRRK2 described in this work follow the specific hypothesis that known interactions between LRRK2 and the cellular trafficking machinery, as well as components of the extra cellular matrix play a role in innate immune processes. In particular work described here aims to elucidate or clarify the role of LRRK2 in TLR trafficking, phagocytosis and chemotaxis. An objective of this work is to build upon past literature that utilised the non-specific LRRK2 inhibitor L2-in-1 by utilising a specific LRRK2 KO system. A role of LRRK2 in innate immune receptor trafficking, phagocytosis or chemotaxis would build upon described functions of LRRK2 in neurons, and provide a new and potentially more relevant understanding of the cellular role of LRRK2 in an innate immune context.
Cellular investigation of LRRK2 is further investigated at a ‘global’ level through utilisation of RNA-seq and metabolomic technology. Work described here aims to use modern ‘-omics’ technology to compare the transcriptomic and metabolomic profiles of cells stimulated with LPS or MDP; activators of the innate immune system. The use of such technologies allows a snapshot of the innate immune response to be captured and analysed for genotypic differences. While RNA-seq analysis facilitates analysis at a truly global scale, a specific objective of the use of metabolomic data is to compare the effect of LRRK2 in an innate immune context to an effect upon ceramide levels recently described in neuronal tissue.
2. Materials and Methods

2.1 Secondary structure prediction

Secondary structure predictions were made using GENESILICO Metaserver 2 (Kurowski and Bujnicki, 2003), which yields secondary structure predictions from jnet (Cuff and Barton, 2000), spinex (Faraggi et al., 2012), spine (Dor and Zhou, 2007), psipred (Jones, 1999). For the design of N-terminal domain boundaries, the region P820-S990 of human LRRK2 (accession number: Q5S007) submitted to the metaserver. For secondary structure prediction of the predicted LRR domain itself, the region S979-A1329 was submitted.

2.2 Molecular biology techniques

PCR reactions mixes were made using specific primers specified for each construct, in combination with a recommended mix of supplied reagents with Vent polymerase PCR kit (New England Biolabs, USA) (dNTPs, ‘Thermopol buffer’ MgSO₄, Vent polymerase), and MiliQ purified water (Merck Milipore, USA). DNA restriction digest reactions were performed using New England Biolabs, USA restriction endonucleases following the manufacturers recommended protocols. Restriction digest reactions were performed for a minimum of 2 hours at 37 °C or left overnight at the same temperature when required to allow restriction digests to proceed to completion. When ligations reactions were required T4 DNA Ligase (New England Biolabs, USA) was used with a typical molar ratio of vector: insert of 1:3 or 1:6. Reaction mixes were made around these ratios using reagents and concentrations recommended by the manufacturer. Reactions were performed at room temperature for 1 hour. Bacterial transformations were made into chemically competent DH5α generated in lab, or DH5α-select gold efficiency cells (Bioline, UK). Bacterial transformation was performed using a volumetric ratio of 1 : 20 DNA : competent cells. DNA was mixed with cells for 30 seconds on ice before being transferred to a 42 °C water bath for 1 minute. Transformations were then incubated for 2 minutes on ice before being supplemented with 500 µl SOC Medium (Thermo Fisher Scientific, USA). Cultures were incubated for 1 hour at 37 °C before being plated on selection medium. DNA was extracted from bacterial cultures using QIAprep Spin Miniprep Kit (Qiagen, Germany). DNA was extracted from agarose gels by extracting DNA bands using a scalpel, followed by Wizard SV Gel and PCR Clean-Up system (Promega, USA), or QIAquick Gel Extraction Kit (Qiagen,
Germany). DNA concentrations were measured using a Nanodrop 1000 (Thermo Fisher Scientific, USA).

2.3 LRR6 baculovirus and protein generation

LRR6 construct was initially cloned from full-length LRRK2 using ‘Ligation independent cloning’ (LIC) primers LIC For_E980 and LIC Rev_M1335 (Table 1)

Table 1: Primers used for LIC cloning of LRR6

<table>
<thead>
<tr>
<th>LIC Primer</th>
<th>DNA Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIC For_E980</td>
<td>TACTTCCAATCCAATGCAGAGAGAGAATATATTACA</td>
</tr>
<tr>
<td>LIC Rev_M1335</td>
<td>TTATCCACTTCCAATGTTATTACATTCCGGTTATAAGGCAC</td>
</tr>
</tbody>
</table>

PCR was performed using Vent DNA polymerase (New England Biolabs, USA) by the following PCR conditions:

1. 94 °C – 1 minute
2. 94 °C – 45 seconds
3. 65 °C – 45 seconds
4. 72 °C – 2 minutes 45 seconds
5. Repeat from step 2 24x

Resulting PCR product as well as LIC pFastBac-1 transposition vectors (Bac-to-Bac; Invitrogen): ‘pFastBac His6 MBP N10 TEV LIC’ cloning vector (4C) (plasmid 30116, Addgene, USA) and ‘pFastBac His6 TEV LIC’ cloning vector (4B) (plasmid 30115, Addgene, USA) were digested using SspI (New England Biolabs, USA), and treated with T4 DNA polymerase (New England Biolabs, USA) in the presence of 5 mM dCTP or dGTP (New England Biolabs, USA) for PCR product and LIC vectors respectively. T4 polymerase treated LRR6 and each T4 treated LIC pFastBac-1 transposition vector were allowed to anneal, generating MBP-LRR6 and His-LRR6 constructs for bacmid generation. Bacmids and recombinant baculoviruses were generated according to the Bac-to-Bac procedure (Invitrogen, USA). 10 L of Spodoptera frugiperda Sf9 cells at approximately 1.3x10⁶ cells/ml were infected with MBP-LRR6 encoding baculovirus at a molarity of infection of one, and harvested two days later. 2 L of Sf9 cells were similarly infected with His₆-LRR6 encoding baculovirus but harvested three days later.
2.4 Cell pellet harvesting, cell lysis, and Ni affinity purification

Protein cultures generated using baculovirus were pelleted at 4,000 rpm for 10 mins at room temperature in a JLA8.100 rotor (Beckman Coulter, USA), supernatant was discarded, and cell pellet split into 5 x 2 L pellets of MBP-LRR6 and 4x 0.5 L His-LRR6 for flash freezing in liquid nitrogen and storage at -80 °C.

2 L pellets of MBP-LRR6 were thawed and resuspended in a basic lysis buffer of 80 ml of 20 mM Tris pH 7.5. This buffer and all subsequently described buffers were supplemented with reducing agents or additives as indicated in each experiment. 2 ml of BugBuster (Merck & Co, USA) and 1 ml of protease inhibitor cocktail (Merck & Co, USA) was then added. Lysis was performed on ice by Vibra-Cell VCX130 ultra-sonicator with 13 mm horn (Sonics & Materials Inc, USA). Lysed cells were incubated, rolling at 4 °C, before soluble extract was isolated by centrifugation at 16,000 rpm at 4 °C for 90 mins using an F0850 rotor (Beckman Coulter, USA). Supernatant was supplemented with 20 ml of 5 x lysis additions (20 mM Tris pH 7.5, 2.5 M NaCl, 100 mM Imidazole). This volume was adjusted based on the original volume of soluble extract to give a 1x final concentration of lysis additions. All volumes were adjusted based on original size of harvested pellet.

Supplemented soluble extracts were loaded onto a Ni chelated 5 ml HiTrap Chelating HP column (GE Healthcare, USA) pre-equilibrated in Ni Binding Buffer (20 mM Tris pH 7.5, 500 mM NaCl, 20 mM Imidazole). Washing and elution was performed on an ÄKTA FPLC system (GE Healthcare, USA), elution was performed over a linear gradient of 10 or 15 column volumes from Ni binding buffer to Ni elution buffer (20 mM Tris pH 7.5, 200 mM NaCl, 250 mM Imidazole).

2.5 Anion exchange chromatography

Pooled fractions of eluted MBP-LRR6 from Ni affinity purification was spun down, then supernatant diluted 1:10 in 20 mM Tris pH 8.0, 75 mM NaCl in order to raise pH and lower NaCl concentration. Diluted protein was loaded onto a 5 ml HiTrap Q HP (GE Healthcare, USA) pre-equilibrated in Q binding buffer (20 mM Tris pH 8.0, 100 mM NaCl). The column was eluted on an ÄKTA FPLC system with a 10 column volume gradient to 50 % Q elution buffer (20 mM Tris pH 8.0, 1 M NaCl), followed by a step to 100 % Q elution buffer for 5 column volumes.
2.6 Heparin affinity purification

Pooled fractions of eluted MBP-LRR6 from Ni affinity purification were incubated with Tobacco Etch Virus protease (TEV) overnight at 4 °C. Precipitant was spun down, then supernatant diluted 1:5 in 20 mM Tris pH 7.5 to lower NaCl concentration. Diluted protein was loaded onto a 5 ml HiTrap Heparin HP (GE Healthcare, USA) equilibrated in Tris_Heparin binding buffer (20 mM Tris pH 7.5, 65 mM NaCl). The column was eluted on an ÄKTA FPLC system (GE Healthcare, USA) with a 10 column volume gradient to 100% Tris_Heparin elution buffer (20 mM Tris pH 7.5, 1 M NaCl).

Samples loaded and eluted from heparin in Tris buffer was pooled and dialysed using Spectra/Por molecular porous membrane tubing 6-8,000 molecular weight cut off (Spectrum Inc, USA) in 5 L of Phosphate_Heparin binding buffer (20 mM NaPO₄, pH 7.5, 50 mM NaCl) mixed slowly overnight at 4 °C. Precipitant was spun down, then supernatant loaded onto a pre-equilibrated 5 ml HiTrap Heparin HP (GE Healthcare, USA). The column was eluted on an ÄKTA FPLC system (GE Healthcare, USA) with a 10 column volume gradient to 100% Phosphate_Heparin elution buffer (20 mM NaPO₄ pH 7.5, 1 M NaCl).

2.7 Analytical size exclusion chromatography

A Superdex 200 10/300 GL (GE Healthcare, USA) column was equilibrated with GF_buffer (10 mM NaPO₄ pH 7, 250 mM NaCl). Samples of heparin purified MBP-LRR6 were prepared by taking 3 ml of protein and concentrating 3x to 1 ml using a Vivaspin 6 30,000 MWCO concentrator (GE Healthcare, USA), or incubating a 1 ml sample with TEV protease overnight at 4 °C. Samples were run with 1.2 column volumes of GF_buffer at 0.5 ml/min.

2.8 SDS-PAGE and native gels

Proteins were generally analysed by 10 % SDS-PAGE polyacrylamide gels using the Bolt Minigel tank (Life Technologies, USA). Samples were boiled and reduced using 2-mercaptoethanol unless stated otherwise, and run at 200 V for 50 mins. Gels were stained with Coomassie InstantBlue (Expedeon Ltd, UK).

6 % polyacrylamide native PAGE gels were cast at pH 9.4 and samples loaded in SDS free loading dye. Samples were run for the indicated amount of time at 100 V using the Bolt Minigel tank (Life Technologies, USA). Gels were stained with Coomassie InstantBlue.
2.9 Western blotting

Proteins from SDS-PAGE gel were transferred to Hybond-C Nitrocellulose membrane (GE Healthcare, USA) using a Bolt Mini Blot Module (Life Technologies, USA) in Tris Glycine transfer buffer. Membranes were blocked in 3 % milk in ‘phosphate buffered saline, 1 % Tween’ (PBST) overnight, rolling at 4 °C. Membranes were probed with indicated antibodies, either monoclonal mouse anti-human IgG Fc specific (GG-7 clone; Sigma-Aldrich, USA) or mouse anti-His6 (BD Biosciences, USA) at dilutions of 1:2500 and 1:300 respectively for 1 hour, rolling at room temperature. Membranes were washed for 15 mins, 10 mins, 5 mins in PBST then incubated with an horseradish peroxidase conjugated goat-anti-mouse secondary antibody (A4416; Sigma) diluted 1:3000 in 3 % milk-PBST for 1 hour rolling at room temperature. Membrane washes were repeated before incubation with ECL reagent (GE Healthcare, USA) and development on Hyperfilm ECL (GE Healthcare, USA).

2.10 Identification of leucine rich repeats by sequence analysis and VLR-fusion construct generation

Computational identification and annotation of individual leucine rich repeats was performed using the LRRfinder webserver (Offord et al., 2010). A region encompassing K951-M1335 of human LRRK2 (Accession number: Q5S007) was submitted to the webserver for this purpose.

LRRK2–VLR fusion constructs encompassing LRRK2 residues Asn-992 (LRR1) to Leu-1302 (LRR14) were generated as fusions with residues Ala-23 to Leu-82 and Asn-133 to Thr-201 of hagfish VLR B.61 (termed VLRN and VLRC respectively). A ‘Receptor for Advanced Glycation Endproducts’ (RAGE) secretion signal fusion with VLRN and VLRC with TEV cleavable Fc-His6 fusion (Gangloff et al., 2013) were cloned into the pFastBac-1 transposition vector (Bac-to-Bac; Invitrogen, USA) using BamHI with NheI restriction sites and NheI with NotI restriction sites respectively (New England Biolabs, USA). Full length LRRK2 was used as a PCR template using combinations of primers (table 2) to generate various LRR constructs as described in results.
Each VLR construct therefore contains a CAAGCA leader sequence followed by a NheI site for restriction digest (5’-3’). PCR was performed using Vent DNA polymerase (New England Biolabs, USA) by the following PCR conditions:

1. 94 °C – 4 minutes
2. 95 °C – 30 seconds
3. 75 °C – 30 seconds
4. 72 °C – 2 minutes 45 seconds
5. Repeat from step 2 34x

Each LRR PCR product was fused between VLRN and VLRC using NheI digestion (New England Biolabs, USA), combined with Antarctic phosphatase (New England Biolabs, USA) treatment. Orientation of each insert was determined by sequencing using an external primer. RAGE-VLRN-LRR-VLRC-TEV-Fc-His₆ constructs were then cloned into a pCDNA3 vector using BamHI and NotI restriction digest (New England Biolabs, USA), then finally transferred to pMT/V5-His A vector using KpnI and NotI (New England Biolabs, USA).

### 2.11 S2 Cell Test Expression of VLR-fusion constructs

S2 cells were plated in a 6-well dish at $3 \times 10^6$ cells/well in insect-xpress medium (Lonza Plc, UK) supplemented with 10% filtered, heat-inactivated Foetal Bovine Serum (Life Technologies, USA). Once cells had settled, media was removed and replaced with 2 ml serum free insect-xpress media. 3 µg of each DNA construct in pMT/V5-His A vector was added to 100 µl of serum free insect-xpress media and filter sterilised through a 0.22 µm CoStar SpinX filter (Sigma-Aldrich, USA), then transferred to a bijou. 8 µl of Cellfectin II reagent (Invitrogen, USA) was added to 100 µl of serum free insect-xpress media per transfection reaction, and left for 5 minutes at room temperature. 100 µl of media:cellfectin mix was added drop-wise to each Bijou bottle containing DNA for transfection. Transfection

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequence 5’-3’</th>
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<tr>
<td>LRR1 For (N992)</td>
<td>CAAGCAGCTAGCAATGAACTAAGAGATATTGAT</td>
</tr>
<tr>
<td>LRR2 For (N1021)</td>
<td>CAAGCAGCTAGCAATGCACTCACGAGCTTTCCA</td>
</tr>
<tr>
<td>LRR12 Rev (L1252)</td>
<td>TGCTTGGCTAGCAAGATGCTTTCTCTACTCT</td>
</tr>
<tr>
<td>LRR13 Rev (V1275)</td>
<td>TGCTTGGCTAGCGACATCCAGATGTCAGATT</td>
</tr>
<tr>
<td>LRR14 Rev (L1302)</td>
<td>TGCTTGGCTAGCCAGTTCATCCAAAGGAAGATC</td>
</tr>
</tbody>
</table>

Table 2: Primers used for cloning of VLR fusion constructs
mixes were left at room temperature for 30 minutes, then topped up to 1 ml with serum free insect-xpress media. Media was gently removed from S2 cells and replaced with 1 ml transfection mix. Cells were incubated at 27 °C overnight, then transfection mix was removed from cells and replaced with fresh insect-xpress media supplemented with 10 % FCS. Several hours later, 10 µl of 100 µg/ml sterile CuSO₄ was added to each well of transfected cells to induce protein expression. 24 hours later, cells were displaced and gently spun down at 0.2 g for 5 mins. Cell pellets were harvested in 100 µl of loading dye and flash frozen, while an aliquot of supernatant was prepared for SDS-PAGE analysis, with the rest flash frozen in liquid nitrogen.

50 µl Recombinant Protein A Sepharose Fast Flow (GE Healthcare, USA) slurry was spun in a Pierce Spin Column (Thermo Fisher Scientific, USA) at 500 g for 30 secs. Protein A beads were washed in in 1 ml PBS, then spun again at 500 g for 1 min. Washed beads were added to thawed 2 ml test expression supernatant and incubated with gyration at 4 °C for 90 mins. Supernatant was then transferred back to a spin column to separate protein bound beads by centrifugation at 500 g until all beads were collected. Beads were subsequently re-suspended in 50 µl of non-reducing loading dye, and split into two tubes, one of which was subsequently reduced with 1 µl 2-mercaptoethanol.

2.12 Mice, genotyping and routine cell culture

WT C57BL/6J mice were obtained from Charles River, UK. LRRK2⁻/⁻ B6.129X1(FVB)-Lrrk2tm1.1Cai/J mice was obtained from The Jackson Laboratory, United States (Parisiadou et al., 2009). All mice strains were bred independently. All work involving live animals complied with the University of Cambridge Ethics Committee regulations and was performed under the Home Office Project License number 80/2572. DNA from Ear snips of LRRK2⁻/⁻ B6.129X1(FVB)-Lrrk2tm1.1Cai/J mice was isolated for genotyping using the Phire animal tissue digest PCR kit (Thermo Fisher Scientific, USA). Genotyping PCR was carried out in accordance with recommendations by The Jackson Laboratory utilising the recommended genotyping primers (table 3) and reaction conditions. Genotyping PCR products were run on a 1 % agarose gel made using ultra pure agarose (Thermo Fisher Scientific, USA) in a horizontal electrophoresis system (Bio-Rad Laboratories, USA), and visualised using GelRed nucleic acid stain (Biotium, USA) and a Gel Doc XR+ Gel documentation system (Bio-Rad Laboratories, USA).
Routine tissue culture was performed in a containment level 2 laboratory in a sterilized tissue culture flow hood. Such tissue culture hoods were routinely wiped with 70% ethanol both before and after work, as well as subjected to UV radiation after each use in order to maintain sterility. During tissue culture work sterile and autoclaved disposable plastic pipettes and pipette tips were used and disposed of into a Virkon (DuPont, USA) to maintain sterility. Sterility of cultures was checked by routine visual inspection and light microscopy. Cell culture media was replaced as indicated, or every three days for routine work such as with cell lines.

For the differentiation and culture of ‘primary bone marrow derived macrophages’ (pBMDMs), mice were killed between 8 and 16 weeks of age by cervical dislocation, skin was sterilized with 70% ethanol, and legs removed. Under sterile conditions, the tibia and femur were isolated, cleaned of muscle, and the proximal and distal epiphysis cut away. Bone marrow was flushed out of the bone using primary growth media (‘Dulbecco's modified Eagle's medium’ (DMEM) (Thermo Fisher Scientific, USA) supplemented with 10% ‘foetal calf serum’ (FCS) (Thermo Fisher Scientific, USA), 20% L929 conditioned media and 8 mM L-glutamine (Sigma-Aldrich, USA)). Isolated cells were centrifuged at 300 g for 10 min at 15 °C, and re-suspended in 60 ml of growth media, and allowed to grow at 37 °C in 5% CO₂. Cells were supplemented with a further 60 ml of growth media after 2 days, and media replaced every 3 days. All experiments were performed on cells between 6 and 11 days after initial bone marrow isolation. Live cell counts were performed using a haemocytometer with trypan blue staining (Sigma Aldrich, USA).

Immortalised WT and TLR4⁻/⁻ BMDM cell lines were originally obtained from Professor Doug Golenbock, University of Massachusetts Medical School. TLR4⁻/⁻ cells were maintained in ‘Roswell Park Memorial Institute 1640’ (RPMI) medium (Thermo Fisher Scientific, USA) supplemented with 10% FCS, and 2 mM L-glutamine.

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>9940</td>
<td>CTCTGAGAGCAGGAGCGT</td>
</tr>
<tr>
<td>9941</td>
<td>TGCCCTCCTGAGACATTTCAGCC</td>
</tr>
</tbody>
</table>
2.13 Cytokine bead array

1x10^5 cells/well were plated in a 96-well tissue culture plate (Corning, USA) a day prior to treatment, and left to adhere overnight at 37 °C in 5 % CO2. Cells were then treated in triplicate with 100 ng/ml ultrapure LPS from E. coli O111:B4 (InvivoGen, USA), 50 ng/ml recombinant murine Ifn-γ (Peprotech, UK), 10 µg/ml MDP (InvivoGen, USA) or 75 µM alpha-synuclein oligomers (αSyn Oligos) as indicated for each experiment. αSyn Oligos were supplied by Dr Craig Hughes, the preparation of oligomers is detailed elsewhere (Hughes et al., submitted for publication). LPS was sonicated prior to application to cells. After incubation for the indicated amount of time at 37 °C in 5 % CO2, supernatants were transferred to a round-bottomed 96-well tissue culture plate and stored at -80 °C. Thawed triplicate supernatants were later pooled and analysed using a ‘Mouse Th1/Th2 10plex Kit FlowCytomix’ (eBioscience, USA) bead array kit following the manufacturers instructions. Bead array was analysed on an Attune NxT acoustic focusing cytometer (Life Technologies, USA).

2.14 RNA isolation and qRT-PCR

2.5x10^6 cells/well were plated in a 12-well tissue culture plate (Corning, USA) a day prior to RNA extraction, and left to adhere overnight at 37 °C in 5 % CO2. Cells were then treated with 100 ng/ml ultrapure LPS from E. coli O111:B4 (InvivoGen, USA), 50 ng/ml recombinant murine Ifn-γ (Peprotech, UK), 10 µg/ml MDP (InvivoGen, USA) or 75 µM alpha-synuclein oligomers (αSyn Oligos) as indicated for each experiment. αSyn Oligos were supplied by Dr Craig Hughes, the preparation of oligomers is detailed elsewhere (Hughes et al., submitted for publication). LPS was sonicated prior to application to cells. After 2 hours incubation at 37 °C in 5 % CO2, cells were washed in ‘phosphate buffered saline’ (PBS) (Thermo Fisher Scientific, USA) and scraped from tissue culture wells at 4 °C. RNA was isolated using RNeasy mini kit (Qiagen, Germany) in combination with QIAshredder cell homogenization (Qiagen, Germany) following the manufacturers instructions. To remove genomic DNA, extracted RNA was DNase treated using TURBO DNA-free kit (Applied Biosystems, USA). Resulting RNA was analysed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). ‘Quantitative reverse transcription polymerase chain reaction’ (qRT-PCR) was performed using SensiFAST SYBR No-ROX One-Step Kit (Bioline, UK) following the manufacturers instructions and appropriate primers selected based on data
submitted to the primer bank database (table 4) (Spandidos et al., 2009). qRT-PCR reactions were performed using a Rotor-Gene Q (Qiagen, Germany), and quantification of fold-changes of transcript were calculated using cycle threshold values accounting for reaction efficiency (Pfaffl, 2001).

### Table 4: Primers for qRT-PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward primer</th>
<th>DNA sequence 5’-3’</th>
<th>Reverse primer</th>
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<tr>
<td>LRRK2</td>
<td>ATCTCACCCCTTCATGCTTTCTG</td>
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<td>TLR4</td>
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<tr>
<td>TLR2</td>
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<td>AGGCCTCTCCCTCTATTGTATT</td>
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2.15 Flow cytometry of cell surface proteins

1x10^6 cells/well were plated in 12-well tissue culture plates and left to adhere overnight at 37 °C in 5 % CO₂. Where appropriate, cells were treated with 200 ng/ml ultrapure LPS from E. coli O111:B4 or 10 µg/ml MDP, and incubated at 37 °C in 5 % CO₂ for the indicated amount of time for each experiment. LPS was sonicated prior to application to cells. Following any incubations, cells were washed 2 x with PBS at 4 °C. Cells were then scraped into MACS buffer (PBS supplemented with 2 % FCS, 1 mM EDTA (Merck and Co, USA)) and spun at 300 g for 6 minutes in a conical-bottom 96-well plate (Thermo Fisher Scientific, USA). Cells were re-suspended in MACS buffer supplemented with 1:100 rat anti-mouse CD16/CD32 functional grade purified (93 clone; eBioscience, USA) and incubated at 4 °C for 15 minutes. Cells were spun at 300 g for 6 minutes, then re-suspended in MACS buffer supplemented with an appropriate antibody for fluorescent analysis (table 5). Staining was performed for 30 mins at 4 °C. Cells were then centrifuged at 300 g for 6 mins and re-suspended in MACS buffer 3 x to remove unbound antibody before finally being spun at 300 g for 6 minutes, and re-suspended in MACS buffer supplemented with 2 % methanol-free formaldehyde (Thermo Fisher Scientific, USA) to fix. Fixed cells were stored at 4 °C overnight, then analysed using a BD Accuri C6 flow cytometer (BD Biosciences, USA) for single stain experiments, or an Attune NxT acoustic focusing cytometer (Life Technologies, USA) for F4/80, CD11c, CD11b triple labeling experiments.

Table 5: Flow cytometry antibodies

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2.16 Phagocytosis assays

1 µm yellow-green fluorescent labeled carboxylate-modified beads (L4655; Sigma-Aldrich, USA) were used for all bead phagocytosis assays. Appropriate dilution of beads was ascertained by counting on a haemocytometer in combination with a FV1200 confocal microscope (Olympus Corporation, Japan). For all bead phagocytosis assays, 1x10^6 cells/well were first plated in a 12-well tissue culture plates and left to adhere over night at 37 °C in 5 % CO₂. Cells could subsequently be incubated with inhibitors and appropriately diluted beads. For visualisation of bead opsonisation, 2 µl of stock beads were diluted into 20 µl of PBS with a range of dilutions of CF640R mouse anti fluorescein antibody (clone 1F8-1E4; Thermo Fisher Scientific, USA) as indicated in the experiment. Beads were incubated at 37 °C for 1 hour, with occasional shaking. Stained cells were immediately mounted on microscope slides using VECTASHIELD antifade mounting medium (Vector Laboratories, UK), and imaged on an FV1200 confocal microscope (Olympus Corporation, Japan).

For initial optimisation of beads/cell ratio for experiments, 50 µl of beads were diluted 1:10 in PBS (Thermo Fisher Scientific) and centrifuged at 8000 rpm in a Heraeus Biofuge Pico centrifuge (Thermo Fisher Scientific, USA) for 1 minute. 400 µl of PBS was carefully removed 100 µl at a time to leave beads unperturbed, then re-suspended in a further 400 µl of PBS. Washed beads were diluted into primary growth media 3:1000 for 10 beads/cell, and 3:100 for 100 beads/cell. 1 ml of diluted beads was added to each well where appropriate, and incubated for the indicated amount of time at 37 °C in 5 % CO₂. Cells were then washed and fixed for analysis by flow cytometry as follows: Cells were then transferred to ice, and washed 3x with PBS at 4 °C. PBS was then removed and replaced with MACS buffer into which cells were scraped. Cells were transferred to a conical-bottom 96-well plate (Thermo Fisher Scientific, USA) and spun at 300 g for 6 minutes. Cells were then re-suspended into MACS buffer supplemented with 2 % methanol-free formaldehyde (Thermo Fisher Scientific, USA) to fix, and stored at 4 °C overnight. Fixed cells were then analysed using a BD Accuri C6 flow cytometer (BD Biosciences).

For optimised bead phagocytosis experiments, 10 µl of beads were diluted 1:10 in PBS, or for experiments utilising opsonised beads, 10 µl of beads were diluted into 80 µl of PBS with 10 µl of CF594 mouse anti-fluorescein antibody (clone 1F8-1E4; Sigma-Aldrich, USA). Beads were incubated at 37 °C for 1 hour, with occasional shaking. Beads were then
diluted with a further 400 µl of PBS to wash, and centrifuged at 8000 rpm in a Heraeus Biofuge Pico centrifuge (Thermo Fisher Scientific, USA) for 5 minutes. 400 µl of PBS was carefully removed 100 µl at a time to leave beads unperturbed. Beads were then diluted at a ratio of 3:1000 into primary growth media supplemented with either 1 % dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA), or 1 mM cytochalesin D (Thermo Fisher Scientific, USA) dissolved in 1 % DMSO (final concentration: 10 µM) as indicated in the experiment. Before the application of beads, cells were incubated in primary growth media supplemented with 1 % DMSO or 1 mM cytochalesin D dissolved in 1 % DMSO (final concentration: 10 µM) for 30 mins at 37 °C in 5 % CO₂. Where appropriate, this media was replaced with 1 ml of bead containing media in which cells were incubated for 2 hours at 37 °C in 5 % CO₂. Cells were then washed and fixed for analysis by flow cytometry as previously described.

For bacterial phagocytosis assays, appropriate dilution of log-growth phase alpha-select E. coli expressing ‘green fluorescent protein’ (GFP) to give a final ‘multiplicity of infection’ (MOI) of 10 was ascertained by the counting of bacterial colonies derived from streaked bacterial cultures on Lennox ‘Luria broth’ (LB) agar (Sigma-Aldrich, USA) plates supplemented with 100 µg/ml ampicillin (Sigma-Aldrich, USA). For full experiments, 0.2x10⁶ cells/well were first added to an 8-well chamber slides and allowed to adhere over night at 37 °C in 5 % CO₂. On the same day, a starter culture of E. coli was inoculated in LB (Sigma-Aldrich, USA) supplemented with 100 µg/ml ampicillin and grown for 17.5 hours at 37 °C with shaking. The next day, 500 µl of E. coli culture was inoculated into 5 ml of fresh LB-amp pre-warmed to 37 °C, and incubated at 37 °C with shaking for 2 hours to reach log-phase growth. E. coli culture was then centrifuged at 5000 g for 10 minutes at room temperature. E. coli were re-suspended in 5 ml of primary growth media. 0.5 ml of this culture in primary growth media was then diluted into a final volume of 10 ml of primary growth media. 2 ml of this media was then diluted into 2 ml of primary growth media supplemented with either 1 % DMSO, or 1 mM cytochalesin D dissolved in 1 % DMSO (final concentration: 10 µM). Before the application of E. coli cultures, pBMDM cells were incubated in primary growth media supplemented with 1 % DMSO or 1 mM cytochalesin D dissolved in 1 % DMSO (final concentration: 10 µM) for 30 mins at 37 °C in 5 % CO₂. Where appropriate, this media was replaced with 400 µl of E. coli culture containing media in which cells were incubated for 2
hours at 37 °C in 5 % CO₂. Cells were then transferred to ice and washed 5x with PBS at 4 °C to remove non-internalised bacteria. Cells were then fixed by incubation in the dark with PBS supplemented with 4 % methanol-free formaldehyde for 15 minutes at room temperature. Cells were washed 3x in PBS, and mounted for imaging using VECTASHIELD antifade mounting medium containing DAPI (Vector Laboratories Ltd, UK). Slides were imaged on an FV1200 confocal microscope (Olympus Corporation, Japan), with quantification of bacteria/cell performed using the ‘find maxima’ function of Fiji for ImageJ (Schindelin et al., 2012; Schindelin et al., 2015).

2.17 Chemotaxis assays

100 µl of cells were added to the membranes of 8 µm polycarbonate membrane tissue culture treated polystyrene Costar transwells (6.5 mm insert) (Corning, USA). Prior to addition to transwells, cells were counted and diluted to an appropriate concentration so that the indicated number of cells will be transferred to transwell membranes as indicated for each experiment. 600 µl of ‘primary growth media’ was added to the well beneath each transwell, and incubated in a humidified chamber at 37 °C in 5 % CO₂ for 2 hours. At this point, transwell inserts were transferred to fresh 12-well tissue culture plates containing either fresh primary cell media or primary cell media supplemented with 100 µM ADP (Sigma-Aldrich, USA) as appropriate. Cells were placed within a humidified chamber and incubated at 37 °C in 5 % CO₂ for the indicated amount of time for each experiment. At the end of the incubation period, transwells were moved to a fresh 12-well tissue culture plate, and media carefully removed from above the transwell membrane. 200 µl PBS was added to the transwell membrane and swabbed with a Q-tip (Johnson & Johnson, USA) to remove cells that have not migrated into the membrane. PBS was again removed from the transwell and replaced with a further 200 µl PBS; this process was repeated 3x before cells within the membrane were fixed by addition of 200 µl methanol-free 4 % formaldehyde in PBS. At the same time, media was carefully removed from the original wells in which transwells were incubated, and replaced directly with 200 µl methanol-free 4 % formaldehyde in PBS. Cells in the transwell membrane and in the tissue culture well were incubated this way for 15 minutes at room temperature in the dark, after which formaldehyde was removed and cells again washed 3x in 200 µl PBS. Cells were then stained with 200 µl of 5 µg/ml Hoechst in
PBS ad imaged by immufluorescent microscopy. Cell counts were performed using the ‘find maxima’ function of Fiji for ImageJ (Schindelin et al., 2012; Schindelin et al., 2015).

2.18 RNA sequencing and transcriptomic data analysis

Bone marrow was isolated from 16-week old female mice housed in the same facility for this study. 3x10^6 cells/well were plated in Greiner 6-well tissue culture plates (Sigma-Aldrich, USA) a day prior to RNA extraction and left to adhere over night at 37 °C in 5 % CO₂. Where appropriate, cells were then treated with 100 ng/ml ultrapure LPS from E. coli O111:B4, or 10 µg/ml MDP. LPS was sonicated prior to application to cells. After 2 hours incubation at 37 °C in 5 % CO₂, cells were washed in PBS, then scraped into PBS at 4 °C. RNA was isolated using RNeasy mini kit in combination with QIAshredder cell homogenization following the manufacturers instructions. To remove genomic DNA, extracted RNA was DNase treated using TURBO DNA-free kit. Resulting RNA was analysed using a Nanodrop 1000 spectrophotometer. Samples with A_{260/230} < 1.8 were further purified with RNeasy MinElute Cleanup Kit (Qiagen, Germany). Samples were then flash frozen in liquid nitrogen, and transferred to ‘Cambridge Genomic Services’ where RNA was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, USA). RNA integrity was verified using 2100 Bioanlyser (Agilent Genomics, USA), and mRNA library preparation was performed using TruSeq Stranded mRNA Library Prep Kit (Illumina, USA) with quality control by 2200 Tapestation (Agilent Genomics, USA). High output sequencing runs of single-end 75 bp read length were performed on NextSeq500 (Illumina, USA) using NextSeq 500/550 High Output v2 Kit (75 cycles) (Illumina, USA). A minimum read depth of 18x10^6 reads per sample was achieved. Read pre-processing, mapping with quality control was performed using a standard pipeline by ‘Cambridge Genomic Services’. Ensembl Mus_musculus.GRCm38.dna.primary_assembly.fa (release 84) reference genome file was used to do the mapping of reads, using the annotated transcripts from the ensembl Mus_musculus.GRCm38.84.gtf. Differential gene expression analysis was performed using DESeq2 (Love et al., 2014). Analysis was performed as a ‘paired comparison experiment’ for each treatment group as comparisons between genotype are made between different samples of different mice (unpaired), while comparisons of treated vs untreated samples are made using samples from the same mice (paired). A target frame and design matrix were adapted from an analogous scenario laid out in the EdgeR user guide section 3.5:
“Comparisons both between and within subjects” (Chen et al., 2008; Robinson et al., 2009). This analysis enabled simple differential gene expression analysis between genotypes, and 2-parameter analysis to compare responses of each genotype to innate immune stimuli by interrogation of a targets frame. This targets frame identifies each sample as belonging to a mouse (mouse.n), and each of these mice as being treated with LPS, MDP, or left untreated (media) (table 6).

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Table 6: Targets frame for samples in DEseq2 analysis

Pathway analysis was performed by calculating gene enrichment against the Reactome database (www.reactome.org) of biological signaling pathways (Joshi-Tope et al., 2005). To explore the data thoroughly, differential gene expression datasets were filtered at multiple levels of statistical significance of differential gene expression before being analysed for pathway enrichment. Datasets filtered for differential gene expression adjusted p value < 0.01, 0.05 and 0.1 were submitted for pathway enrichment analysis using CARD.
(www.card.niaid.nih.gov) (Dutta et al., 2016). RNA sequencing reads were converted for CARD compatibility by Sam Katz (PhD student, Department of Veterinary Medicine, University of Cambridge). Enriched pathways shared between WT and LRRK2 KO macrophages were identified using only pathways identified at differential gene expression adjusted p < 0.01 and pathway enrichment adjusted p < 0.01. To robustly identify genotype specific enriched pathways; pathway enrichment at all three differential gene expression confidence levels was filtered for those pathways that were uniquely statistically significantly enriched in only one LRRK2 genotype: at pathway enrichment adjusted p < 0.05 and/or 0.1. For each treatment, pathways were scored by resilience as to how frequently they were calculated to be statistically significantly enriched across the six configurations of pathway analysis performed.

2.19 Metabolomics

Bone marrow was isolated from 16 week-old female mice housed in the same facility for this study. Macrophages were derived in the previously described method, but from bone marrow frozen at -80 °C after extraction. 1.5x10⁶ cells/well were plated in 12-well tissue culture and left to adhere over night at 37 °C in 5 % CO₂. Where appropriate, cells were then treated with 100 ng/ml ultrapure LPS from E. coli O111:B4, 10 µg/ml MDP, or serum starved by replacement of primary growth media with ‘Earle's Balanced Salt Solution’ (EBSS) (Thermo Fisher Scientific, USA). LPS was sonicated prior to application to cells. After 2 hours incubation at 37 °C in 5 % CO₂, cells were washed in PBS, then high-performance liquid chromatography’ (HPLC) grade methanol (Sigma-Aldrich, USA) was used to precipitate proteins and dissolve lipids. Cells were incubated for 10 minutes at room temperature, before being scraped and transferred to tubes, then vortexed and sonicated thoroughly to achieve a homogenous solution. Samples were then flash frozen in liquid nitrogen, and transferred to Helene Mobbs (PhD student, Dept. Biochemistry, University of Cambridge) for further preparation, mass spec analysis, and peak picking. Briefly, samples were thawed and sonicated for 15 minutes, then centrifuged for 5 minutes at 12,000 g or until the crashed protein formed a pellet. The resulting liquid was transferred and dried under nitrogen. The sample was re-suspended in 50 µl of HPLC grade methanol and sonicated for 5 minutes. 100 µl of HPLC grade water (Sigma-Aldrich, USA) was added and the sample vortexed.
A well described ‘intact lipid method’ was adapted for mass spec analysis of lipids in this study (Ament et al., 2016). This analysis was performed using a Xevo G2 ‘Quadrupole Time of Flight’ (QToF) mass spectrometer with a Z-spray ‘electrospray ionisation’ (ESI) source (Waters Ltd, UK), coupled to an ACQUITY Ultra Performance Liquid Chromatography system with an Acquity CSH C18, 1.7 mm (2.1 x 100 mm) column (Waters Ltd, UK). Precise chromatographic conditions (appendix table 1), and conditions for mass spectrometry (appendix table 2) are described in the appendix. Peak picking was performed using a custom R code described in the thesis of Helene Mobbs (in preparation). This code was originally compiled by Dr Zoe Hall of the lab of Professor Jules Griffin (Dept. Biochemistry, and MRC Human Nutrition Research, University of Cambridge).
3. Expression and purification trials of the LRR domain of LRRK2

3.1 Aims

- Express and purify the LRR domain of LRRK2 in order to carry out biophysical studies
- Optimise the expression and purification of the ‘MBP-LRR6’ construct
- Explore different expression systems and novel construct design for LRR expression

3.2 Background and initial work

From a structural perspective alone, LRRK2 is extremely complex. A catalytic core bearing both GTPase activity and kinase activity is surrounded by a series of repeat structures (fig 12) (Mills et al., 2014). The catalytic core of LRRK2 has been the focus of much attention due to the clustering of disease causing SNPs within these domains, and the fact that enzymatic readouts are a relatively simple method for assessing the functional impact of an amino acid mutation (Corti et al., 2011). However, proteins are three-dimensional structures subject to evolutionary pressures not just within enzymatic domains, but as a complete unit. In spite of this, the repeat structures flanking the catalytic core of LRRK2 have been relatively under-studied. As an example of the importance of non-enzymatic domains of LRRK2, it has been shown that the presence of the WD40 domain adjacent to the kinase domain, at the C-terminus of the protein, is essential to kinase activity (fig 12) (Jorgensen et al., 2009). Human LRRK2 expression in zebrafish has also been used to demonstrate that deletion of the WD40 domain leads to a levodopa responsive locomotor defects (Sheng et al., 2010). This suggests functional significance of the structural domains of LRRK2, and is an illustration of how structure and function are inter-linked. Another example of a structure-function relationship is in the biological significance of ligand binding. Interactions mediated through domains with no inherent enzymatic activity can dictate the localisation of enzymes, as well as substrate recruitment and co-ordination towards enzymatic domains.
The domains either side of the catalytic core of LRRK2 are an LRR domain at the N-terminus of the GTPase domain, and WD40 domain at the C-terminus of the Kinase domain (fig 12). Indeed, every domain outside of the catalytic core is predicted to be a repeat structure. Repeat structures are thought to be beneficial in evolutionary terms, as recombination allows the simple generation of new variants. This is exploited in the evolution of LRR-containing disease resistance genes in plants, through a process known as effector-triggered immunity (DeYoung and Innes, 2006). In comparison to LRR domains, WD40 repeats are relatively stable as they are formed of toroidal folds (simple, non-interleaved super-secondary structure units folded into a closed structure), which are halfway between the extended solenoid structures formed by LRRs, and globular proteins such as kinases; thus generating a ‘happy-medium’ between flexibility and stability (Chaudhuri et al., 2008; Stirnimann et al., 2010).

Given the clear involvement of LRRK2 in inflammatory disease, initial interest was placed in the LRR domain of LRRK2. LRR domains play a fundamental role in the detection of PAMP and DAMP molecules by innate immune proteins best exemplified by the TLR family of PRRs, but are also found on other receptors such as NLR family of PRRs (Botos et al., 2011). The TLR family consists of ten members in humans, with the greatest source of amino acid sequence variability between receptors identified at LRR ectodomains (Barreiro et al., 2009). The LRR domains of these receptors are responsible for ligand binding, therefore, variability between these domains accounts for differential ligand binding between

Figure 12: LRRK2 domain organisation. Boundaries adapted from Corti et al., 2011. LRRK2 catalytic core is highlighted within a red box. ARM = Armadillo repeats, ANK = Ankyrin repeats, LRR = Leucine rich repeats, Roc = Ras of complex proteins, COR = C-terminal of Roc.
receptors that is fundamental to their function. X-ray crystallography allows the building of atomic resolution models of protein structures, and protein-ligand interactions. TLR crystal structures were initially solved of TLR1-TLR2, and TLR2-TLR6 heterodimers (Jin et al., 2007; Kang et al., 2009); as well as a TLR3 homodimer (Liu et al., 2008), and TLR4 homodimer with MD2 co-receptors (Kim et al., 2007). Subsequently, the LRR ectodomains of TLR5 (Yoon et al., 2012), TLR7 (Zhang et al., 2016), TLR8 (TANJI et al., 2013), and TLR9 (Ohto et al., 2015) have been solved largely through use of non-human orthologues of TLRs, as well as the discovery of new VLR fusion technologies for LRR expression (Jin and Lee, 2008). Elucidating the atomic resolution structures of the TLRs has revealed the ligand binding mechanisms, dimerisation interfaces, and, when un-ligated structures are also available, conformational changes of TLRs induced by ligand-binding. With this in mind, it is interesting that PSI-pBLAST analysis identifies bovine TLR3 (uniprot accession: QSTJ59) as having the highest homology of the TLRs to the predicted LRR domain of LRRK2 with an e-value of 5e-7 at the time of writing. The highest homology to a human TLR by the same analysis was again, TLR3 (uniprot accession: O15455) with an e-value of 1e-06. TLR3 is localised to endosomal membranes, and recognises 40-50 base pair lengths of double-stranded RNA (Liu et al., 2008).

LRRs are characterised by stretches of 20-29 amino acid repeats, with a highly variable but leucine-rich 11 amino acid repeat of LxxLxLxxNxL, where ‘L’ is typically leucine but can be substituted for another hydrophobic residue such as isoleucine, valine, or phenylalanine. ‘N’ is classically asparagine, but can also be cysteine, threonine or serine (Enkhbayar et al., 2004). The initial discovery of the structure of the porcine ribonuclease inhibitor in 1993 laid the groundwork for our understanding of LRR domains, as this structure consisted of an extremely regular consensus sequence (Kobe and Deisenhofer, 1993). From the ribonuclease inhibitor structure, it was observed that each repeat forms a structural unit consisting of a β-strand and an α-helix linked by unstructured loops. These repeats stack so that an overall solenoid is formed which curves like a horseshoe. The concave side of this solenoid stacks so that the β-strands of each repeat form a hydrogen-bond stabilised parallel β-sheet (fig 13). Repeated asparagine or cysteine residues form an ‘asparagine ladder’ with continuous hydrogen bonding between backbone carbonyl groups across the length of the LRR solenoid. With the discovery of subsequent LRR containing structures, it has become clear that variability in the helical elements which decorate the
convex side of the solenoid help to determine the curvature of the overall domain. The amount of curvature provided by each repeat depends on the fold of that helix. For example, $3_{10}$ helices have a smaller radius than a classical $\alpha$-helix, and therefore LRRs containing $3_{10}$ helices have lower curvature (Enkhbayar et al., 2004). It is also possible to find short segments of $\beta$-sheet, simple unstructured loops, or even polyproline II helices within this variable region of the LRR structure.

Figure 13: The regular LRRs of the ribonuclease Inhibitor. The Porcine Ribonuclease inhibitor demonstrates extremely regular leucine rich repeats, with a single $\beta$-strand and $\alpha$-helix in each repeat; forming a horseshow shaped super structure. Domains are coloured blue to red from $N$ to $C$-termini of the protein amino acid sequence. PBD code: 2BNH
In order to better understand the fold and ligand binding potential of the LRR domain of LRRK2, efforts have been made to express the domain recombinantly. It was the long-term goal to use protein expressed in this way for ligand binding assays, and crystallisation with a view towards determining an X-ray structure. Initial work towards expressing soluble protein revolved around exploring potential domain boundaries of the LRR domain of LRRK2. As mentioned, LRR folding results in an extended solenoid with hydrophobic residues packed towards a hydrophobic core. The means by which this extended structure is sealed on each side in order to protect the hydrophobic core is highly variable between LRR proteins; but that the core is not exposed is fundamental to LRR stability (Bella et al., 2008). It is thought that exposure of the hydrophobic core of these non-globular domains is at least partly responsible for what makes LRR proteins notoriously difficult to handle. Consequently, early work on LRR protein expression revolved around exploring domain boundaries for LRR construct design in an attempt to obtain a soluble and stable construct (appendix poster: Levy MRes 2013). The Roc domain of LRRK2 remains the only segment of LRRK2 to have been successfully crystallised to date (Deng et al., 2008). This Roc domain lies at the C-terminus of the LRR domain, thus providing a good C-terminal domain boundary of the LRR domain. In comparison, the N-terminus of the LRR domain is disordered with predicted Ankyrin repeats but no confirmed boundary. Interpro classifies the LRR domain of human LRRK2 to begin just prior to residue 1000 of the protein (fig 14a). Uniprot classifies the N-terminal LRR of this domain to begin at residue 983 (table 7).
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Table 7: Uniprot Family & Domains. Taken from ‘Domains and Repeats’ of Human LRRK2 (Q5S007)
**Figure 14: LRR construct design.**

**a:** Interpro visualisation of predicted LRRK2 domains. ARM = Armadillo repeats, ANK = Ankyrin repeats, LRR = Leucine rich repeats, Roc = Ras of complex proteins, COR = C-terminal of Roc. **b:** Schematic of LRR construct design. The construct LRR6 contains an N-terminus at E980, only three amino acids before the predicted LRR domain boundary at Y983 made by Uniprot. The crystal structure of the LRRK2 Roc domain informs the use of a C-terminal domain boundary at M1335 for the LRR6 construct.
As part of an earlier degree, initial success was achieved in the expression of a construct termed LRR6 with an N-terminal boundary at E980, very close to the Uniprot predicted N-terminus of the LRR domain (fig 14b). Expression of the LRR6 construct was trialed in different protein expression systems, with the presence and absence of a ‘tobacco etch virus’ (TEV) protease cleavable ‘maltose binding protein’ (MBP) solubilisation tag (fig 15a). Bacterial and insect cell expression systems were trialed. Specifically, BL21(DE3) and Rosetta(DE3)pLysS strains of *E. coli*, were used, as well as *Spodoptera frugiperda* (Sf9) and Tn5B1-4 (High Fives) insect cell lines. Attempts to express LRR6 in bacterial cell culture resulted in very low expressions levels of protein, which required chaotropic agents such as urea to dissolve. This held true for both BL21(DE3) and Rosetta(DE3)pLysS strains of *E. coli*, suggesting that codon usage was not responsible for poor expression. In comparison, soluble protein was obtained using insect cell expression systems, with Sf9 cells proving most amenable to downstream purification (Table 8). As part of this earlier work, a multi-step purification of Sf9 expressed MBP-LRR6 was described. It was noted that a large amount of protein was lost during anion exchange chromatography, suggesting LRR6 is not amenable to this form of purification (fig 15b). Interestingly however resulting purified protein could be successfully bound to, and eluted from heparin, a negatively charged glycosaminoglycan used for the purification of DNA binding proteins (fig 16). Such binding suggested that the LRR6 construct was correctly folded in spite of poor purification characteristics by anion exchange chromatography, and also reinforced the idea that the LRR domain of LRRK2 may carry out a DNA binding function as suggested by the previously described homology to TLR3 (MRes work summarised in appendix poster). If proven true, such binding would suggest a role for LRRK2 as a PRR of the innate immune system.
Table 8: Expression trials of LRR constructs. (Levy, MRes 2013)

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Figure 15: MBP-LRR6 construct design and purification. a: MBP-LRR6 construct design. MBP is His<sub>6</sub> tagged and can be cleaved from LRR6 construct by TEV protease. b: Summary gel from the optimised purification protocol of the MBP-LRR6 construct. Significant protein losses were observed during anion exchange chromatography. 10% SDS-PAGE, coomassie blue staining (Levy, MRes 2013).
Figure 16: Purified TEV cleaved MBP-LRR6 binds heparin, suggesting DNA binding properties.

3.3 Results

Work performed previously showed promising purification and solubility characteristics for the construct MBP-LRR6 as well as an affinity for heparin, which suggested a potential DNA binding role for the LRR domain of LRRK2 (appendix poster). In order to build upon this work, and progress to biophysical analysis of the LRR domain, it was deemed necessary to generate fresh P1 baculovirus for MBP-LRR6 expression. The initial aim of biophysical work was to reproduce and build upon the previously observed purification characteristics of LRR6. Unfortunately, new batches of the MBP-LRR6 construct did not reproduce the previously observed solubility of the construct. Here, work on the LRR6 construct, as well as the design, test expression, and proof-of-principle purification of novel VLR-capped constructs is described.

3.3.1 Purification trials of baculovirally prepared MBP-LRR6

Fresh P1 baculovirus was generated in an attempt to maximise yield of MBP-LRR6. Protein harvested from Sf9 cells infected with freshly generated MBP-LRR6 baculovirus yielded protein of approximately 80 kDa; the expected molecular mass of the MBP-LRR6 construct. Ni affinity purification was performed, utilising the affinity of the poly-histidine tag on the N-terminus of the protein for nickel ions. Elution of MBP-LRR6 from Ni²⁺ chelating column was optimised to not only increase concentration of Imidazole from 20 mM to 250 mM in order to outcompete the binding interaction between His₆-tagged protein and Ni-chelated column, but also to decrease the concentration of NaCl from 500 mM to 200 mM (fig 17a). This decrease in NaCl concentration was introduced as binding to the column was performed at a high salt concentration, taking advantage of the observation that higher NaCl concentrations decreased sample viscosity. One explanation for this effect is that high salt concentrations may outcompete DNA for interaction with MBP-LRR6. Evaluation of eluted protein by SDS-PAGE analysis verified successful purification of MBP-LRR6, with a concentrated band of protein at the constructs expected of 85 kDa eluted in a single peak (fig 17b). SDS-PAGE also revealed a major contaminant of < 25 kDa was consistently purified away from MBP-LRR6 at low concentrations of NaCl at this stage (fig 17b). These preparations of MBP-LRR6 yielded a total of 2 mg protein per liter of culture at this stage when eluted by this optimised protocol.
Nickel-affinity purified protein was next pooled for application to an anion exchange column. MBP-LRR6 has a theoretical isoelectric point of 5.86, making the protein negatively charged at neutral pH. Pooled protein was diluted into 75 mM NaCl at pH 8 in order to increase the negative charge of the protein, and decrease the competition for binding presented by Cl⁻ ions. Protein was then loaded onto an anion exchange column.
Figure 17: Optimised Ni affinity purification of MBP-LRR6. **a:** Elution profile of 2 L Sf9 prep of MBP-LRR6 on HiTrap Chelating HP 5 ml column. Peaks annotated 1 and 2. **b:** Purification analysed by 10 % SDS-PAGE gel, Coomassie staining.
MBP-LRR6 eluted from the anion exchange column as a broad peak over 30 ml of the linear gradient of NaCl (fig 18a). This behaviour is indicative of heterogeneity in the protein sample, as a range of NaCl concentrations are able to outcompete the ionic interaction between the anion exchange matrix and the charged binding surface of MBP-LRR6. This fact is confirmed by SDS-PAGE analysis, identifying a band of the expected mass of MBP-LRR6 across the broad elution peak, as well as in the tail of the elution peak (fig 18b). This result shows that anion exchange chromatography is not a suitable technique for purification of fresh MBP-LRR6. The ineffectiveness of anion exchange chromatography for MBP-LRR6 purification was observed for protein preparations used here, and also in preparations described in previous work (Levy, MRes 2013). Earlier work at a smaller expression scale had shown affinity of LRR6 for heparin. The extremely tight elution profile of LRR6 in this study suggested that this interaction could be utilised after Ni affinity purification in order to bypass poorly resolving anion exchange chromatography.

Nickel affinity purified MBP-LRR6 was cleaved to its constituent domains of MBP and LRR6 by incubation with TEV protease overnight, then diluted in order to lower NaCl concentration, before being loaded onto heparin. Elution from the heparin column with a gradient of NaCl resulted in very efficient separation of the cleaved MBP tag, which failed to bind heparin (fig 19b). Binding of LRR6 to heparin was observed with a 40 kDa band corresponding to the expected molecular mass of LRR6 detected in eluates by SDS-PAGE (fig 19b). Protein was seen to elute from heparin in three peaks, suggesting the separation of two major species from LRR6 (fig 19a), however SDS-PAGE analysis of these peaks showed protein at the expected mass of LRR6 across all three of these peaks (fig 19b). This behaviour is reminiscent of what was observed by anion exchange chromatography, with LRR6 protein appearing to have a range of different strengths of interaction, again suggesting heterogeneity. Additionally, significant amounts of contaminant bound to heparin as well as the TEV protease, showing that heparin binding is not a specific enough purification technique to replace anion exchange chromatography (fig 19b).
Figure 18: Poor purification of MBP-LRR6 by anion exchange chromatography. a: Broad elution spectrum of Ni affinity purified MBP-LRR6 from HiTrap Q 5 ml column with increasing NaCl concentrations. b: 10% SDS-PAGE gel analysis of eluted protein reveals presence of MBP-LRR6 across the broad peak, Coomassie staining.
One significant variable between early indications of heparin binding and the currently described experiments that may account for the difference in behaviour of LRR6 on heparin is buffer conditions. To investigate this, pooled samples eluted from heparin were dialysed from a Tris buffer at pH 7.5, to a phosphate buffer at the same pH. However, a very similar elution profile to that seen in Tris buffer was observed (fig 19c). Three major elution peaks were present, all containing different contaminants, as well as LRR6 at 40 kDa (fig 19d). It was also clear that the amount of soluble protein was decreasing appreciably with each step in the purification procedure.
Figure 19: Purification of cleaved MBP-LRR6 by heparin affinity. 

a: Elution spectrum of Ni affinity purified MBP + LRR6 from HiTrap Heparin 5 ml column with increasing NaCl concentrations in Tris buffer. 

b: 10% SDS-PAGE gel analysis of eluted protein reveals presence of LRR6 across the spectrum of eluted protein in Tris buffer, Coomassie staining.

c: Elution spectrum of protein purified by HiTrap Heparin 5 ml column with increasing NaCl concentrations in phosphate buffer.

d: 10% SDS-PAGE gel analysis of eluted protein reveals presence of LRR6 across the spectrum of eluted protein in phosphate buffer, Coomassie staining.
To investigate the folding of MBP-LRR6, Ni affinity purified MBP-LRR6 was run on a native PAGE gel to assess basic physical properties of the protein including its size, charge and shape. After two hours, the majority of protein was seen in the well of the gel, giving an indication that the protein has formed an aggregate too large to enter the polyacrylamide matrix (fig 20a). Discrete bands were also seen, possibly demonstrating the existence of oligomeric species of MBP-LRR6. Native gels were cast at pH 9.4, while the theoretical pI of the MBP-LRR6 construct is 5.86, indicating that the effect observed is a product of protein shape and size rather than a charge effect. The sequence of LRR6 shows the presence of nine cysteine residues which could be responsible for aggregation by forming intermolecular disulphide bonds in the oxidizing conditions to which they are exposed following cell lysis. Fresh MBP-LRR6 purified in the presence of 10 mM 2-mercaptoethanol demonstrated that interaction of MBP-LRR6 with the Ni affinity column was indistinguishable from that seen under non-reducing conditions. This MBP-LRR6 isolated under reducing conditions was then run on native PAGE gel to investigate the effect of reducing agent on physical properties of MBP-LRR6. Again, results with MBP-LRR6 isolated under reducing conditions were indistinguishable from that isolated under non-reducing conditions (fig 20b). This indicates that disulphide bonding is not causing MBP-LRR6 to aggregate. It is possible that the protein is partially folded, leading to hydrophobic aggregation.

To gain a better understanding of this protein aggregation, size exclusion chromatography was applied. MBP-LRR6 purified using heparin affinity was seen to elute at 8 ml, corresponding to the expected void volume of the column, indicating that large aggregates of MBP-LRR6 are present (fig 20c). Concentration of MBP-LRR6 by a factor of three increased the proportion of this void volume peak over other components of the gel filtration profile, indicating that the proportion of aggregated protein increases as the concentration of MBP-LRR6 increases. Concentrations of protein were high enough in this sample to verify the presence of 80 kDa MBP-LRR6 in the 8 ml void volume peak by SDS-PAGE (fig 20d). TEV cleavage of the sample overnight incompletely cleaved 80 kDa MBP-LRR6 into 40 kDa and 45 kDa bands, corresponding to the expected masses of LRR6 and MBP respectively (fig 20e). Cleavage caused an increased proportion of an elution peak at 16 ml, identified by SDS-PAGE to be MBP (fig 20f), and a smaller, unresolved peak at 21 ml which could not be identified by SDS-PAGE.
Figure 20: Analysis of MBP-LRR6 physical properties. 

a, b: Coomassie stained native gel analysis of Ni affinity purified MBP-LRR6 isolated under a: non-reducing conditions, b: reducing conditions.

c: Analytical size exclusion analysis of Ni and heparin affinity purified MBP-LRR6 using S200 HiLoad10/300 GL column. Annotated with peak names.

d: 10% SDS-PAGE gel of 3x concentrated MBP-LRR6 after analytical size exclusion, Coomassie staining.

e: TEV cleavage analysis by 10% SDS-PAGE, Coomassie staining.

f: 10% SDS-PAGE gel of TEV cleaved MBP-LRR6 after analytical size exclusion, Coomassie staining.
3.3.2 Expression and Purification of His-LRR6

A His-tagged LRR6 construct was generated in parallel with MBP-LRR6 in order to compare expression and purification characteristics of protein without a bulky MBP fusion tag. Test expression of the construct was promising with a large band at the expected mass for His-LRR6 of 40 kDa detectable by western blotting in harvested pellets of Sf9 cells, from three days after baculoviral infection. Significant cleavage products were also detected at around 35 kDa, and another at low molecular weight (fig 21a). A significant amount of His-LRR6 was also detected in supernatants of harvested Sf9 cultures, indicating that protein was being released from cells lysed following baculoviral infection. A 500 ml prep of Sf9 cells was harvested then protein extracted and purified using the established protocol for MBP-LRR6 Ni affinity purification.

The elution profile for His-LRR6 consisted of a single, extended elution peak (fig 21b). Analysis of the eluted proteins by SDS-PAGE gel show that this peak does not contain His-LRR6 but is a contaminant eluting at low concentrations of imidazole, similar to that seen for MBP-LRR6. Small amounts of His-LRR6 were seen to be eluted at a slightly higher concentration of imidazole as MBP-LRR6 of approximately 125 mM (fig 21c), however the lack of overexpressed, undegraded, soluble protein in this system, meant this construct was not pursued any further.
Figure 21: Expression and purification trials of His-LRR6. 

a: Time course evaluation of His-LRR6 expression in Sf9 cell pellets. Western blot, anti-His<sub>6</sub> primary antibody, 10 s exposure.

b: Elution spectrum of Ni affinity purified His-LRR6 isolated from Sf9 cell pellets MOI: 1 Day 3.

c: 10 % SDS-PAGE gel analysis of eluted protein reveals presence of 40 kDa His-LRR6 after peak of protein elution indicating a lack of overexpressed soluble protein, Coomassie staining.
3.3.3 VLR-LRR Fusion Construct

As discussed previously, one potential cause of protein aggregation is hydrophobic interactions between incorrectly folded proteins. A remarkable development in the expression of LRR proteins was made in 2007 with the generation of fusion proteins between LRRs and the hagfish ‘variable lymphocyte receptor’ (VLR) to form ‘VLR-LRR’ fusion constructs. These fusion constructs have been demonstrated to improve LRR protein expression, solubility and crystallisability (Jin and Lee, 2008b). Such characteristics of fusion proteins have led to the structures of a range of LRR containing proteins being solved, including TLRs 5 and 6 (Jin et al., 2007; Kim et al., 2007; Kang et al., 2009; Yoon et al., 2012). The technique has also been successfully employed in our lab to investigate and solve the prototypical drosophila Toll receptor structure (Gangloff et al., 2013; Lewis et al., 2013).

The highly conserved LxxLxLxxNxL motif of LRR proteins, where ‘L’ is normally leucine, but is commonly replaced by other hydrophobic amino acids, can be used to generate precise fusions of hagfish LRR-containing VLR ‘caps’ to the LRR repeats of LRRK2. N and C-terminal caps from hagfish protein ‘VLR B.61’ have been shown to successfully cap the regular 23-24 amino acid repeats of extracellular LRR containing proteins (Kim et al., 2007; Lewis et al., 2013). As discussed previously, the specific characteristics of each repeat can affect physical properties of the LRR solenoid, for example: longer repeats will have a larger diameter than shorter repeats. This is important as it means different repeats will be capped by the hagfish VLR with different degrees of success. Such design considerations require a more precise understanding of the LRR repeats that make up the LRR domain. Submission of the amino acid sequence of the predicted LRR region of human LRRK2 to ‘LRRfinder’ webserver identified 12 LRR repeats, however it was noted that two of these predicted repeats were far longer than would be expected for a normal LRR protein (table 9). Secondary structure prediction was performed using Genesilico Metaserver2 and the resulting secondary structure predictions were manually scanned for predicted β-strands aligning with amino-acid sequence regions approximating to the LRR consensus sequence of LxxLxLxxNxL (fig 22). Computationally predicted LRR consensus motifs aligned perfectly with predicted β-strands, and two LRR-like motifs were manually detected in regions of unclear secondary structure, potentially explaining why computational methods had not predicted an LRR at these positions (fig 22). Manually assigning these sequences as LRR consensus
motifs results in regularly spaced predicted LRR repeats of between 20 and 29 amino acids, as would be expected for an LRR fold (table 10). These predicted repeats would also maintain a uniform pattern of repeating β-strands and helices that would be expected to form the concave and convex surfaces of the LRR solenoid respectively.

In order to maximise the possibility of generating a successfully capped VLR-LRR construct, different LRR-VLR fusions were generated, each truncating and capping different repeats of the LRR domain. The LRR repeats of LRRK2 contain an irregular repeat at each end of the domain; LRR1 and LRR14 (table 10). LRR1 has a repeat length of 29 amino acids, the very-upper end of what would be expected within an LRR domain, and the LRR consensus motif of LRR14 ends in polar aspartic acid rather than the expected hydrophobic residue. Therefore, constructs were designed to truncate the LRR domain of one, or both of these irregular repeats. Construct VLR1 is designed to cap the full length of the LRR domain, VLR2 truncates the irregular repeat LRR14, and VLR3 truncates both LRR1 and LRR14. Constructs VLR4 was designed to cap the domain between LRR2 and LRR12 - two very regular repeats (table 11). Each VLR fusion construct was cloned into baculoviral and Schneider 2 (S2) cell expression vectors; pFastBac-1 and pMT/V5-His A respectively. Each construct contains an N-terminal ‘receptor for advanced glycation endproducts’ (RAGE) secretion signal, and a C-terminal TEV cleavable Fc-His\textsubscript{6} tag (fig 23).
Table 9: Predicted LRRs. Results of LRR predictor webserver. Identified LxxLxLxxNxL motif highlighted in yellow.

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Figure 22: Manually identified LRR repeats. Metaserver2 alignment of secondary structure predictions of C-terminal half of LRRK2 LRR domain. H = helix, E = β-strand. LxxLxLxxNxL motifs identified by LRRPredictor highlighted in yellow, manually identified LxxLxLxxNxL motifs highlighted in purple.
Table 10: LRRK2 LRR identification. LxxLxLxxNxL sequence annotated and separated from variable loop regions of each repeat. Each repeat is defined by its beta-sheet. * indicates a highly irregular repeat.

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<td>MTILKLSONKF SCIPEAILNLPH</td>
<td>23</td>
</tr>
<tr>
<td>LRR10</td>
<td>LRSLDMSSNDI QYPGPAHWKSLN</td>
<td>24</td>
</tr>
<tr>
<td>LRR11</td>
<td>LRELLFASNQI SIIDLSEKAYLWSR</td>
<td>25</td>
</tr>
<tr>
<td>LRR12</td>
<td>VEKLHLSNKL KEIPPEIGCLEN</td>
<td>23</td>
</tr>
<tr>
<td>LRR13</td>
<td>LTSLDVSYNLE LRSFPNEMGKLSDKIW</td>
<td>27</td>
</tr>
<tr>
<td>LRR14*</td>
<td>LPLDELHNLNF FKHIGCACKDII</td>
<td>23</td>
</tr>
</tbody>
</table>
Table 11: VLR fusion constructs. Four VLR fusions were designed, each truncating individual LRRs as identified in table 10. Fusions were generated after Asn residue of repeat.

<table>
<thead>
<tr>
<th>VLR Fusion Construct Name</th>
<th>LRR Repeats Included in Construct</th>
<th>Full length mass</th>
<th>TEV cleaved mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLR1</td>
<td>LRR1 - LRR14</td>
<td>79.8 kDa</td>
<td>51.8 kDa</td>
</tr>
<tr>
<td>VLR2</td>
<td>LRR1 - LRR13</td>
<td>76.6 kDa</td>
<td>48.6 kDa</td>
</tr>
<tr>
<td>VLR3</td>
<td>LRR2 - LRR13</td>
<td>73.2 kDa</td>
<td>45.2 kDa</td>
</tr>
<tr>
<td>VLR4</td>
<td>LRR2 - LRR12</td>
<td>70.7 kDa</td>
<td>42.7 kDa</td>
</tr>
</tbody>
</table>

Figure 23: VLR fusion construct design. Each LRR domain is fused to N and C terminal VLR caps (VLRN and VLRC), and an N terminal RAGE secretion signal. Fc fusion tag is attached by a TEV cleavable linker. Approximate masses annotated.
Transient expression of VLR-LRR constructs was first trialed in S2 cells, as this expression system does not require the lengthy process of generating baculovirus. A construct termed ‘TollN6-VLR’ was included in expression trials as a positive control (Gangloff et al., 2013), as this protein has been shown to give good expression in the S2 system (Lewis, personal communication). SDS-PAGE gels showed that constructs VLR2, VLR3 and VLR4 gave expression in the S2 system, with VLR3 giving by far the best expression, only slightly less than the TollN6-VLR positive control expression. Lack of expression of the VLR1 construct verifies that the irregularity of LRR14 makes it unsuitable for the applied VLR cap, with remarkable improvement in expression also observed upon truncation of irregular repeat LRR1. Bands for these proteins, detected by western blotting against the Fc tag, were seen to run with an apparent mass of around 150 kDa, running slightly larger, but corresponding approximately to VLR construct dimers of expected mass ranging from 140 - 160 kDa, and 126 kDa TollN6-VLR (fig 24a).

As constructs were observed to run at a slightly higher mass than expected, attention was focused on the TollN6-VLR hybrid construct, which has been successfully expressed previously in the lab. The aim of this experiment was to verify overexpression of the construct, and that detection by western blot was not a high molecular weight background signal detected by the anti-Fc antibody. 2 ml of TollN6-VLR test expression culture was obtained and purified by protein A affinity chromatography, exploiting the Fc tag of the construct. Protein purified on protein A beads was run on SDS-PAGE to reveal a clean band at the expected mass of the TollN6-VLR construct of 63 kDa under reducing conditions (fig 24b). Western blot analysis verified this band to be the TollN6-VLR construct via detection of the Fc tag of the construct (fig 24c). A band corresponding to 28 kDa Fc tag was also detected suggesting proteolytic cleavage of the Fc tag linker of the construct in cell supernatant during incubation. Under non-reducing conditions, a large signal was detected at high molecular mass and lost under reducing conditions for the Protein A purified sample (fig 24c). This signal at high molecular weight at a greater mass than that observed during test expressions (fig 24a). The large molecular weight signal was found in the unbound fraction of protein as well as appearing to be concentrated in the non-reducing bound fraction of protein (fig 24c), however no high molecular weight protein contaminant can be detected by Coomassie staining. This approach demonstrated that TollN6-VLR could be expressed in S2 cells, and purified from supernatant using Protein A beads in a single step.
Figure 24: VLR capped construct test expressions. Western blots probed with anti-Fc primary antibody a: Test expression non-reducing conditions, 15 mins exposure, α-Fc antibody. b, c: Test purification of Toll₆-VLR from test expression supernatant, non-reduced samples except where stated otherwise, b: Coomassie stain. c: Western blot 5 min exposure, α-Fc antibody.
3.4 Discussion

Work described here details two parallel approaches to generate soluble protein of the LRR domain of LRRK2. This includes an approach using a routine domain boundary prediction and optimisation of purification; as well as a VLR fusion approach, which makes use of new VLR-LRR fusion technology. The former of these approaches focused on the construct MBP-LRR6 after promising initial findings (figs 15, 16, appendix poster).

Results obtained from fresh MBP-LRR6 baculovirus mirrored that of previous work in terms of protein solubility and behaviour during nickel chromatography. Predictable behaviour and sharp elution from a nickel chelating column suggests that if protein is aggregating, as suggested during downstream purification and analysis, it may be doing so after initial nickel binding steps. In an attempt to investigate this, nickel affinity purified protein was analysed by native gel electrophoresis, where MBP-LRR6 formed the major component of the constituent protein of the 0.8 mg/ml sample, as determined by SDS-PAGE analysis. This analysis suggested that even immediately after elution from the nickel chelating column, a large aggregate with smaller oligomeric species forms; seen as protein stuck in the well and forming evenly distributed bands below this point. Analytical gel filtration of protein purified by nickel and heparin affinity chromatography confirmed that purified protein is forming a large species that enters the column void volume. The proportion of protein eluting from the void volume was increased by concentration of purified protein, reaffirming that protein aggregation is occurring that is responsive to concentration effects. Analysis by SDS-PAGE of species identified and purified during analytical gel filtration, confirms that the aggregated protein is MBP-LRR6, with a band corresponding to the expected mass of full length MBP-LRR6 that can be TEV cleaved to MBP and LRR6. Protein aggregation explains the poor behaviour of the construct on anion exchange media and heparin chromatography. Instead of aggregation over time, aggregation of MBP-LRR6 may occur from initial protein expression. Such aggregation may have been masked from nickel affinity chromatography by virtue of the fact that nickel binding makes specific use of the His6 tag at the N-terminus of the protein, while subsequent purification methods are reliant on the overall fold of the protein. What is puzzling is that while behaviour on anion exchange media was common between previously prepared MBP-LRR6 and protein generated from fresh baculovirus, only previous
experiments utilising protein generated from old baculovirus performed predictably and cleanly on heparin; eluting as a single species, as a single sharp peak on the heparin elution profile (fig 16). Specific conditions for protein binding to heparin were investigated using dialysis into different buffers with no notable improvement. One variable between old and new MBP-LRR6 preps is the prep scale and initial lysis conditions. As discussed previously, the lysis and purification conditions of new MBP-LRR6 was optimised with higher salt concentrations than had been used previously. This was necessary to decrease total extract viscosity to facilitate loading onto Ni chelating column for initial purification, however it is possible these initial lysis conditions have an effect on the protein; although this is not reflected in the behavior of the proteins during nickel affinity chromatography. The possibility that disulphide linkages between free cysteines was responsible for aggregation, especially after exposure to the oxidizing effect of the Ni rich chelating column was investigated, even though this had not been an issue to obtain heparin binding protein previously. The introduction of the reducing agent, 2-mercaptoethanol, had no noticeable effect on protein yield, or aggregation as analysed by native gel electrophoresis. Ultimately, it would appear that MBP-LRR6 folding suffers from batch variation between preparations; an inherent weakness of baculoviral protein expression in insect cells. Other LRR constructs with an extended N-terminus may prove more stable, and warrant expression trials.

While batch-to-batch variation is an issue with baculoviral protein expression, such an expression system was considered necessary following the limited success of bacterial systems for LRR-containing Toll and TLR construct expression by our lab, and LRRK2 LRR expression by others (Vancraenenbroeck et al., 2012). Protein production strains of *E. coli* are optimised for the generation of large quantities of protein very rapidly, with crude measures such as the reduction of growth temperature used to control this process and allow for efficient folding (Rosano and Ceccarelli, 2014). In contrast, insect cell expression systems make use of higher order cellular quality control mechanisms for protein production, and carry out some post-translational modifications that may be important for folding of LRR6, as is known for other human proteins (Kost et al., 2005). Sf9 and Tn581-4 cell lines require baculoviral transfection, a process that makes insect cell expression significantly less reproducible than bacterial cell expression. On top of this, insect cell doubling time is roughly 24 hours under optimum conditions, compared to 20 minutes for *E.coli*; this makes insect cell expression a much more drawn out process. One method to
limit batch effects for the expression of constructs such as MBP-LRR6 is the use of S2 cells. This consideration was taken into account for the generation of VLR-LRR hybrid constructs. S2 cells are an immortalised drosophila cell line that may be either stably or transiently transfected for inducible protein expression. S2 cells have recently been used for the elucidation of the TLR8 crystal structure (Tanji et al., 2013). Bulk maintenance of stably transfected S2 cultures in selective media does come at considerable expense, and is comparable in methodology to mammalian protein expression in stably transfected HEK293 cells. Expression in HEK293 cells represents a future direction in which LRR expression may be taken, as constructs were cloned from human cDNA, and so may require cofactors, chaperones, post-translational modifications, and folding quality-control mechanisms that are not available even in insect cells. Dependence of LRRK2 on chaperones for stabilisation has been demonstrated via an interaction with ‘heat-shock protein 90’ (HSP90) and 14-3-3 (Wang et al., 2008; Nichols et al., 2010). While the LRR6 construct excludes the S910 and S935 major interaction sites of 14-3-3, a more extended N-terminal domain boundary could be designed to incorporate both of these phosphorylation sites (Stevers et al., 2017). Expression of a construct containing residues S910 and S935 may benefit from co-expression of the 14-3-3 chaperone. A short peptide containing S910 and S935 was sufficient to bind 14-3-3 during in vitro assays, suggesting the absence of S1444 in these constructs may not entirely negate potential stabilising effects of 14-3-3 binding (Stevers et al., 2017). Interaction of LRRK2 has also been shown with ‘heat shock chaperone 70’ (HSC70), a constitutively expressed eukaryotic chaperone, directly involved in protein folding at the ribosome (Young et al., 2004; Orenstein et al., 2013). An alternative approach for LRR protein expression, as proved successful in the expression of TLR ectodomains, is to re-clone the LRR domain of LRRK2 from alternative species. Expression of non-human orthologous LRRK2 may be performed independently, or in combination with use of mammalian protein expression.

A recent molecular model of the full-length LRRK2 protein has been developed through the use of biochemical restraints provided by evidence such as the Roc domain crystal structure (Deng et al., 2008), negative-stain electron microscopy, biochemical crosslinking, homology modelling, and small angle X-ray scattering to predict the organisation and packing of the full length LRRK2 dimer (Guaitoli et al., 2016). Strikingly, this model of LRRK2 has revealed how tightly packed the various domains of LRRK2 are
predicted to be, forming a compact bundle with many domain-domain interactions as opposed to following a ‘bead-on-a-string’ archetype (fig 25). Such a model suggests that small conformational changes in one domain from ligand or protein-binding events could have dramatic effects on LRRK2 function, and that potential protein-binding domains, such as LRR or WD40 domains, can, for example, bring substrates into close proximity for phosphorylation by the kinase domain. This model has subsequently had a level of verification provided by a low-resolution 16 Å electron microscopy study, revealing a similar ‘envelope’ to that which had been modeled. Unfortunately the resolution provided by this study was too low to accurately place individually modeled domains (Sejwal et al., 2017).

Figure 25: Structural model of dimeric LRRK2 model reveals a compact architecture involving distant interdomain contacts. Taken directly from Guaitoli et al., 2016. ARM = Armadillo repeats, ANK = Ankyrin repeats, LRR = Leucine rich repeats, Roc = Ras of complex proteins, COR = C-terminal of Roc, KIN = Kinase.

From a biophysical point of view, the close interactions between domains could help explain difficulties in expressing individual domains for structural analysis. This is because physiologically expressed full-length protein would be expected to be stabilised by interdomain interactions, while ectopically expressed individual domains will not. Indeed, an attempt in 2012 to express the LRR domain of LRRK2 necessitated the use of zwitterionic detergents to overcome aggregation speculated to occur due to exposed hydrophobics of the domain. This detergent, ‘Empigen BB’ formed large micelles to stabilize these exposed hydrophobic patches, but in doing so compromised their ability to perform useful biophysical analysis on the soluble protein obtained (Vancraenenbroeck et al., 2012). Questions must also be raised over the usefulness of protein generated this way, as the need to artificially mask hydrophobic patches does suggest at least a degree of protein misfolding, which would not reflect LRRK2 in its native state. One hindrance of studying a non-
enzymatic protein domain is the inability to experimentally test the function of purified protein to ascertain the correctness of folding. The construct used by Vancraenenbroeck et al was designed with an N-terminus at Y983 of LRRK2; only three residues away from the E980 of the LRR6 construct described in the present study, and a C-terminus lying very close to the predicted 14th LRR of LRRK2 made in this study.

It is possible that MBP-LRR6 as well as the construct of Vancraenenbroeck et al. experienced a lack of solubility through the exposure of the hydrophobic core of the LRR solenoid at the terminal repeats which were not appropriately capped by neighboring domains or secondary structure elements. The use of VLR-capped LRR constructs provides an alternative to trial and error probing of such domain boundary considerations or the use of strong additives such as Empigen BB (Jin and Lee, 2008b). The strength of VLR capping as a technique, is the introduction of a known and well-characterized element to seal the individual LRRs to which the fusion is made. The introduction of a VLR cap is not expected to perturb the 3D architecture of the LRRs to which the VLR has been fused. Evidence for this comes from initial use of VLR fusions in the generation of TLR4, TLR2 and TLR1 hybrids with VLR capping structures. Multiple hybrids were generated of these proteins, using different VLR capping structures. Resulting crystal structures showed very little variation in LRR structure between different hybrids for each TLR molecule (Kim et al., 2007; Jin and Lee, 2008b). VLR hybrids have previously been used to express, solubilise and crystallise LRR domains from extracellular, but not intracellular proteins. Differences in LRR regularity account for varying success of the application of VLRs to LRR-containing proteins. This is reflected in different truncations of the LRRK2 LRR domain by the VLR-LRR constructs described here; as the VLR3 construct, which corresponds to fusion of one of the most regular predicted LRRs, resulted in the greatest protein expression of constructs trialed, while constructs incorporating the highly irregular N-terminal repeat gave almost no protein expression. Further work is now required to build upon this.

While VLR capped constructs may overcome issues with LRR domain boundary prediction, another approach is to attempt full-length protein expression. Such expression is made difficult for extremely large proteins such as LRRK2 by proteolytic degradation, and the need to optimise expression conditions under which the whole protein may be successfully folded. These difficulties of expressing full-length LRRK2 are demonstrated in a
recent study using a mammalian HEK293 expression system (Sejwal et al., 2017). Negative stain transmission electron microscopy revealed that expressed full-length LRRK2 aggregated into globular particles of various sizes without the use of detergents for stabilisation of hydrophobic surfaces, and that even in the presence of stabilising detergent, heterogeneity of LRRK2 protein could still be observed. In spite of these limitations, this study represents a big step forward in the expression of full-length LRRK2. Refinement of this approach may well pave the way for LRRK2 structural studies, especially given recent advances in the generation of high-resolution structures by cryo-electron microscopy of macromolecules (Frank, 2017). Having said this, such results also demonstrate that bulk expression of full length LRRK2 is currently not able to generate protein homogenous enough for biophysical studies. In comparison, expression of full-length LRRK2 as part of ‘standard’ tissue culture experiments typically results in yields incompatible to biochemical and structural analysis techniques such as Biacore ligand binding assays, or X-ray crystallography. These considerations in conjunction with the inherent difficulties associated with the expression of proteins as large as LRRK2 account for the focus paid upon individual domain expression during this project. The on-going establishment of a non-adherent HEK293 cell line, in combination with a WAVE bioreactor will make the mammalian bulk expression of full-length LRRK2 within the lab a viable option for the near future.

Purification of the LRR6 construct proved unsuccessful, however results described here do verify that VLR capped proteins can be successfully expressed and purified in an insect cell expression system. More specifically, careful analysis of the LRRs of the LRRK2 LRR domain has allowed the cloning of VLR capped constructs for insect cell expression. Truncation of irregular repeats found at the extremities of the LRR domain improved expression levels, corresponding with a predicted improvement of fit for the VLR cap to the LRR. These constructs have been cloned into expression vectors for S2 cells, or baculoviral expression in Sf9 cells. Together with the more conventional constructs designed through exploration of LRR domain boundaries; a range of constructs have been generated for the continued expression and purification trials of the LRR domain of LRRK2, and initial steps towards protein characterisation of protein expression have been taken. These constructs and the LRR characterisation that has gone into the design of constructs will facilitate progress towards physical analysis of the LRR domain of LRRK2.
4. Investigating the role of LRRK2 in immune receptor trafficking and cytoskeletal dependent processes using primary macrophages

4.1 Aims

- Characterise primary WT and LRRK2 KO bone marrow derived macrophages for LRRK2 expression and innate immune inducibility.
- Assess the role of LRRK2 on expression and trafficking of TLR4 and TLR2.
- Utilise LRRK2 KO macrophages to clarify the role of LRRK2 on phagocytosis, in a range of innate immune contexts.

4.2 Background

LRRK2 is expressed in both cells of the innate immune system and the nervous system (Maekawa et al., 2010). There is a growing appreciation of the overlap between the functions of these two systems, which have historically been considered to be largely separated; a concept termed ‘immune privilege’. It is now known that both resident immune cells in the brain, and infiltrating immune cells circulating from the blood play a role in moulding the brain in health, but also in disease (Wolf et al., 2017).

The fact that LRRK2 mutations are a genetic cause of Parkinson’s disease, and that the protein has apparent functions in both a neuronal and immunological context, makes it a particularly interesting case study of neuroinflammation. That said, the wide role of LRRK2 may also be confounding; many functions of LRRK2 have been described, but it is unlikely that all of these are truly the route of LRRK2 mutation pathogenicity – so are mutations of LRRK2 more relevant to Parkinson’s disease pathology in an inflammatory or neuronal context? The discovery that the commonly used LRRK2 kinase inhibitor, L2in1, is highly non-specific and inhibited off-target innate immune signalling proteins such as ERK5 suggested that disease relevance may be found in neuronal cells; where many described LRRK2 functions still hold true (Luerman et al., 2014). That said, interesting immunological effects of LRRK2 have subsequently been described that are independent of L2in1, many of
which have been discussed previously. Work described here attempts to explore ways in which LRRK2 interactions and cellular effects discovered biochemically or in a neuronal cell context may be relevant to immunological processes. This was achieved by investigating the role of LRRK2 in immune receptor trafficking and phagocytosis.

**LRRK2 regulates vesicular trafficking in neurons**

One reported function of LRRK2 is in the process of vesicular trafficking. As discussed previously, neuronal morphology and functional specialisation places extreme demands on many aspects of cell biology. Such demands include vesicular trafficking, as proteins and lipids generated at the cell body require transport along extremely extended neuronal axons, to synapses at the cell periphery (Millecamps and Julien, 2013). The strongest link between LRRK2 and vesicular trafficking may lie in the process of autophagy. Autophagy is a process that is fundamentally reliant on a functional vesicular trafficking system; with the autophagosome essentially a highly specialised vesicle optimised for protein degradation. The formation of the autophagosome relies upon maturation processes involving the initial nucleation of a phagophore, elongation around a target molecule, and targeted delivery of endosomal and lysosomal vesicles during maturation (Glick et al., 2010). LRRK2 has been associated with changes in autophagic markers, as well as resulting substrate degradation and neuronal morphological changes; these processes have been discussed in detail previously (Herzig et al., 2011; Sánchez-Danés et al., 2012; Tong et al., 2012). Here LRRK2 interactions that modulate the trafficking events that underpin autophagy and vesicular transport as a whole will be discussed.

Vesicular trafficking is regulated by small GTPases known as Rab proteins. Rab GTPases attach to vesicular membranes by a covalently bonded lipid tail, and modulate the trafficking of the vesicle to which they are attached. Combinations of Rab proteins attach to vesicles over time, resulting in the programming of a path through which that vesicle will travel. For example, early endosomes expressing Rab5 can mature into Rab7 expressing late endosomes, which, in the context of autophagy, may fuse with the autophagosome, or may be recycled to the golgi in a
process involving Rab9 and the retromer complex (Lombardi et al., 1993; Vieira et al., 2003). In the other direction, Rab8 is involved in regulating vesicle transport from the golgi network to the plasma membrane (Huber et al., 1993). There are over 60 known members of the Rab family of GTPases, demonstrating the overall complexity of this process (Stenmark, 2009). LRRK2 was initially shown to interact with Rab5 (Shin et al., 2008; Yun et al., 2015), then later Rab32 was identified as an interaction partner (Waschbüsch et al., 2014). Subsequently, interactions have been shown with a whole range of Rab proteins. A phosphoproteomic screen has identified a subset of Rabs as LRRK2 ligands. Phosphorylation of these Rabs was increased by the G2019S pathogenic mutation of LRRK2 (Steger et al., 2016). Identified Rabs in this study included Rab10, Rab8a, Rab1a and Rab1b which each contain a conserved Thr73 phosphorylation site at the GTPase switch II region for LRRK2 phosphorylation. Rabs with a serine residue at this switch II region were less efficiently phosphorylated; a finding which is in line with previous reports of LRRK2 substrate preference for threonine residues (Kamikawaji et al., 2009; Webber et al., 2011). Rab7L1 has also been identified as a further LRRK2 interaction partner (Beilina et al., 2014). This is interesting as Rab7L1 has been identified as a risk factor for Parkinson’s disease independently of LRRK2; suggesting the possibility of a common pathogenic mechanism (Nalls et al., 2014). Furthermore, deficiency of Rab7L1 or LRRK2 leads to common golgi sorting defects, and a deficiency of VPS35; which can otherwise be immunoprecipitated with LRRK2 (MacLeod et al., 2013). VPS35 is a component of the retromer complex, involved in endosomal/golgi sorting, and has been linked to a rare form of Parkinson’s disease independently of LRRK2 (Vilariño-Güell et al., 2011; Zimprich et al., 2011; Zavodszky et al., 2014). The LRRK2-Rab interactions described above have been linked to perturbations of autophagy (Beilina et al., 2014; Kuwahara et al., 2016) synaptic vesicle endocytosis (Shin et al., 2008; Arranz et al., 2015), neuron axonal elongation (Kuwahara et al., 2016), and the retromer protein-sorting pathway (MacLeod et al., 2013).

A further connection between LRRK2 and vesicular dynamics exists in EndophilinA. LRRK2 has been shown phosphorylate EndophilinA at S75, a residue thought to be important for EndophilinA interaction with the cell membrane.
EndophilinA is enriched at pre-synaptic terminals, where its phosphorylation is thought to induce or detect membrane curvature, and drive vesicular formation. Either an increase or a decrease in EndophilinA S75 phosphorylation by LRRK2 is thought to adversely affect vesicle formation and synaptic endocytosis (Matta et al., 2012; Arranz et al., 2015). Interestingly, EndophilinA S75 phosphorylation by LRRK2 has subsequently been shown to induce not just endocytosis, but also macroautophagy by allowing the docking of autophagic proteins at presynaptic terminal membranes curved by EndophilinA (Soukup et al., 2016).

**TLR localisation is regulated by trafficking events**

Vesicular trafficking is important in all cell types, including those of the innate immune system. For instance, one level of regulation of TLR signalling is receptor localisation and trafficking. At the most basic level TLRs 1, 2, 4, 5 and 6 are found at the cell surface for the detection of extracellular bacterial PAMPs (Gay et al., 2014), while TLRs 3, 7, 8, and 9 are localised to the endosomal compartment for the recognition of nucleic acids (Gay et al., 2014). Of these, work here focuses on TLR4 and TLR2.

TLR4 is localised at the cell membrane as a heterodimer with MD2, and is internalised to the endosome upon ligand binding (Akira and Takeda, 2004). At the cell surface, TLR4 signals through the signalling adaptor proteins Myd88 and ‘MyD88-adapter-like’ (MAL). Within the endosomal compartment, signalling is achieved through the ‘TIR domain-containing adaptor protein inducing IFNβ’ (TRIF) adaptor protein. The result of this is that changes of TLR4 localisation affect downstream signalling, with immunological implications (Kagan et al., 2008). The trafficking of TLR4 in particular has been of particular interest since the characterisation of ‘transmembrane emp24 domain-containing protein 7’ (TMED7) as an adaptor for TLR4 packaging at the endoplasmic reticulum, for trafficking towards the cell membrane via the golgi apparatus (Liaunardy-Jopeace et al., 2014). Termination of TLR4 signalling is achieved by internalisation of receptor complexes into lysosomes for degradation upon ligand binding (Husebye et al., 2006). Rab10, a reported substrate for LRRK2, regulates trafficking of TLR4 from the golgi to the
plasma membrane; an important process required to replenish surface TLR4 levels (Wang et al., 2010). Rab11a has been shown to be fundamental to TLR4 trafficking dynamics, in particular for localisation of TLR4 to *E. coli* phagosomes, and the resulting ‘interferon regulatory factor 3’ (IRF3) signalling with downstream interferon-β induction (Husebye et al., 2010). CD14, a pattern recognition receptor for LPS, is also implicated in regulating TLR4 endocytosis, and IRF3 activation (Zanoni et al., 2011). It is interesting to note that Rab8a, another reported LRRK2 ligand, has been suggested to regulate Phosphoinositide 3-kinase (PI3K)/Akt signalling induced by cell surface localised TLR4 (Luo et al., 2014). These examples demonstrate a pattern recognition receptor modulating TLR4 trafficking, while a Rab GTPase modulates TLR4 signalling directly from the cell surface; suggesting TLR signalling and localisation are intimately linked (fig 26).
**Figure 26: Simplified scheme of TLR4 vesicular trafficking and implications for inflammatory signalling.** TLR4 signals via Mal/Myd88 at the cell surface, and TRAM/TRIF in endosomal compartments. Trafficking of TLR4 is regulated by Rab proteins, with implications on inflammatory signalling, but also signal termination via proteosomal degradation of TLR4 complexes, and replenishment of TLR4 to the cell surface.
In comparison to TLR4, the dynamics of TLR2 trafficking are little understood. TLR2 can form functional heterodimers with TLR1 and TLR6, and interacts with a variety of non-TLR accessory proteins that influence ligand recognition. Overall, these factors contribute to an exceptionally broad TLR2 ligand specificity. Lipoproteins and lipopeptides are considered canonical TLR2 ligands, however a plethora of further proteins and polysaccharides have also been described (Oliveira-Nascimento et al., 2012). TLR2 is classically considered to signal from the cell surface, however internalisation and trafficking of TLR1/2 and TLR2/6 heterodimers to the golgi has been shown to occur in response to binding of triacylated lipoproteins, or either of lipoteichoic acid (LTA) or diacylated lipoproteins respectively (Triantafilou et al., 2006). Therefore, there is some similarity between TLR2 and TLR4 dynamic trafficking (Latz et al., 2002). For TLR2 however, evidence suggests that internalisation is not required for TLR2 signalling (Triantafilou et al., 2004; Triantafilou et al., 2006). Indeed, recent evidence suggests that while TLR2 heterodimers are endocytosed after binding LTA or bacteria, this process is actually inhibitory to TLR2 signalling (Nilsen et al., 2008). Of particular interest to neuroinflammation and Parkinson’s disease, the inflammatory response to alpha-synuclein oligomers is thought to be mediated through TLR1/2 heterodimers (Daniele et al., 2015), with amelioration of inflammation evident in TLR2 knock down animals (Kim et al., 2014). It has also been observed that TLR2 inhibition can prevent alpha-synuclein accumulation by promoting autophagy, and clearance of alpha-synuclein aggregates (Kim et al., 2015).

To summarise, there is extensive involvement of LRRK2 in modulating the cellular trafficking machinery, in particular via interactions with a range of Rab proteins. This machinery is involved in the trafficking of TLRs generally, but also the highly dynamic trafficking of TLR4 in response to ligand recognition that occurs as part of the macrophage innate immune response. TLR2 similarly undergoes trafficking upon ligand recognition as part of a less well-characterised process. It stands to reason that LRRK2 may modulate TLR localisation and dynamics through interactions with the cellular trafficking machinery.
**LRRK2 regulates the cytoskeleton and cytoskeletal dynamics in neurons**

An extensively documented function of LRRK2 in neurons is the regulation of axonal elongation and axonal branching. Perturbation of this process is thought to be neurotoxic due to the extreme energetic demands placed in particular on dopaminergic neurons (Cavanagh, 1984; Bolam and Pissadaki, 2012). Axonal elongation and cell morphology more generally are dependent on the cell cytoskeleton, a network consisting of three elements: intermediate filaments, microtubules, and actin (Fletcher and Mullins, 2010). Neurofilaments are a class of intermediate filaments in neurons in which microtubules are embedded. It is along these microtubules that the majority of vesicular transport proceeds. Advances in super-resolution microscopy have revealed that regularly spaced rings of actin exist along the length of the axon, linked by the protein adaptor, spectrin (Zhong et al., 2014). These structures are relatively static, providing a physical framework for neurons. During neurogenesis, dynamic actin structures are responsible for continual guidance of the extending neuronal axon in response to environmental cues (Strasser et al., 2004). A further neuronal phenotype of LRRK2 perturbation can be seen in a reduction in the number of dendritic spines in neurons expressing G2019S LRRK2 (Winner et al., 2011; Häbig et al., 2013). Dendritic spines form the postsynaptic part of most excitatory synapses, and like the extending axon, are primarily composed of highly dynamic actin. Mechanisms of actin regulation are described as “integral to the formation, maturation, and plasticity of dendritic spines and to learning and memory” (Hotulainen and Hoogenraad, 2010). That perturbations to the actin of dendritic spines alone affects so many neuronal and behavioural properties demonstrates how important the interactions of LRRK2 with the cytoskeleton may be. LRRK2 has been shown to interact both directly and indirectly with the cellular cytoskeleton.

Direct interactions have been shown between LRRK2 and microtubules, facilitated by the Roc domain of LRRK2 (Gandhi et al., 2008). Parkinson’s disease associated mutant forms of LRRK2 have been described as preferentially forming filamentous structures linked to microtubules; interestingly the commonly described G2019S mutant was an exception to this (Kett et al., 2012). Later work has found
that genetic knockout of LRRK2 led to increased microtubule acetylation, resulting in neuronal growth cone defects (Law et al., 2014). In another study, LRRK2 containing GTPase mutations showed increased association with deacetylated microtubules, and associated deficits in axonal transport. Acetylation of microtubules via inhibition or genetic knockdown of cellular deacetylases was sufficient to rescue both LRRK2 microtubule association, and axonal transport (Godena et al., 2014). β-tubulin was also found to be phosphorylated by LRRK2, leading to microtubule stabilisation (Gillardon, 2009). Further LRRK2 associations exist with the cytoskeleton through interaction with cytoskeletal adaptor proteins. Tau decorates microtubules under homeostatic conditions, resulting in a microtubule stabilising effect and enhanced axonal transport. Tau can be found to be hyper-phosphorylated and associated with Lewy body pathology during Parkinson’s disease (Lei et al., 2010). Multiple reports suggest LRRK2 contributes to Tau hyper-phosphorylation (Kawakami et al., 2012; Bailey et al., 2013) while also promoting accumulation and aggregation of Tau into toxic protein inclusions (MacLeod et al., 2006; Guerreiro et al., 2016). A further report suggests phosphorylation of Tau is promoted by the G2019S over-active kinase mutant of LRRK2 via GSK3β, but did not find evidence showing Tau to be directly phosphorylated by LRRK2 (Lin et al., 2010).

In contrast to direct microtubule interactions, LRRK2 influences actin dynamics indirectly, through a number of actin interacting proteins and dynamic regulators. The ERM proteins, Ezrin, Moesin and Radixin, have been discussed previously as substrates for LRRK2, and are the basis for the generation of ‘LRRKtide’, an artificial substrate for LRRK2 (Jaleel et al., 2007). The ERM family of proteins are phosphorylated by LRRK2 at a conserved residue responsible for the ability to bind F-actin (Arpin et al., 2011). Therefore, LRRK2 is considered a modulator of ERM protein function by affecting physical association of the actin cytoskeleton with the plasma membrane. ERM proteins are found at actin-rich filopodia, where they control actin dynamics within the filopodia, and thus modulate neurite outgrowth (Paglini et al., 1998). The G2019S over-active kinase mutation of LRRK2 increases ERM protein association with F-actin, and inhibits neurite outgrowth, while LRRK2 KO achieved the opposite effect (Parisiadou et al., 2009). LRRK2 also binds strongly to the Rho
GTPase Rac1, and more weakly to Cdc42, resulting in activation of downstream processes (Chan et al., 2011). Rho GTPases activate signalling cascades beginning with the ‘p-21 activated kinase’ (PAK) family, which result in modulation of the actin cytoskeleton. For example, downstream activation of the MAPK proteins ERK1 and ERK2 modulate actin polymerisation and cell spreading (Smith et al., 2008). While activation of LIM kinase acts to inhibit the action of the cytoskeletonally associated protein coflin, ultimately blocking actin depolymerisation and leading to membrane ruffles (Edwards et al., 1999). The interaction between Rho GTPase proteins and LRRK2 is affected by disease causing mutations of LRRK2, with the G2019S mutation weakening the interaction and activation of Rac1. Co-expression of Rac1 with G2019S LRRK2 was sufficient to rescue G2019S-LRRK2 induced neurite shortening in SH-SY5Y cultures, demonstrating relevance to neuronal toxicity (Chan et al., 2011). Another group has demonstrated the opposite relationship between LRRK2 and Rac1, with knockout of LRRK2 resulting in Rac1 activation, perturbing neuronal branching and dendritic spine prevalence (Schreij et al., 2015). In spite of phenotypic effects of these associations, LRRK2 is not thought to directly phosphorylate Rho GTPases (Moehle et al., 2015). A direct association of LRRK2 with PAK6 has also been demonstrated (Civiero et al., 2015). Finally, the only guanine nucleotide exchange factor (GEF) of LRRK2 reported so far is ARHGEF7 (Haebig et al., 2010). Interaction between ARHGEF7 and LRRK2 was perturbed by R1141C mutation. An interaction between LRRK2 and Cdc42 was also reported in this study. ARHGEF7 acts upon Rac1 and Cdc42, catalysing the release of GDP for GTP, as well as interacting with downstream PAK kinases. Knockout of LRRK2 leads to increased branching and an enhanced number of neuronal growth cones, along with mislocalisation of ARHGEF7 within those growth cones (Häbig et al., 2013).

The cytoskeleton is vital to many aspects of innate immune cell function

The direct interaction of LRRK2 with microtubules, and interaction with multiple levels of actin dynamic regulators strengthens the idea that regulation of cytoskeletal dynamics may be a primary physiological role of LRRK2. As with vesicular trafficking however, the importance of a regulated cytoskeleton is not limited to a neuronal context.
Cytoskeletal dynamics underpin many of the functions of innate immune cells, from migration to sites of inflammation, to active processes such as phagocytosis. Macrophages and monocyte precursors are highly motile cells, travelling long distances in the blood and within tissues. Within tissues, macrophages sense environmental cues, resulting in efficient migration to sources of inflammation and disease (Shi and Pamer, 2011). This high level of motility of macrophages is in stark contrast to what is seen in neuronal cells, where after neurogenesis and motility of the growth cone, the physical position of axons is fixed (Ghashghaei et al., 2007). Motility of cells is chiefly governed by dynamic changes of the cytoskeleton. The actin cytoskeleton plays a prominent role at the leading edge of motile cells. Lamellipodia are thin sheet-like structures formed of a network of highly branched actin filaments. This branching pattern is facilitated by the actin-related protein-2/3 (ARP2/3) complex, which nucleates actin polymerisation from an existing actin fibre in conjunction with the WASP/WAVE family of proteins (Machesky et al., 1999; Takenawa and Suetsugu, 2007). While both filopodia and lamellipodia are structures formed of actin, lamellipodia are formed of thin actin sheets, while actin in filopodia forms tight parallel bundles that push against the cell membrane to produce finger-like protrusions (Fletcher and Mullins, 2010). Filopodia and lamellipodia are linked, as filopodia are found embedded within, and protruding from, the branched lamellipodial actin network (Svitkina et al., 2003). The extended nature of filopodia allow macrophages to probe the environment for pathogens and chemoattractant gradients; a function enhanced by the enrichment of extracellular receptors within these structures (Mattila and Lappalainen, 2008). Actin structures and dynamics are therefore functionally involved in the innate immune response by exerting a level of control upon macrophage migration and environmental sensing.

Filopodia are also involved in the active process of phagocytosis; a process by which extracellular material is taken up by a cell. Phagocytosis facilitates macrophage killing of extracellular pathogens, and clearance of cellular debris. Debris clearance in particular bears importance in the context of degenerative disease such as Parkinson’s, where reactive microgliosis and immunogenic protein aggregate formation correlate with pathology. Phagocytosis at face value appears to
be a simple process of particle engulfment, however massive coordination of cellular machinery is in-fact involved (Niedergang and Chavrier, 2004; Swanson, 2008). Mechanically, filopodia-like extensions are required to extend around particles to be engulfed as part of this process, and a phagocytic cup is formed underneath material to be phagocytosed. This phagocytic cup is a structure formed by actin rearrangement that induces membrane curvature, and excludes membrane proteins at the site of contact with material to be phagocytosed (Lee et al., 2007). Formation of membrane structures for phagocytosis can be triggered by activation of receptors, and in particular Fc-receptors, at the cell surface. Receptor engagement results in signalling to trigger actin rearrangement. Such signalling involves Rho GTPases including Rac1 and Cdc42; interaction partners of LRRK2 (Beemiller et al., 2010; Rougerie et al., 2013). Cdc42 regulates the induction of the ARP2/3 complex, classically through activation of WASP; leading to the formation of lamellipodia sheets, and filopodia protrusions (Nobes and Hall, 1995; Park and Cox, 2009). Rac1 signalling overlaps with that of Cdc42, however during Fcy receptor-mediated phagocytosis, Rac1 is distributed differentially to Cdc42. That is, Rac1 is present throughout the phagocytic cup, while Cdc42 is restricted to the leading edge of the cell (Hoppe and Swanson, 2004). This suggests distinct roles of Rho GTPase proteins during phagocytosis, and demonstrates an intricacy in Rho GTPase signalling that has immunological importance (fig 27). Phagocytosis could therefore be affected by perturbation of LRRK2 interaction with Rho GTPases. Interestingly, Rac1 activation

![Diagram](image.png)

**Figure 27:** Phagocytosis by microglia is important in both health and disease.
can be stimulated by the growth of microtubules, resulting in the formation of lamellipodia (Waterman-Storer et al., 1999). This highlights the interconnectivity between the microtubule and actin networks which are often studied in isolation (Etienne-Manneville, 2004).

The role of LRRK2 in phagocytosis is confused by conflicting data. Early reports of LRRK2 function in macrophages using the inhibitor L2in1 suggested a direct role for LRRK2 in phagocytosis. These experiments used the HIV tat protein to stimulate BV-2 immortalised microglia to phagocytose latex beads or neuronal axons (Marker et al., 2012). In comparison, a more recent report comparing phagocytosis of zymosan-coated beads by thioglycolate-elicited primary macrophages (overexpressing WT or G2019S mutant LRRK2) showed no effect of the G2019S LRRK2 mutation on bead phagocytosis (Moehle et al., 2015). ShRNA was used to knock down LRRK2 in RAW264.7 cells by a further group. Results from this experiment indicated that LRRK2 did not affect phagocytosis of beads stimulated by extensive LPS treatment (Schapansky et al., 2014). Primary macrophages derived from LRRK2 KO mice are a better model to definitively test the role of LRRK2 in phagocytosis owing to the specific nature of using a genetic model compared to chemical inhibitors, and knockout of LRRK2 compared to knock down. Further to this, extraction of primary cells by thioglycolate has been suggested to lead to physiological changes in macrophages that may perturb their physiology (Hoover and Nacy, 1984; Ray and Dittel, 2010). This is similarly true for cell lines such as BV-2 or RAW264.3 cells which have undergone an immortalisation process. pBMDM cells are routinely used as a ‘gold standard’ model for mouse macrophages, as they undergo a reproducible maturation process from bone marrow, analogous to haematopoiesis (Weischenfeldt and Porse, 2008).

**Summary**

Neuronal processes such as axonal transport and neurite extension, as well as autophagy, are perturbed by LRRK2 knockout or pathogenic mutation. Direct and indirect interactions between LRRK2 and a range of cellular machinery including Rab proteins and cytoskeletal dynamic regulators are thought to underpin the role of LRRK2 in these processes. Many innate immune cell functions rely upon the same
cellular machinery that LRRK2 has been shown to interact with in neurons and other cell types. Work detailed in this chapter is performed using macrophages, and aims to explore the role of LRRK2 in such processes. A focus is placed upon the expression and trafficking of innate immune receptors, as well as with the process of phagocytosis. Experiments to study the involvement of LRRK2 in innate immune receptor trafficking are novel, while studies of the role of LRRK2 in phagocytosis are designed to clarify, and build upon conflicting existing data in the LRRK2 literature.

4.3 Results

4.3.1 Effective knockout of LRRK2 in primary macrophages derived from B6.129X1(FVB)-Lrrk2tm1.1Cai/J mice

To investigate the role of LRRK2 in innate immunity, a genetic system was employed utilising LRRK2 KO mice. The use of this system is key to overcoming limitations of some previous work in the field, which utilised the non-specific LRRK2 inhibitor L2in1 (Deng et al., 2011). Heterozygous B6.129X1(FVB)-Lrrk2tm1.1Cai/J were bred, and offspring genotyped to obtain a homozygous mating pair from which LRRK2 KO mice could be derived (fig 28a). Bone marrow from homozygous B6.129X1(FVB)-Lrrk2tm1.1Cai/J mice was obtained and differentiated into primary bone marrow derived macrophages (pBMDM) for these experiments. The LRRK2 gene is not removed in B6.129X1(FVB)-Lrrk2tm1.1Cai/J mice, but rather interrupted by specific excision of exon 2, resulting in a premature stop codon in exon 3 of LRRK2 mRNA (fig 28b) (Parisiadou et al., 2009). LRRK2 mRNA containing a premature stop codon will be degraded by nonsense-mediated decay at the ribosome, this is reflected by a reduction of 49 % in LRRK2 mRNA levels in macrophages derived from B6.129X1(FVB)-Lrrk2tm1.1Cai/J mice when compared to WT mice as measured by qRT-PCR (fig 28c). Therefore mRNA levels verify that the LRRK2 gene has been perturbed successfully in LRRK2 KO pBMDMs.
Figure 28: LRRK2 knockout in B6.129X1(FVB)-Lrrk2tm1.1Cai/J mice. 

a: Representative genotyping PCR result. Ladder sizes in base pairs (bp) indicated in white text. Expected results: WT = 145 bp, LRRK2 KO = 200 bp, Heterozygote = both. b: Simplified schematic of LRRK2 knockout by the generation of a premature stop codon within LRRK2 exon 3 (Parisiadou et al., 2009). Resulting mRNA will be degraded by nonsense-mediated decay. c: Decreased LRRK2 expression in pBMDMs derived from B6.129X1(FVB)-Lrrk2tm1.1Cai/J mice. qRT-PCR with beta-actin and GAPDH as housekeeping genes. Error bars are S.E.M. of three independent experiments.
4.3.2 LRRK2 transcription is responsive to inflammatory stimuli in pBMDM cells

Having established successful knockout of LRRK2 in pBMDM cells, the transcription of LRRK2 in WT macrophages was next investigated in response to inflammatory stimuli. WT macrophages were incubated with a range of inflammatory stimuli; including LPS, Ifn-γ, MDP and αSyn oligos. After two hours of stimulation, RNA extraction and qRT-PCR analysis was performed. An increase of over two-fold was observed upon treatment with LPS, and more modest 1.5-fold changes upon stimulation with Ifn-γ or αSyn oligos (fig 29). While the LRRK2 transcriptional response to LPS was greatest, it was also the most variable. A subsequent RNA sequencing experiment confirmed 3.5-fold induction of LRRK2 upon LPS stimulation. An MDP treatment was included as, despite several functional connections to LRRK2, there is no existing data for the effect of NOD2 activation on LRRK2 expression. αSyn oligos were similarly tested as there is joint involvement of LRRK2 and alpha-synuclein in many cellular processes: including inflammation, protein transport, mitochondrial function, autophagy, and proteosomal degradation (Liu et al., 2012). Neither MDP nor αSyn oligos had a noticeable effect on LRRK2 transcription at two hours (fig 29). These results indicate that LRRK2 transcription is affected by specific perturbations to the innate immune system in pBMDMs, but that this responsiveness is modest.

Figure 29: LRRK2 transcript levels upon innate immune activation. qRT-PCR with beta-actin and GAPDH as housekeeping genes. Errors bars are S.E.M. of three independent experiments for LPS, Ifn-γ and MDP. Two independent experiments for αSyn oligos. paired two-tailed t-tests compared to a value of 1.
4.3.3 LRRK2 has no obvious effect on cytokine secretion in response to inflammatory stimuli

Given that LRRK2 transcription is responsive to inflammatory stimuli, a bead array was performed in order to measure the secretion of inflammatory cytokines in response to inflammatory stimuli. LPS, IFN-γ, MDP and αSyn oligos were again used to stimulate WT and LRRK2 KO macrophages for 2 hours, 6 hours and 24 hours. At these time points supernatants were removed and assayed for inflammatory cytokine content. Although results were from a single experiment, it was noted that no obvious differences were observed between WT and LRRK2 KO macrophages, in line with previous reports (fig 30). Specifically, of the inflammatory stimuli applied, only LPS and αSyn oligos led to a notable increase in cytokine secretion by macrophages, with Ifn-γ and MDP stimulated macrophages resulting in only slightly increased levels of cytokine secretion compared to unstimulated macrophages, a baseline of 200 pg/ml was ascertained from such unstimulated cells. One exception to this is at 24 hours, where Ifn-γ stimulation resulted in a slight increase in TNFα secretion, but no obvious difference between WT and LRRK2 KO macrophages. LPS stimulation resulted in increased secretion of Il-1α, Il-6, Il-10 and TNFα especially at 6 and 24 hour time points. Again, no differences were observed between LRRK2 genotypes that was consistent across multiple time points. Similar observations were made upon stimulation with αSyn oligos with cytokine secretion peaking at 6 hours and remaining elevated by 24 hours, with no obvious LRRK2 genotype dependent differences observed. After 2 hours of stimulation with αSyn oligos, only TNFα secretion was seen to be elevated, with secretion apparently elevated in WT macrophages compared to LRRK2 KO equivalent cells with 380 pg/ml detected in WT supernatants compared to 170 pg/ml in those of LRRK2 KO cells (fig 30). This observation was time point specific however, and was not observed at 6 hours, when TNFα levels were much more significantly elevated, at 20,000 pg/ml.
Figure 30: Preliminary experiments show no effect of LRRK2 knockout on cytokine secretion in response to simple inflammatory stimuli. Cells were stimulated as indicated for each row of graphs and supernatant cytokine concentrations measured at the time point indicated for each column of graphs. TNFα concentrations occasionally exceeded detection limits as indicated by ‘max’. A single independent experiment was performed.
4.3.4 LRRK2 has no effect on TLR4 expression or trafficking dynamics

Up-regulation of LRRK2 transcription upon LPS stimulation verifies an association between TLR4 and LRRK2 in pBMDM cells, while the fact that no observed differences were made in cytokine secretion between WT and LRRK2 KO macrophages suggests that this may manifest at a different level. Indeed, as discussed previously, some of the earliest evidence of the involvement of LRRK2 in innate immune signalling came from an association of LRRK2 with TLR4 signalling in macrophages. However, while the effect of TLR4 signalling on LRRK2 has been studied, little has been shown on the effect of LRRK2 on TLR4. As LRRK2 is upregulated upon LPS treatment, LRRK2 KO and WT pBMDMs were treated with LPS to establish if transcription of TLR4 was affected. Cells were treated with LPS for two hours and mRNA extracted for qRT-PCR analysis. A decrease of TLR4 transcription after LPS stimulation was detected, with a 30 % attenuation in transcription observed as part of a negative-feedback loop; confirming that derived pBMDM cells are behaving as expected (Nhu et al, 2006). Importantly, no significant difference was seen in this attenuation of TLR4 transcription between WT and LRRK2 KO macrophages (fig 31). This indicates that LRRK2 has no effect on transcription of TLR4.

**Figure 31: TLR4 transcript levels upon LPS stimulation.** qRT-PCR with beta-actin and GAPDH as housekeeping genes. Errors bars are S.E.M. of three independent experiments, unpaired two-tailed t-tests between genotypes.
In order to compare TLR4 endocytosis between LRRK2 genotypes, the propensity of TLR4 to undergo endocytosis in response to LPS was first measured using flow cytometry on WT pBMDM cells. Cells were left unstimulated, or were stimulated with a range of LPS doses for one or two hours, then stained for surface TLR4. Mean fluorescent intensity (MFI) measurements were used to calculate the percentage of endocytosis as described in the following equation:

$$\text{endocytosis(\%)} = \left( \frac{\text{Untreated MFI}_{\text{TLR}} - \text{Untreated MFI}_{\text{iso}} - (\text{MFI}_{\text{TLR}} - \text{MFI}_{\text{iso}})}{\text{Untreated MFI}_{\text{TLR}} - \text{Untreated MFI}_{\text{iso}}} \right) \times 100$$

Results demonstrated a clear increase in endocytosis with increasing concentrations of LPS, with more endocytosis observed after two hours of incubation than after one hour (fig 32a,b). TLR4 endocytosis was seen to begin to plateau between 100 ng/ml and 200 ng/ml LPS after two hours incubation, so it was not considered necessary to explore higher LPS concentrations or incubation durations (fig 32b).
To measure cell surface levels of TLR4, fixed pBMDM cells were stained using one of two anti-TLR4 antibody clones: MTS510 or Sa15-21. The specificity of these clones to TLR4 was verified using unstimulated pBMDM cells derived from TLR4 KO mice. No shift in fluorescent intensity was observed between cells stained for surface TLR4 using MTS510 or Sa15-21 and cells stained with appropriate isotype control antibodies (fig 33a). In comparison, WT pBMDM cells clearly showed an increase in fluorescence intensity when stained with either MTS510 or Sa15-21 antibodies as compared to appropriate isotype controls (fig 33b). These results indicate high specificity of anti-TLR4 antibodies and successful application of flow cytometry to the measurement of cell surface TLR4.

Figure 32: Optimisation of TLR4 endocytosis conditions. TLR4 stained with Sa15-21 antibody a: WT pBMDMs incubated in LPS for one hour in a single independent experiment. b: WT pBMDMs incubated in LPS for 2 hours. Error bars are within symbols; these are S.D. of two independent experiments.
Figure 33: Verification of anti-TLR4 antibody binding specificity. Representative FACS histograms. Unfilled red peak is TLR4 stained cells, filled grey peak is isotype control staining. a: TLR4 KO pBMDM cells stained with MTS510 or Sa15-21 clones as indicated. b: WT pBMDM cells stained with MTS510 or Sa15-21 clones as indicated.
The effect of LRRK2 knockout on TLR4 endocytosis was then assayed by comparing surface TLR4 levels between WT and LRRK2 KO pBMDM cells following LPS stimulation. LRRK2 KO had no significant effect on resting surface TLR4 levels in pBMDM cells as measured using the MTS510 antibody (fig 34a). The epitope for MTS510 on TLR4 is reported to be hidden prior to receptor endocytosis, perhaps during LPS induced dimerisation (Akashi et al., 2003); a step which precedes endocytosis. Therefore, the use of MTS510 is thought to give a better indication of steps leading to endocytosis, rather than TLR4 endocytosis itself. For simplicity, the term endocytosis will be used for the LPS induced loss of TLR4 signal as measured by MTS510 in this report. After one hour of LPS stimulation, 35 % of cell surface TLR4 was endocytosed. This increased to around 75 % after two hours of LPS stimulation (fig 34b). No significant differences in TLR4 endocytosis were observed between WT and KO macrophages upon stimulation with LPS when staining with the MTS510 antibody (fig 34b). This provides an indication that the early steps induced by LPS binding which lead to TLR4 endocytosis are not influenced by LRRK2. The Sa15-21 antibody is thought to give a clear indication of TLR4 endocytosis itself, owing to an epitope which is distinct from that of MTS510 and that can still be recognised after LPS binding (Akashi et al., 2003). In agreement with MTS510 data, there were no significant differences in resting cell surface TLR4 expression between unstimulated WT and LRRK2 KO macrophages as measured using the Sa15-21 antibody (fig 34c). After one hour of LPS stimulation, 50 % of TLR4 was seen to have been endocytosed, increasing to 70 % after two hours (fig 34d). It is interesting that more TLR4 endocytosis was observed using Sa15-21 antibody than using the MTS510 antibody after one hour, however this disparity was lost after two hours of incubation with LPS (fig 34b,d). No differences in TLR4 endocytosis were observed between LRRK2 WT and KO macrophages using the Sa15-21 antibody (fig 34d).

All together, these data provide a clear indication that LRRK2 has no effect on resting surface levels TLR4, or LPS induced TLR4 changes such as TLR4 transcription or dynamic trafficking.
Figure 34: LRRK2 has no effect on TLR4 dynamic trafficking or resting surface expression. pBMDM cells incubated with 200 ng/ml LPS for indicated lengths of time or unstimulated if not indicated. Error bars are S.E.M. Statistics are unpaired two-tailed t-tests between genotypes where relevant, otherwise paired two-tailed t-tests compared to a value of 1. a,b: Cells stained with MTS510 anti-TLR4 antibody, three independent experiments. c,d: Cells stained with Sa15-21 anti-TLR4 antibody, five independent experiments.
4.3.5 LRRK2 perturbs TLR2 transcription in response to innate immune stimulation, but not cell surface expression

Before investigating cell surface expression levels of TLR2, transcription of TLR2 was assessed following innate immune stimulation by LPS, Ifn-γ, MDP and αSyn oligos; all stimuli except Ifn-γ were seen to cause up-regulation of TLR2 transcription (fig 35a). LPS stimulation caused the greatest increase in TLR2 transcription with a ten-fold change compared to untreated cells. MDP and αSyn oligos caused a slightly more modest increase of between five and ten-fold. Interestingly, MDP stimulation resulted in a significant difference in TLR2 transcription between LRRK2 KO and WT pBMDM cells (fig 35a). Specifically, LRRK2 KO macrophages experienced a mean 10-fold induction of TLR2 transcription, compared to 6.3-fold for WT macrophages across three experiments. Whilst statistically significant differences in the induction of TLR2 transcription were not observed for other treatments, it was noted that LRRK2 KO macrophages also experienced a greater increase than WT equivalent cells after incubation with either LPS or αSyn oligos. No difference in TLR2 transcription was observed between LRRK2 genotypes in untreated cells across three experiments, suggesting that observations made relate to innate immune responses, and not underlying differences under resting conditions (fig 35b).
Figure 35: TLR2 transcription is modulated by LRRK2 in response to immune activation. qRT-PCR with beta-actin and GAPDH as housekeeping genes. Errors bars are S.E.M. of two independent experiments for αSyn oligos, three independent experiments for all other conditions. 

a: Statistics are unpaired two-tailed t-tests between genotypes. 

b: TLR2 transcription in untreated cells. Statistics are paired two-tailed t-tests compared to a value of 1.
Given the apparent effect of LRRK2 on the MDP response and TLR2 transcription, flow cytometry was used to measure cell surface levels of TLR2 over a longer time-course of MDP stimulation. In agreement with transcriptional data, no differences in cell surface levels of TLR2 were observed between unstimulated pBMDM cells (fig 36a). MDP stimulation resulted in a modest increase in levels of TLR2 observed at the cell surface between six and 24 hours, with a 1.5-fold increase in TLR2 observed compared to untreated cells. At two hours, a small refractory period was observed, where TLR2 surface levels were slightly diminished. No statistically significant differences were observed between WT and LRRK2 KO macrophage surface TLR2 levels in response to MDP stimulation over the length of the time-course (fig 36b).

![Graph](image)

**Figure 36:** LRRK2 has no effect on TLR2 surface expression in resting or MDP treated macrophages. Error bars are S.E.M. of three independent experiments. a: Unstimulated pBMDM stained for surface TLR2. b: pBMDM cells incubated with 10 μg/ml MDP for indicated lengths of time.
4.3.6 Interaction of LRRK2 with cytoskeletal components does not affect phagocytosis

To comprehensively test for a role or LRRK2 in phagocytosis, a series of phagocytic targets were applied to pBMDM cells in culture, starting with fluorescein labelled, carboxylate modified latex beads. First, optimisation of the number of target beads incubated per macrophage was established over a time-course of incubation. Using either 10 or 100 latex beads per WT pBMDM, the number of phagocytic events undertaken per cell (to an upper limit of 5) could be distinguished using flow cytometry; with each bead observable as a small jump in fluorescent intensity (fig 37a). The number of cells which had undergone a phagocytic event was seen to increase with time for both 10 and 100 beads per cell. Neither condition reached a plateau over the maximum two-hour incubation period. After two hours, around 50 % of cells had undergone a phagocytic event when incubated with 10 beads per cell, while around 90 % of cells had undergone a phagocytic event when incubated with 100 beads per cell (fig 37b). Although a greater proportion of cells were phagocytically positive at 100 beads per cell, even within half an hour the majority of phagocytically positive cells had already taken up 5+ beads. Above five beads, the ability of flow cytometry to measure the number of phagocytic events occurring was compromised. In comparison, after two hours, the majority of cells had phagocytosed 1-5 beads and so could still be resolved when incubated with only 10 beads per cell. Therefore, a two-hour incubation with 10 beads per cell was taken forward for future experiments (fig 37c).
Figure 37: Phagocytosis of carboxylate modified latex beads. 

a: Representative FACS histograms of WT cells after two hours incubation with 10 or 100 beads per cell as indicated. Numbers indicate the number of beads corresponding to the labelled peak. Blue histogram is cells incubated with beads, grey histograms are cells only. 

b: Proportion of cells which have undergone a phagocytic event. Error bars are S.D. calculated from cell counts in a single experiment. 

c: Proportion of cells which have undergone a specific number of phagocytic events as indicated to the right of each dataset. Error bars are S.D. calculated from cell counts in a single experiment.
WT and LRRK2 KO macrophages were next incubated with 10 beads per cell for two hours and phagocytic events were measured by flow cytometry. To account for the possibility that beads may stick to the surface of cells without being truly phagocytosed, as well as to verify that the process under observation was truly actin dependent, cells were pre-incubated and then maintained in either cytochalasin D, a potent inhibitor of actin polymerisation, or a mock ‘dimethyl sulfoxide’ (DMSO) treatment. WT and LRRK2 KO macrophages treated with only DMSO showed clear phagocytosis of fluorescent beads, again with 1-5 beads being clearly distinguishable on flow cytometry histograms. In contrast, WT and LRRK2 KO macrophages treated with cytochalasin D showed a marked decrease in phagocytosis (fig 38a). Around 35 % of cells were seen to be phagocytically active after two hours of incubation during this round of experiments. No significant differences were seen in phagocytosis of beads between WT and LRRK2 KO genotypes (fig 38b). This was further confirmed by closer inspection of the distribution of cell number within phagocytically positive cells, where the proportion of cells containing each number of beads (up to five beads) was close to identical (fig 38c). These results show that LRRK2 has no effect on the propensity of macrophages to phagocytose negatively charged latex beads.

Next, phagocytosis of opsonised beads was tested. Macrophages are professional phagocytes, expressing Fcγ receptors and ‘complement receptor 3’, which can induce phagocytosis of material. Fcγ receptor engagement and clustering by antibody coated particles leads to activation of the Rho type GTPases: Cdc42 and Rac1 (Hoppe and Swanson, 2004). The precise involvement of Cdc42 and Rac1 on Fcγ receptor mediated phagocytosis are incompletely understood, but as LRRK2 interactors may show an LRRK2 phenotype not seen without Fcγ receptor engagement. In order to achieve this, fluorescein-labelled beads were shaken and incubated with a range of concentrations of anti-fluorescein IgG. Visualisation by confocal microscopy revealed a halo of IgG surrounding beads. An increasing intensity of halo fluorescence was observed with increasing concentrations of IgG (fig 39). All tested concentrations of IgG displayed successful opsonisation. A concentration of 0.2 mg/ml of anti-fluorescein IgG was taken forward for future experiments.
**Figure 38: Phagocytosis of carboxylate-modified latex beads.** Error bars are S.E.M. across three experiments. **a:** Representative FACS histograms of WT or LRRK2 KO pBMDM cells after two hours incubation with 10 beads per cell. Numbers indicate the number of beads corresponding to the labelled peak. Coloured histograms DMSO treated, grey histograms are cytochalesin D treated. **b:** Proportion of cells which have undergone a phagocytic event. **c:** Proportion of cells which have undergone a specific number of phagocytic events.
Opsonised beads were incubated with cells as in previous experiments with un-opsonised beads; a two-hour incubation with 10 opsonised beads per cell. Once again, phagocytosis could be visualised by flow cytometry with clear peaks visible in flow cytometry histograms for up to 3 beads, and lower resolution peaks visible up to 5 beads. A greater proportion of cells containing 5+ beads were observed in both WT and LRRK2 KO macrophages, revealing increased phagocytic activity towards opsonised beads. Cytochalesin D again demonstrated that observed phagocytosis was an actin polymerisation dependent process (fig 40a). Around 45% of cells were seen to be phagocytically active, an increase of 10% upon that measured using un-opsonised beads. No significant differences were seen in phagocytosis of beads between WT and LRRK2 KO genotypes (fig 40b). The most significant difference compared to un-opsonised beads was the proportion of cells containing 5+ beads, an increase from 5% to around 15%. However, no significant differences in the proportion of cells containing any particular number of beads were observed using opsonised beads (fig 40c). These results indicate that LRRK2 has no effect on the propensity of a macrophage to phagocytose opsonised latex beads.

Figure 39: Fluorescein bead opsonisation. Representative confocal images of beads opsonised with increasing concentrations of anti-fluorescein IgG antibody. Beads are 1 μm.
Figure 40: Phagocytosis of IgG opsonised latex beads. Error bars are S.E.M. across three experiments. **a:** Representative FACS histograms of WT or LRRK2 KO pBMDM cells after two hours incubation with 10 beads per cell. Numbers indicate the number of beads corresponding to the labelled peak. Coloured histograms DMSO treated, grey histograms are cytochalesin D treated. **b:** Proportion of cells which have undergone a phagocytic event. **c:** Proportion of cells which have undergone a specific number of phagocytic events.
A final assay was performed using green fluorescent protein (GFP) expressing *E. coli*; a physiological target for macrophage phagocytosis. *E. coli* are non-invasive Gram-negative bacteria which express an array of PAMPS, including LPS and flagellin. These PAMPS are recognised by macrophage surface receptors, causing a pro-phagocytic phenotype (Ribes et al., 2010). Log-phase *E. coli* were diluted to a MOI of 10 into DMSO or cytochalesin D containing media. Macrophages were then incubated with *E. coli* for two hours before being washed and fixed for microscopy. Confocal microscopy revealed that cytochalesin D treatment had been effective in limiting phagocytosis of *E. coli*, confirming that this assay was successfully measuring an actin-dependent process (fig 41a). At least 500 macrophages were counted in each quantified experiment with an average of 1.5-2 bacteria detected per macrophage. No statistically significant difference in *E. coli* phagocytosis was observed between WT and LRRK2 KO macrophages (fig 41b).

Between these phagocytosis assays, it is clear that LRRK2 has no effect on phagocytosis in primary macrophages. This finding suggests that an earlier report of LRRK2 modulating phagocytosis is most likely an off-target effect of the L2in1 LRRK2 inhibitor.
Figure 41: Phagocytosis of GFP expressing *Escherichia coli*. a: Representative images of pBMDM cells after incubation with GFP *E. coli* for two hours. Nuclei stained in blue, green fluorescence is GFP *E. coli*. Scale bar is 25 μm. b: Quantification of *E. coli* phagocytosis in DMSO or cytochalesin D treated cells as ratio of bacteria per macrophage. Error bars are S.E.M. across three experiments for DMSO, a single experiment was quantified for cytochalesin D.
4.3.7 Measuring the effect of LRRK2 on chemotaxis using Boyden chambers

Having observed no effect of LRRK2 upon phagocytosis, an attempt was made to quantify the effect of LRRK2 interactions with the cellular cytoskeleton on chemotaxis towards the chemoattractant ADP. A Boyden chamber assay was established towards this purpose. Cells were plated on the Boyden chamber membrane, and the tissue culture well below this membrane was treated with ADP. After a period of incubation to allow for chemotaxis to occur, migration both into and through the membrane was quantified by staining and quantifying macrophage nuclei within the membrane, and at the bottom of the tissue culture well. A range of parameters were tested in this experimental setup to establish optimum conditions under which to perform comparative chemotaxis assays between LRRK2 genotypes. First, a range of numbers of WT macrophages were plated on the membrane. After a 24 hour incubation, a significant effect of ADP could be observed upon chemotaxis of macrophages in chambers with 25,000, 50,000 and 100,000 cells plated. Quantification showed a clear increase in cell number could be observed as having migrated into the Boyden chamber membrane, as well as through the membrane and into the bottom of these tissue culture wells when incubated with ADP (fig 42a). The separation in cell numbers counted between ADP treated samples and untreated samples increased with the number of cells plated, with a mean of 958 cells counted in the membrane of ADP treated cells, and only 158 cells counted in untreated Boyden chambers. An effect could also be observed in the tissue culture well, however the cell numbers were much lower and more variable by this method of quantification, with a mean of 85 cells counted in ADP treated samples, and only 8 in untreated samples. The parameter of 100,000 plated cells was taken forward to further optimisation, with cells treated with ADP for a range of different incubation times. Cells were incubated with ADP for 6, 18, 24, or 30 hours and chemotaxis was quantified as before (fig 42b). An observable effect of ADP upon chemotaxis was observable immediately at 6 hours in the Boyden membrane with a mean of 614 cells counted in ADP treated chambers, compared to 99 cells in untreated cells. The absolute difference in quantified cell numbers in the Boyden membrane did not vary greatly up to 24 hours with a mean of 1180 cells counted in ADP treated chambers,
and 379 counted in unstimulated chambers, however this difference decreased after 24 hours. The effect of chemotaxis in cells counted at the bottom of the tissue culture well increased with time, becoming observable at 16 hours and increasing up to the longest incubation time of 30 hours. This suggests that chemotaxis was still going at this late time point. With these considerations in mind, a time point of 24 hours with 100,000 cells played was deemed appropriate for experiments.

**Figure 42: Chemotaxis assay optimisation.** Quantification of cells migrating into the Boyden membrane, or to the bottom of tissue culture well as indicated in the presence of absence of ADP chemoattractant. **a:** Optimisation of cell numbers for chemotaxis assay. 24 hour incubation. S.E.M. across three independent experiments. **b:** Optimisation of incubation time over which chemotaxis can occur. 100,000 cells/chamber plated. S.D of triplicate wells from a single experiment.
Upon taking optimised parameters forward for full experiments with WT and LRRK2 KO macrophages, the previously observed effect of ADP upon chemotaxis was no longer observable. Specifically, a higher number of macrophages was quantified within the membrane and tissue culture well of unstimulated Boyden chambers than in previous experiments (fig 43). A mean of around 1000 cells was counted within the membrane of unstimulated chambers, with only between 1000 and 1300 cells counted in ADP treated chambers. The number of cells counted at the bottom of tissue culture wells under both stimulated and unstimulated conditions was also significantly increased in these experiments compared to optimisation experiments.

Furthermore, no notable differences were observed between WT and LRRK2 KO macrophage chemotaxis into the Boyden membrane. A difference could be observed in chemotaxis to the bottom of the tissue culture membrane with a mean of 513 WT macrophages compared to only 283 LRRK2 KO macrophages in the presence of ADP, however these differences were also reflected in the elevated number of cells quantified in unstimulated chambers, with a mean of 158 WT macrophages counted compared to 38 LRRK2 KO macrophages. This suggests inaccuracies in cell counting upon initial plating of cells within Boyden membranes may outweigh any observable

![Graphs showing cell count in membrane and well](image)

**Figure 43: Chemotaxis assays.** Quantification of cells migrating into the Boyden membrane, or to the bottom of tissue culture well as indicated in the presence of absence of ADP chemoattractant. 24 hour incubation, 100,000 cells plated. S.E.M. across three independent experiments
effect of genotype on chemotaxis. Thus full experiments showed unacceptable variability from previous optimisation experiments, and a hugely decreased observed effect of ADP on measured chemotaxis. This was particularly true upon quantification of cells in the Boyden membrane. Further optimisation will be required before Boyden chamber assays are suitable for quantification of the effect of LRRK2 upon chemotaxis.

4.4. Discussion

The aim of this work was to investigate potential roles of LRRK2 in the dynamic trafficking of TLRs and dynamic cytoskeletal process of phagocytosis. The generation of pBMDMs facilitated these studies through specificity brought about by genetic knockout of LRRK2. The use of a genetic mouse model has important advantages over the use of knockdown systems or chemical inhibitors used by others in this field. B6.129X1(FVB)-Lrrk2tm1.1Cai/J mice contain a premature stop codon in exon 3 of LRRK2 mRNA, resulting from excision of a portion of exon 2 in the DNA of these mice (Parisiadou et al., 2009). This means that while mRNA derived from the LRRK2 gene may be expressed and detected in cells obtained from these mice, this mRNA will undergo nonsense-mediated decay at the ribosome rather than translation to mature LRRK2; as such these mice are an LRRK2 KO genotype in line with published results (Parisiadou et al., 2009; Kervestin and Jacobson, 2012). Perturbation of LRRK2 DNA was confirmed by genotyping of mice, and the process of degradation of LRRK2 mRNA was reflected in a 50 % decrease in LRRK2 mRNA detected by qRT-PCR; an observation which could be confirmed by RNA sequencing. These considerations provided unequivocal evidence that LRRK2 mRNA was perturbed in LRRK2 KO mice. In comparison, the use of several currently available anti-LRRK2 antibodies failed to specifically stain LRRK2 in our hands, resulting in a large amount of background signal by immunofluorescent microscopy.

In WT primary macrophages, LRRK2 transcription could be induced by activation of innate immune receptors, most notably by activation of TLR4 with LPS. While at least a two-fold increase in LRRK2 transcription was observed in all mice tested, the extent of this increase did vary between mice more than other stimuli
tested. Confirmation of inducibility of LRRK2 expression by LPS stimulation was later achieved through RNA sequencing. The variability of LRRK2 inducibility observed using qRT-PCR perhaps reflects the nature of the literature, where there are mixed reports as to whether LPS induces LRRK2 transcription at all (Dzamko et al., 2012; Moehle et al., 2012). Within this context, it is particularly interesting to observe that such a dramatic increase in LRRK2 transcription in response to LPS stimulation. Reports of Ifn-γ inducibility of LRRK2 expression are very consistent (Gardet et al., 2010; Thévenet et al., 2011; Kuss et al., 2014), therefore it was surprising that only a modest increase in transcription as observed. This is most likely a consequence of the relatively short two-hour incubation with Ifn-γ that was applied. In comparison, an almost three-fold increase in LRRK2 transcription has been reported as measured by microarray after 24 hours of stimulation of mouse macrophages with Ifn-γ (Depke et al., 2014). LRRK2 transcription was also assessed after stimulation with MDP and αSyn oligos. These innate immune activators were included due to their close functional relationship to LRRK2. NOD2 and LRRK2 are both risk factors for Crohn’s disease (Liu and Lenardo, 2012), sort lysozyme in Paneth cells (Zhang et al., 2015), and are reported to jointly modulate IL-6 production (Yan and Liu, 2017). Alpha-synuclein and LRRK2 have been suggested to demonstrate synergy in their neurotoxic effects, through involvement in common pathways such as autophagy, neuronal trafficking and proteosomal degradation (Liu et al., 2012). Further to this, alpha-synuclein also activates immune receptors including TLR1/2 heterodimers (Kim et al., 2014; Daniele et al., 2015), and TLR4 (Fellner et al., 2013). In spite of this, neither MDP, nor alpha-synuclein had a clear effect on LRRK2 transcription. The use of alpha-synuclein is more problematic than other stimuli applied in these experiments as the activity of alpha-synuclein on innate immunity varies depending on the precise nature of aggregation; which is variable between batches and with time (Kim et al., 2014; Tosatto et al., 2015). Access to a consistent and carefully controlled source of aggregated alpha-synuclein would allow a clearer indication of macrophage responses to the proteins oligomeric form. These results utilise LRRK2 KO mice to answer basic immunological questions about LRRK2 function without the uncertainty of non-specific effects of inhibitors such as L2in1. Here it was demonstrated that LRRK2 expression in primary macrophages derived from these
mice is responsive to Ifn-γ, and that transcription of LRRK2 is enhanced by LPS stimulation at an early time point. LRRK2 transcriptional enhancement was specific to these stimuli, as MDP stimulation had no effect on transcription, while alpha-synuclein had no clear effect. While LRRK2 could be induced by LPS and Ifn-γ stimulation, no clear effect was observed upon LRRK2 knockout on cytokine secretion in response to either LPS or Ifn-γ stimulation, nor other innate immune stimuli including MDP, and αSyn oligos.

Assays to measure surface levels of TLR4 and TLR2 were successfully set up and applied to measure the effect of LRRK2 on the dynamic trafficking of TLR4, and TLR2 expression levels.

The association between TLR4 and LRRK2 signalling has been extensively discussed. This relationship, combined with comparatively well-defined vesicular dynamics, make TLR4 a perfect candidate for a receptor whose trafficking may be influenced by LRRK2. Perturbation of TLR4 trafficking could have profound effects on the balance between Myd88 and TRIF signalling, or termination of the TLR4 response to LPS (Kagan et al., 2008; Husebye et al., 2010). Such changes in the balance of signalling would have subsequent effects on inflammatory transcription programmes downstream of TLR4. Knockout of LRRK2 had no effect on either resting levels of TLR4, or TLR4 transcription in response to LPS. This means that while TLR4 signalling regulates LRRK2 transcription; reciprocal regulation does not appear to occur. Measurements of macrophage TLR4 endocytosis were in line with observations made by other groups. TLR4 endocytosis ‘saturated’ at about 70 %, where increased LPS had no greater effect on surface levels of TLR4. This required 10 x less LPS to achieve than was used by another group (100 ng/ml vs 1 ug/ml, after one hour incubation) (Zanoni et al., 2011). This observation could be a reflection of the increased responsiveness of primary macrophages compared to macrophage cell lines. Ultimately there were no detectable differences in TLR4 at the cell surface between WT and LRRK2 KO macrophages, either under resting conditions or following LPS stimulation. This finding was consistent using two distinct anti-TLR4 antibodies; MTS-510 and Sa15-21. These antibodies indicated a comparable overall level of endocytosis despite binding different TLR4 epitopes, and followed the same
trend of increasing endocytosis with time. While it would appear that LRRK2 does not affect TLR4 localisation, TLR4 signalling does appear to modulate LRRK2 localisation. Indeed phosphorylation of LRRK2, which occurs downstream of TLR4 activation, has even been reported to regulate LRRK2 extracellular release through exosomes, with exosomal release reported in a diverse range of cellular contexts; both inside and outside of the brain (Fraser et al., 2013). In an innate immune context, activation of TLR4 reportedly causes LRRK2 dimerisation and membrane localisation (Schapansky et al., 2014). Therefore, TLR4 signalling modulates LRRK2 trafficking, but as with transcriptional responses, LRRK2 does not appear to affect TLR4 trafficking. Taken together, multiple lines of evidence now suggest that while TLR4 and LRRK2 do interact, LRRK2 acts only downstream of TLR4.

While TLR4 is the pattern recognition receptor most intimately associated with LRRK2, TLR2 is highly involved in Parkinson’s disease more generally. TLR2 is reported as enriched in the Parkinson’s brain, and in pathologically relevant neuronal regions such as the substantia nigra (Dzamko et al., 2017). A recent genetic discovery has even suggested that polymorphisms in TLR2 may be a risk factor for the sporadic disease (Wu et al., 2015). This would be logical as TLR2 detection of alpha-synuclein as a DAMP is thought to mediate neuroinflammation in Parkinson’s disease. In primary macrophages, TLR2 transcription was strongly induced by stimulation with LPS, MDP and αSyn oligos. LPS induction resulted in around a 10-fold increase in transcription, exactly in line with other reports for two hours of stimulation (Nhu et al., 2006). Interestingly, LRRK2 KO macrophages stimulated with MDP resulted in a significantly enhanced transcription of TLR2 compared to WT cells. This appears to represent a trend, as stimulation with LPS and αSyn oligos similarly resulted in greater TLR2 transcription, but did not reach statistical significance. It would appear that LRRK2 acts to inhibit TLR2 transcription specifically in response to inflammatory stimuli, as no differences were observed in TLR2 transcription in unstimulated macrophages. The similarity in TLR2 transcription in unstimulated macrophages provides reassurance that observed differences upon innate immune stimulation are not the result of a systematic error. Similarly, while Ifn-γ failed to noticeably stimulate transcription of TLR2, more overall TLR2 transcription was
observed in WT Ifn-γ stimulated macrophages than in LRRK2 KO equivalent cells. A 24-hour time course of TLR2 cell surface expression was performed to investigate the effect of enhanced transcription of TLR2 on TLR2 protein levels at the cell surface. No differences were observed across this time course. Potential explanations for this include that LPS stimulation results in peak TLR2 transcription only after six hours, meaning that at later time point TLR2 transcription in WT macrophages may catch-up with that of LRRK2 KO equivalent cells (Nhu et al., 2006). It would therefore be interesting to look at a more complete time course of TLR2 transcription in response to inflammatory stimuli to see if this is the case, or whether differences in TLR2 transcription are maintained over time. It is also possible that differences in TLR2 transcription were not observed at the cell surface due to a compensatory mechanism in TLR2 trafficking to maintain homeostatic levels of TLR2 at the cells surface in the absence of LRRK2. To address this, total TLR2 levels, and TLR2 distribution could be compared between LRRK2 genotypes by immunofluorescent microscopy upon stimulation with MDP. While the functional significance of TLR2 endocytosis is not understood, TLR2 trafficking dynamics could be investigated by flow cytometry in response to stimulation by LTA or perhaps even *Staphylococcus aureus* (Stuart et al., 2005). Such measurements would represent TLR2 trafficking dynamics, and would complement data collected for TLR4 in response to LPS.

For a complete understanding of the role of LRRK2 in phagocytosis, different phagocytic targets were used; each introducing a higher level of signalling complexity to the system. An initial assay using carboxylate modified fluorescent beads was used to optimise experimental parameters and to compare phagocytic uptake of simple large molecules. Flow cytometry was able resolve up to five beads in a macrophage through discernable jumps in MFI. Applying 10 beads per cell was optimal to achieve bead phagocytosis by WT macrophages within this resolvable range. Application of beads under these conditions revealed no differences in uptake between LRRK2 genotypes. Around 35% of cells were seen to be phagocytically active after two hours of incubation during this round of experiments; a slight decrease from that seen during optimisation. This most likely reflects variation in the
dilution of beads to 10 beads per cell, but could also be affected by pre-incubation and maintenance of cells in DMSO during experiments. Variability in, and between experiments is a weakness of this assay as the ratio of beads to cell is affected by small differences in cell plating density and bead dilution. Using the same stock of beads minimises this technical issue for comparing phagocytosis between genotypes, but not across different experiments where a new dilution of beads is prepared. With this in mind, repeat experiments showed remarkably strong reproducibility across replicate experiments. The uptake of different materials by cells is an area of on-going research in biophysics. Biophysical properties such as charge, shape and hydrophobicity affect cellular uptake of molecules by cells, with negatively charged microparticles taken up more efficiently than positively charged microparticles (Fröhlich, 2012). Opsonisation of beads was next performed in order to measure the effect of LRRK2 on Fcγ receptor mediated phagocytosis. Activation of phagocytosis receptors led to an increased proportion of cells to be phagocytically active compared to non-opsonised beads used previously. Further to this, the proportion of cells containing 5+ beads was tripled when using opsonised beads compared to non-opsonised. This enhancement of phagocytosis indicates that opsonisation was successful, and that resulting macrophage stimulation could be successfully detected by flow cytometry assay. Resolution was lost compared to non-opsonised beads due to this increased proportion of cells containing 5+ beads, however it remained clear that LRRK2 has no effect on the uptake of these opsonised beads.

A final level of complexity was introduced through the use of live E. coli as a phagocytic target. While E. coli are not antibody coated, they do express a plethora of PAMPs on their cell surface including LPS and flagellin for the activation of TLRs; thus stimulating macrophage activation (Ribes et al., 2010). Importantly, this stimulation occurs via a different mechanism to that explored previously, and more physiologically than in experiments performed using extensive LPS pre-incubation of cells (Schapansky et al., 2014). It should be noted that while the use of bacteria is more relevant to LRRK2 in the context of Crohn’s disease than Parkinson’s, alpha-synuclein aggregates which form in Parkinson’s disease are immunogenic, stimulating at least TLR4 and TLR2, thus displaying similarities to E. coli that are not
modelled in latex bead uptake. Analysis of bacterial phagocytosis was performed using confocal microscopy due to the non-uniform nature of *E. coli* in size and fluorescent intensity, which would not register as discrete jumps in MFI that were used to quantify bead phagocytosis by flow cytometry. To compensate adequately for uncertainty associated with quantification of phagocytosis by microscopy, a minimum of 500 cells were counted for each genotype per experiment. No significant differences in bacterial phagocytosis were measured in this analysis. These results show that LRRK2 has no effect on the phagocytosis of targets under a range of different conditions and innate immune stimuli. Thus, using a genetic LRRK2 KO experimental system, further validation is provided to assertions made using LRRK2 knockdown macrophages (Schapansky et al., 2014), and LRRK2 overexpressing G2019S immortalised cells (Moehle et al., 2015). Work described here has goes beyond the scope of these studies performed by other groups, by looking at the effect of LRRK2 KO on the uptake of phagocytic targets triggered by different levels of innate immune signalling. Importantly, these collective studies of phagocytosis using different cells and genetic systems refute findings made using the L2in1 inhibitor, which suggests that results obtained using the L2in1 system were flawed by off-target effects (Marker et al., 2012). One potential further phagocytosis assay, as mentioned in the context of vesicular trafficking, would be to examine the cellular uptake of *Staph. aureus* – a Gram-positive bacterium. Such an assay would not only be interesting in order to measure TLR2 trafficking dynamics, but also in the activation of NF-κB signalling downstream of TLR2 via Rac1; an LRRK2 interaction partner (Arbibe et al., 2000).

While phagocytosis is an actin dependent process directly involved in immunity, the actin cytoskeleton is similarly important in cell migration in the presence and absence of inflammatory stimuli. Chemotaxis assays were established in an attempt to quantify the effect of LRRK2 upon this process. Optimisation of these assays was highly successful with a clear effect of ADP upon migration into Boyden chamber membranes, and through these membranes to the bottom of tissue culture wells. However, application of optimised conditions for these assays failed to recapitulate the previously observed ability to observe the established
effect of ADP upon chemotaxis; ultimately no conclusions can be drawn from these assays in the current form, with further optimisation required in order to draw meaningful conclusions of the effect of LRRK2 upon chemotaxis. The major difference between optimisation experiments and LRRK2 chemotaxis assays is the scale of experiments, with twice as many samples used required per experiment to account for the LRRK2 KO macrophages, but also inaccuracies introduced during cell counting of two different cell populations. The use of a computerised cell counter may be able to reduce the effect of cell counting inaccuracies upon this assay. An alternative approach to measure the effect of LRRK2 upon cell migration may be to observe cells using live cell imaging. Such an assay would be suitable to observe the effect of LRRK2 upon membrane dynamics under non-pathological conditions, but lacks the immunological chemoattractant component present in chemotaxis assays. Cells may be transfected with lifact, a marker for F-actin visualisation, in the presence or absence of chemotactic stimuli (Riedl et al., 2008). Actin structures can also be fixed and imaged with fluorescently labelled phalloidin.
5. Transcriptomic and lipidomic analysis of LRRK2 function in the innate immune response

5.1 Aims

- Stimulate WT and LRRK2 KO macrophages with LPS or MDP, and apply an RNA sequencing approach to obtain datasets reflecting the role of LRRK2 in macrophage innate immune transcriptional responses.
- Apply differential gene expression analysis on WT and LRRK2 KO datasets to establish baseline differences in gene expression in unstimulated cells.
- Apply pathway analysis to compare between WT and LRRK2 KO datasets and establish broad roles of LRRK2 in innate immune responses to LPS or MDP.
- Apply two-parameter analysis to determine specific differentially responding genes to innate immune stimuli.
- Assess lipidomic changes in stimulated macrophages in order to investigate the role of LRRK2 in ceramide metabolism.

5.2 Background

It is clear that the role of LRRK2 is extremely diverse, with apparent functions described across broad areas of biology. Such diversity breeds complexity that can be difficult to reconcile. As such, the specific pathogenic mechanism of LRRK2 in Parkinson’s and other inflammatory disease remains unclear. Modern approaches allow us to capture a snapshot of biologically complex situations. To this end, a transcriptomic approach was taken in order to better understand the role of LRRK2 in innate immunity. A supplementary metabolomic approach was also taken to allow specific questions to be answered about the effect of LRRK2 on ceramide composition.
Transcriptomics

Innate immune programs need to be activated before the majority of transcriptional changes come into effect. These transcriptional changes can be considered part of the innate immune response. Following results of interest in previous work, an experiment was designed in which WT and LRRK2 KO macrophages would be stimulated with either LPS or MDP.

The relationship between TLR4, LPS and LRRK2 has been extensively discussed. Furthermore, the transcriptional response to macrophage LPS stimulation is well understood, and so provides a good background upon which to establish potential perturbations of a normal response by knockout of LRRK2 (Lu et al., 2008). In comparison, the innate immune response to MDP has been less well characterised. NOD2 is the intracellular receptor of MDP, a PAMP expressed as part of bacterial cell walls (Mo et al., 2012). NOD2 activation leads to nucleotide binding, receptor multimerisation, and complex formation with RIP2 via CARD-CARD interactions (Boyle et al., 2014). An extremely diverse range of signalling results, including MAPK signalling cascades, as well as Nf-κB, and IRF7 transcriptional activation (Correa et al., 2012). Nf-κB activation occurs via IKK family of proteins, which also phosphorylate LRRK2 (Dzamko et al., 2012). Alternate activation can occur via interaction with ‘mitochondrial antiviral signalling’ (MAVS), a mitochondrial protein normally associated with the ‘retinoic acid-inducible gene I’ (RIG-I) mediated antiviral response; this results in ‘TNF receptor associated factor 3’ (TRAF3) mediated signalling (Sabbah et al., 2009). Signalling occurs not just through phosphorylation of downstream proteins, but also via the formation of Lys^{63}-linked polyubiquitin chains (Hasegawa et al., 2008). NOD2 activation induces autophagy and enhances apoptosis; two processes associated with LRRK2 (Ogura et al., 2001; Travassos et al., 2010). Furthermore, LRRK2 appears to play a role in modulating the response to MDP inflammatory signalling; and in Paneth cells, has been demonstrated to form an interaction with NOD2 (Zhang et al., 2015; Yan and Liu, 2017). Finally, NOD2 activation is able to modulate TLR signalling. NOD2 acts to down-regulate the TLR2 inflammatory response in mice (Watanabe et al., 2004;
Watanabe et al., 2006), while modulating inflammation in a dose dependent manner in humans (Borm et al., 2008). A similar role appears to be present in modulation of TLR4 signalling; with NOD2 acting to down regulate induction of IL-12 release when stimulated with large doses of LPS, and up regulate IL-12 production upon stimulation with low LPS dose (Kim et al., 2015).

While LPS stimulation of TLR4 represents a well known innate immune process upon which to assess the effects of LRRK2, MDP stimulation of NOD2 is involved in an array of less well-characterised processes which overlap with described roles of LRRK2.

**Ceramide metabolism**

In parallel to RNA sequencing, metabolites were extracted for analysis. A recent report has shown a role of LRRK2 in ceramide metabolism in brain tissue; with increased levels of ceramide observed in LRRK2 KO cells (Ferrazza et al., 2016). Ceramide is linked to many cellular functions, including autophagy and apoptosis (Pattingre et al., 2009; Aflaki et al., 2012), but is also associated directly with inflammatory processes. For instance, ceramide is able to activate the NLRP3 inflammasome in bone marrow derived macrophages, leading to cleavage of pro-IL1β to its mature, pro-inflammatory form (Vandanmagsar et al., 2011). Ceramide can be formed by *de novo* synthesis, or through salvage pathways from ceramide derivatives such as sphingolipids or sphingosine (Maceyka and Spiegel, 2014). GBA1 encodes the enzyme glucocerebrosidase, which is involved in ceramide salvage through catalysis of glucocerebrosides to ceramide and glucose. Intriguingly, mutations to the GBA1 gene are present in around 7 % of patients suffering form Parkinson’s disease, and carriers of GBA1 mutations have increased risk of developing Parkinson’s (Sidransky et al., 2009). Furthermore, patients lacking GBA1 expression develop Gaucher disease, through over accumulation of glucocerebroside. Severity of this Gaucher disease is linked to the level of GBA1 deficiency, with more severe manifestations of the disease presenting with severe neurological defects including epilepsy and cognitive impairment (Aflaki et al., 2017).
Therefore, ceramide can be linked separately to altered metabolism in neurones via LRRK2, and deficient metabolism in macrophages via the Gaucher and Parkinson’s disease associated gene; GBA1. As a result, it is of interest as to whether increased ceramide levels observed in LRRK2 KO brain tissue are also seen in LRRK2 KO macrophages.

5.3 Results

5.3.1 Cell surface markers show little variation in macrophage markers between triplicate mice

RNA sequencing is a sensitive genome wide method of analysis, with the potential to pick up a lot of noise in macrophages even of the same genotype if not controlled appropriately by considerations such as age-matching mice. As discussed previously, pBMDM cells are derived in tissue culture from bone marrow. This differentiation process takes place over the period of a week in the presence of growth factors including macrophage colony-stimulating factor (M-CSF); and so has the potential to introduce such a source of noise. Therefore, efforts were made to confirm the similarity in nature and purity of macrophage cultures before RNA extraction and RNA sequencing. One day prior to RNA extraction, a portion of differentiated macrophages were prepared for flow cytometry analysis and stained for various cell surface markers: CD11b for cells of the myeloid lineage, F4/80 for mouse macrophages, and CD11c for monocytic-derived cells, including macrophages (Murray and Wynn, 2012). These markers revealed no dramatic differences in the differentiation state of cells, with uniform expression of CD11b, and highly similar expression levels of F4/80 and CD11c. CD11c surface expression in LRRK2 KO macrophages displayed a slightly higher level of variability between cultures than WT equivalent cells (fig 44). Overall, cultures were considered similar enough to proceed with differential gene expression analysis.
**Antigen:**
- CD11b
- F4/80
- CD11c

**Marker:**
- Myeloid cells
- Mouse macrophage
- Monocyte derived cells

**Figure 44: Analysis of macrophage cell markers.** Flow cytometry analysis of macrophage cell surface markers. Each coloured, unfilled peak are stained cells of a macrophage population derived from a different mouse. Filled grey peaks are unstained cells derived from a single mouse.
5.3.2 mRNA sequencing, quality control and read mapping

WT and LRRK2 KO macrophages were next stimulated where appropriate with either LPS, or MDP for two hours, after which RNA was extracted and submitted to ‘Cambridge Genomic Services’ for mRNA sequencing. A mean read depth of over 22.2x10^6 reads/sample was achieved with a range of 16.0x10^6 - 24.9x10^6 reads/sample (Table 12). Reads were of high quality, requiring a mean of less than 0.1 % of reads to be trimmed during quality control. A mean of 87.5 % of reads could be unambiguously mapped to gene encoding regions of the genome. Therefore by comparing the frequency of reads per gene between samples, relative expression of genes can be inferred. The resulting datasets could now be analysed for differential gene expression.

Table 12: RNA sequencing quality control and mapping. Number of reads per sample after each step of quality control and read mapping is indicated.

<table>
<thead>
<tr>
<th>Sample</th>
<th>LRRK2 Genotype</th>
<th>Treatment</th>
<th>Raw</th>
<th>Trimmed</th>
<th>Uniquely mapped to genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KO</td>
<td>LPS</td>
<td>22,350,423</td>
<td>22,337,177</td>
<td>19,756,554</td>
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<tr>
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<td>KO</td>
<td>MDP</td>
<td>20,864,607</td>
<td>20,852,101</td>
<td>18,035,498</td>
</tr>
<tr>
<td>3</td>
<td>KO</td>
<td>Media</td>
<td>24,584,270</td>
<td>24,574,608</td>
<td>21,373,819</td>
</tr>
<tr>
<td>4</td>
<td>KO</td>
<td>LPS</td>
<td>23,543,303</td>
<td>23,526,041</td>
<td>20,817,399</td>
</tr>
<tr>
<td>5</td>
<td>KO</td>
<td>MDP</td>
<td>23,633,755</td>
<td>23,624,109</td>
<td>20,888,105</td>
</tr>
<tr>
<td>6</td>
<td>KO</td>
<td>Media</td>
<td>20,997,179</td>
<td>20,979,364</td>
<td>18,387,880</td>
</tr>
<tr>
<td>7</td>
<td>KO</td>
<td>LPS</td>
<td>28,876,974</td>
<td>28,863,515</td>
<td>24,950,718</td>
</tr>
<tr>
<td>8</td>
<td>KO</td>
<td>MDP</td>
<td>21,917,338</td>
<td>21,889,839</td>
<td>18,953,616</td>
</tr>
<tr>
<td>9</td>
<td>KO</td>
<td>Media</td>
<td>22,083,849</td>
<td>22,073,008</td>
<td>19,232,352</td>
</tr>
<tr>
<td>10</td>
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<td>LPS</td>
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<td>19,746,221</td>
<td>17,475,368</td>
</tr>
<tr>
<td>11</td>
<td>WT</td>
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<td>23,022,129</td>
<td>23,009,814</td>
<td>19,887,489</td>
</tr>
<tr>
<td>12</td>
<td>WT</td>
<td>Media</td>
<td>23,088,368</td>
<td>23,069,013</td>
<td>19,908,328</td>
</tr>
<tr>
<td>13</td>
<td>WT</td>
<td>LPS</td>
<td>20,633,505</td>
<td>20,608,724</td>
<td>18,251,968</td>
</tr>
<tr>
<td>14</td>
<td>WT</td>
<td>MDP</td>
<td>21,367,108</td>
<td>21,352,531</td>
<td>18,693,589</td>
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<tr>
<td>15</td>
<td>WT</td>
<td>Media</td>
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<td>25,549,470</td>
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<td>19,980,006</td>
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<td>18,215,079</td>
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<tr>
<td>18</td>
<td>WT</td>
<td>Media</td>
<td>20,019,319</td>
<td>20,006,085</td>
<td>17,641,459</td>
</tr>
</tbody>
</table>
5.3.3 Differential gene expression

Differential gene expression analysis was performed using DEseq2. Datasets of mapped counts were interrogated for differences in each LRRK2 genotype upon innate immune stimulation, as well as for underlying differences between genotypes in unstimulated cells (fig 45a). DEseq2 determines a statistical model accounting for variance in counts per gene, and base mean of counts allowing the statistical significance of apparent differences in gene expression to be estimated. This is a routinely used approach for differential gene expression analysis. Analysis of the effect of innate immune treatment of cells revealed 5000 differentially expressed genes upon LPS stimulation with an ‘adjusted p-value’ (padj) for multiple tests of < 0.01 for both WT and LRRK2 KO macrophages. 4985 genes were found to be significantly differentially expressed in WT macrophages, and 5354 genes differentially expressed in LRRK2 KO macrophages after LPS treatment. A smaller, yet still substantial number of genes were differentially expressed upon treatment with MDP. In total, MDP treatment resulted in 1483 significantly differentially expressed genes in WT macrophages, compared to 1478 genes in LRRK2 KO macrophages (fig 45b).
Figure 45: Differential gene expression analysis. a: LRRK2 genotype (red), and treatment with innate immune stimuli (blue), are considered separately in these experiments. b: Quantification of differentially expressed genes. KO refers to the LRRK2 KO genotype. Numbers refer to differentially expressed genes between annotated samples (padj < 0.01).
When comparing unstimulated cells for differences in gene expression owing simply to knockout of LRRK2, only eight genes were significantly differentially expressed (table 13). One differentially expressed gene is LRRK2, this in line with earlier qPCR results and confirms the accuracy of RNAseq analysis (fig 29). Other genes include Kif21a: a member of the kinesin family of motor proteins, Camk2b: a calcium/calmodulin responsive protein kinase, Cd59a: a regulator of the membrane attack complex in mice, and Nnt: a NAD(P) transhydrogenase with implications in defence against oxidative stress. Very little is known about the Lrmda gene except that it consists of a region of LRRs. Remaining results are not represented at the protein level and so are unlikely to have relevance to the current study. The gene detected as being of the highest significance, Gm14150, is described as a pseudogene, produced by the incorporation of reverse transcribed mRNA into the genome, while Gm44305 is a retained intron. These are likely not differentially expressed genes, but pre-existing genomic difference between strains (Akagi et al., 2008).

### Table 13: Differentially expressed genes between unstimulated macrophages.

<table>
<thead>
<tr>
<th>Ensembl gene ID</th>
<th>baseMean</th>
<th>Fold change (KO/WT)</th>
<th>Padj</th>
<th>Gene Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSMUSG000000082809</td>
<td>177.98</td>
<td>5.65</td>
<td>2.52E-92</td>
<td>Pseudogene Gm14150</td>
</tr>
<tr>
<td>ENSMUSG00000063458</td>
<td>83.02</td>
<td>0.44</td>
<td>3.99E-20</td>
<td>Lrmda</td>
</tr>
<tr>
<td>ENSMUSG0000022629</td>
<td>31.47</td>
<td>1.93</td>
<td>1.07E-14</td>
<td>Kif21a</td>
</tr>
<tr>
<td>ENSMUSG00000105703</td>
<td>89.25</td>
<td>2.01</td>
<td>1.94E-13</td>
<td>Gm43305</td>
</tr>
<tr>
<td>ENSMUSG0000036273</td>
<td>137.37</td>
<td>0.54</td>
<td>8.10E-10</td>
<td>Lrrk2</td>
</tr>
<tr>
<td>ENSMUSG0000057897</td>
<td>58.28</td>
<td>1.66</td>
<td>1.17E-07</td>
<td>Camk2b</td>
</tr>
<tr>
<td>ENSMUSG0000032679</td>
<td>297.27</td>
<td>1.55</td>
<td>1.02E-04</td>
<td>Cd59a</td>
</tr>
<tr>
<td>ENSMUSG0000025453</td>
<td>784.61</td>
<td>1.43</td>
<td>9.56E-04</td>
<td>Nnt</td>
</tr>
</tbody>
</table>

Genes with padj < 0.01. LRRK2 KO/WT pBMDM cells. Genes not represented at the protein level are displayed in grey.
Visualisation of these transcriptional responses affords an appreciation of the broad characteristics of the MDP and LPS responses, as well as similarities and differences between WT and LRRK2 macrophages in their response to innate immune activation. Transcriptional responses were visualised in two ways: MA plots and Volcano plots. Information provided by these methods of visualisation overlap, but combine to provide a good overview of the transcriptional response.

The shapes of MA plots help to confirm that processing of mRNA counts was performed successfully by the distribution of plotted genes. As the mean number of counts increases, the fold change required to reach a statistically significant difference between treated and untreated conditions decreases. Both LPS and MDP treated cells showed a weighting towards gene up-regulation upon stimulation, with a greater number of genes plotted above log fold change = 0 than below (fig 46). Interestingly, for LPS treated cells, it appears that a greater number of genes are up-regulated strongly in LRRK2 KO macrophages than in WT macrophages (fig 46a,b). Gene distribution otherwise appeared similar upon LPS stimulation, and no obvious bulk differences could be seen in the MDP transcriptional response (fig 46c,d).

‘Volcano plots’ provide a similar verification of mRNA count analysis with statistical significance of differential expression of genes between samples increasing with fold change of that gene between treated and untreated samples, giving a characteristic ‘volcano shape’. This characteristic distribution of genes can be seen for all data sets (fig 47). As was observed in MA plots, it can be seen that there is a greater weighting of up-regulated genes than down-regulated, this time seen through a larger number of genes plotted above log2 fold change = 0 than below. No obvious differences can be seen between WT and LRRK2 KO genotypes for either LPS or MDP treatment.
Figure 46: MA plot visualisation of transcriptional gene responses. Dots represent individual genes. Red indicates padj < 0.01. a,c: WT pBMDM cells. b,d: LRRK2 KO pBMDM cells.
Figure 47: ‘Volcano plot’ visualisation of transcriptional gene responses. Dots represent individual genes. Red indicates padj < 0.01. a,c: WT pBMDM cells. b,d: LRRK2 KO pBMDM cells.
In order to quantify these observations in bulk gene transcription, significantly differentially expressed genes between WT and LRRK2 KO genotypes were filtered for significance, then categorised with regard to whether genes were found to be up or down-regulated uniquely in one genotype, shared between both genotypes, or whether a gene was up-regulated in one genotype and down-regulated in another.

As observed previously in both LPS and MDP experiments, a greater number of genes were up-regulated than down-regulated. LPS treatment led to 3192 genes being up-regulated and 2696 down-regulated (fig 48a). MDP treatment had a smaller effect while 1020 genes were up-regulated, compared to 676 down-regulated (fig 48b). Furthermore, quantification confirmed that a greater number of genes were significantly differentially expressed upon LPS treatment in LRRK2 KO macrophages than WT macrophages. Perhaps the most interesting observation from this analysis is that a single gene was found to be down-regulated in WT macrophages and up-regulated in LRRK2 KO macrophages upon treatment with LPS (fig 48a). Transcription of this gene, Rapgef3 is almost halved upon LPS treatment in WT macrophages while being increased just over 7-fold in LRRK2 KO macrophages; a complete reversal in transcriptional regulation upon loss of LRRK2.
Figure 48: Quantification of up and down-regulated differentially expressed genes upon innate immune stimulation. Scaled Venn diagrams. Gene numbers at padj < 0.01. **a:** LPS treated macrophages. **b:** MDP treated macrophages.
5.3.4 Two-parameter analysis identifies differentially responding genes

Analysis so far could be described as conventional differential gene expression analysis, performed once in WT macrophages and once in LRRK2 KO macrophages for each treatment, followed by manual comparisons between results from these experiments. A more powerful analysis is able to consider the parameter of innate immune treatment and the parameter of LRRK2 genotype together, in order to isolate differences between LRRK2 genotype that are specifically owing to innate immune stimulation (fig 49). This two-parameter analysis provides a method to identify specifically ‘differentially responding genes’ between genotypes. Such a method was adapted from the ‘edgeR Users Guide’ (Chen et al., 2008; Robinson et al., 2009) and revealed eleven differentially responding genes to LPS (Padj < 0.1) (fig 50a,b). All differentially responding genes showed an increased level of transcription upon LPS stimulation in LRRK2 KO cells compared to WT cells. As expected, Rapgef3 was identified as an overwhelmingly differentially regulated gene upon LPS stimulation, reflecting the reversal in regulation from down regulation to significant up-regulation with the loss of LRRK2 observed in the previous analysis (fig 50b). In comparison, MDP treatment identified no significantly differentially responding genes (fig 50c,d). This aligns with the relatively mild and slow acting nature of MDP compared to LPS stimulation, but perhaps more importantly, demonstrates a high stringency of the applied method to identify differentially responding genes, including those identified upon LPS treatment.

![Figure 49: Two-parameter analysis.](image)

LRRK2 genotype, and treatment with innate immune stimuli, are considered together in this analysis to identify a single set of differentially responding genes for each treatment.
Figure 50: Visualisation of differentially responding genes. Fold changes are ligand treated gene expression levels (LRRK2 KO/WT). Dots represent individual genes. Red dots indicate padj < 0.1.
Interestingly, of identified differentially responding genes to LPS, three pro-inflammatory chemokines were identified including Ccl3, Ccl4, and Ccl5 (table 14). In comparison, no classical pro-inflammatory cytokines were identified. As well as chemokines themselves, the chemokine receptor Ccrl2 was identified. ‘Activating transcription factor 3’ (Atf3) appears to be biologically relevant to the role of LRRK2 in innate immunity, as it has previously been identified as a negative regulator of TLR4 (Gilchrist et al., 2006). Interestingly, the ‘hydroxycarboxylic acid receptor 2’ (Hcar2) has been previously linked to neurodegeneration and Parkinson’s disease, with the protein’s ligand niacin used by some as a supplement against the disease. ‘Ankyrin repeat and BTB/POZ domain containing protein-2’ (Abtb2) is a protein which has been linked to inhibition of aggregation of alpha-synuclein in neurons (Roy and Pahan, 2013). ‘Rap guanine nucleotide exchange factor 3’ (Rapgef3) is better known as ‘exchange factor directly activated by cAMP 1’ (EPAC1), and is by far the most significantly differentially regulated gene detected. ‘Tripartite motif-containing protein 25’ (TRIM25) is a ubiquitin ligase involved in the RIG-I pathway for the innate immune response to viral DNA (Sanchez et al., 2016). ‘Cysteine And Serine Rich Nuclear Protein 1’ (Csrnp1) is a transcription factor that is negatively regulated by
axin; which is in turn, a negative regulator of the Wnt pathway (Ishiguro et al., 2001). Finally, ‘MAX Dimerization Protein 1’ (Mxd1) is a transcriptional repressor for Myc binding (Cascón and Robledo, 2012).

To validate differentially responding genes identified by two-parameter analysis, qRT-PCR was applied. This focused on candidate genes that had been identified with strong statistical significance and a clear link to innate immunity. LPS treated WT and LRRK2 KO samples were directly compared for this analysis. A statistically significant increase in mRNA was observed in LRRK2 KO macrophages compared to LPS treated WT macrophages for all genes except that of Rapgef3, as determined by paired two-tailed t-tests (fig 51). While transcription of the Rapgef3 gene was not observed to be statistically significantly different in LRRK2 KO compared to WT macrophages, a p-value of 0.06 was still observed. This was most likely due to a large standard deviation between samples. Fold changes in mRNA of all tested significant genes was seen to be slightly lower by qRT-PCR than by RNAseq.

Figure 51: Transcript levels upon LPS stimulation. qRT-PCR with beta-actin and GAPDH as housekeeping genes. Each point is a different LRRK2 KO pBMDM sample compared to three different WT pBMDM samples. Error bars are S.E.M. Paired two-tailed t tests compared to a value of 1.
5.3.5 Pathway analysis

Two-parameter analysis revealed a relatively small number of differentially regulated genes between LRRK2 genotypes, therefore to complement this, pathway analysis was performed in order to compare innate immune stimulation of WT and LRRK2 KO macrophages on a signalling pathway level. Pathway analysis was performed by calculating gene enrichment against the ‘Reactome’ database of biological signalling pathways.

Pathways shared between WT and LRRK2 KO macrophages demonstrated that analysis was carried out successfully. Expected broad areas of biology such as ‘innate immunity’ were highlighted as shared between WT and LRRK2 KO macrophages. More specific pathways were also highlighted; ranging from ‘toll-like receptor signalling cascades’, to ‘interferon and interleukin signalling’ (appendix tables 3, 4). At the most stringent statistical cut off of differential gene expression, only strongly differentially expressed genes were included, explaining why similar results were observed for LPS treated (appendix table 3), and MDP treated (appendix table 4) macrophages.

In order to identify pathways that were affected by the knockout of LRRK2, pathway analysis was performed three times on the RNAseq dataset, using cutoffs for differential gene expression of padj < 0.01, 0.05 and 0.1. The resulting list of pathways from each analysis was then sampled at a high and low stringency for pathways uniquely enriched in either WT or LRRK2 KO macrophages. Therefore, in total, pathway enrichment analysis was performed six times for each treatment of each genotype (appendix tables 5-8). The reason such extensive analysis was performed was in order to be able to score identified pathways for robustness of identification. With a series of six analyses performed, pathways could be identified between one and six times per genotype: therefore, a score of six indicates robust identification, while pathways occurring only once were considered to be noise.
Table 15: Pathways enriched in a WT or LRRK2 KO specific manner after LPS stimulation. 100 ng/ml LPS. Data sampled at two levels of statistical significance of pathway enrichment. Frequency of occurrence of pathway across sampling is indicated in green. Unique occurrences in grey.

<table>
<thead>
<tr>
<th>WT Macrophages</th>
<th>LRRK2 KO Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Association of TriC/CCT with target proteins during biosynthesis</td>
<td>5 Signaling by NOTCH</td>
</tr>
<tr>
<td>4 PI Metabolism</td>
<td>5 TRAF3-dependent IRF activation pathway</td>
</tr>
<tr>
<td>4 Regulation of actin dynamics for phagocytic cup formation</td>
<td>4 Cytosolic sensors of pathogen-associated DNA</td>
</tr>
<tr>
<td>3 Downstream signal transduction</td>
<td>4 p38MAPK events</td>
</tr>
<tr>
<td>3 G alpha (12/13) signalling events</td>
<td>3 Loss of Nlp from mitotic centrosomes</td>
</tr>
<tr>
<td>3 Hemostasis</td>
<td>3 Loss of proteins required for interphase microtubule organization from the centrosome</td>
</tr>
<tr>
<td>3 Regulation of IFNG signaling</td>
<td>3 Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways</td>
</tr>
<tr>
<td>2 Constitutive PI3K/AKT Signaling in Cancer</td>
<td>3 Regulation of PLK1 Activity at G2/M Transition</td>
</tr>
<tr>
<td>2 GAB1 signalosome</td>
<td>3 The NLRP3 inflammasome</td>
</tr>
<tr>
<td>2 Interleukin-3, 5 and GM-CSF signaling</td>
<td>2 G2/M Transition</td>
</tr>
<tr>
<td>2 Interleukin-6 signaling</td>
<td>2 Inflammasomes</td>
</tr>
<tr>
<td>2 Negative regulators of RIG-I/MDA5 signaling</td>
<td>2 Interleukin-2 signaling</td>
</tr>
<tr>
<td>2 Phospholipid metabolism</td>
<td>2 Mitotic G2-G2/M phases</td>
</tr>
<tr>
<td>2 PI-3K cascade</td>
<td>2 Pre-NOTCH Expression and Processing</td>
</tr>
<tr>
<td>2 PI3K events in ERBB2 signaling</td>
<td>1 Antigen Presentation: Folding, assembly and peptide loading of class I MHC</td>
</tr>
<tr>
<td>2 PI3K events in ERBB4 signaling</td>
<td>1 Cell Cycle, Mitotic</td>
</tr>
<tr>
<td>2 PI3K/AKT activation</td>
<td>1 DEx/H-box helicases activate type I IFN and inflammatory cytokines production</td>
</tr>
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<td>WT Macrophages</td>
<td>LRRK2 KO Macrophages</td>
</tr>
<tr>
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<td>-------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>2 P13K/AKT Signaling in Cancer</td>
<td>1 Downstream signal transduction</td>
</tr>
<tr>
<td>2 PIP3 activates AKT signaling</td>
<td>1 ERKs are inactivated</td>
</tr>
<tr>
<td>2 Role of LAT2/NTAL/LAB on calcium mobilization</td>
<td>1 Extrinsic Pathway for Apoptosis</td>
</tr>
<tr>
<td>2 Synthesis of PIPs at the plasma membrane</td>
<td>1 G alpha (12/13) signalling events</td>
</tr>
<tr>
<td>2 TCR signaling</td>
<td>1 Interleukin receptor SHC signaling</td>
</tr>
<tr>
<td>2 The NLRP3 inflammasome</td>
<td>1 Interleukin-3, 5 and GM-CSF signaling</td>
</tr>
<tr>
<td>1 Downstream TCR signaling</td>
<td>1 Interleukin-6 signaling</td>
</tr>
<tr>
<td>1 ERK/MAPK targets</td>
<td>1 Lysosphingolipid and LPA receptors</td>
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<td>1 Fc epsilon receptor (FCERI) signaling</td>
<td>1 Pre-NOTCH Processing in Golgi</td>
</tr>
<tr>
<td>1 Folding of actin by CCT/TriC</td>
<td>1 Signaling by the B Cell Receptor (BCR)</td>
</tr>
<tr>
<td>1 Interleukin-2 signaling</td>
<td>1 TRAF6 mediated JRE7 activation</td>
</tr>
<tr>
<td>1 Interleukin-7 signaling</td>
<td></td>
</tr>
<tr>
<td>1 Regulation of IFNA signaling</td>
<td></td>
</tr>
<tr>
<td>1 Regulation of signaling by CBL</td>
<td></td>
</tr>
<tr>
<td>1 Role of phospholipids in phagocytosis</td>
<td></td>
</tr>
<tr>
<td>1 Signaling by EGFR in Cancer</td>
<td></td>
</tr>
<tr>
<td>1 Signaling by FGFR in disease</td>
<td></td>
</tr>
<tr>
<td>1 Signaling by FGFR1 fusion mutants</td>
<td></td>
</tr>
<tr>
<td>1 Signaling by PDGF</td>
<td></td>
</tr>
<tr>
<td>1 Signaling by SCF-KIT</td>
<td></td>
</tr>
<tr>
<td>1 TAK1 activates NFkB by phosphorylation and activation of IKKs complex</td>
<td></td>
</tr>
<tr>
<td>1 TRAF6 mediated NF-kB activation</td>
<td></td>
</tr>
</tbody>
</table>
Table 16: Pathways enriched in a WT or LRRK2 KO specific manner after MDP stimulation. 10 ug/ml MDP. Data sampled at two levels of statistical significance of pathway enrichment. Frequency of occurrence of pathway across sampling is indicated in green. Unique occurrences in grey.

<table>
<thead>
<tr>
<th><strong>WT Macrophages</strong></th>
<th><strong>LRRK2 KO Macrophages</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Dissolution of Fibrin Clot</td>
<td>5 FCGR activation</td>
</tr>
<tr>
<td>4 GAB1 signalosome</td>
<td>4 Role of phospholipids in phagocytosis</td>
</tr>
<tr>
<td>3 Downstream Signaling Events Of B Cell Receptor (BCR)</td>
<td>3 Cell surface interactions at the vascular wall</td>
</tr>
<tr>
<td>3 MAP kinase activation in TLR cascade</td>
<td>3 Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell</td>
</tr>
<tr>
<td>3 p38MAPK events</td>
<td>2 Signaling by SCF-KIT</td>
</tr>
<tr>
<td>3 Rho GTPase cycle</td>
<td>1 Cell junction organization</td>
</tr>
<tr>
<td>3 Signaling by Rho GTPases</td>
<td>1 DAP12 interactions</td>
</tr>
<tr>
<td>3 Signal by the B Cell Receptor (BCR)</td>
<td>1 Fc epsilon receptor (FCERI) signaling</td>
</tr>
<tr>
<td>2 Adaptive Immune System</td>
<td>1 FCERI mediated NF-kB activation</td>
</tr>
<tr>
<td>2 Constitutive PI3K/AKT Signaling in Cancer</td>
<td>1 GAB1 signalosome</td>
</tr>
<tr>
<td>2 Inflammasomes</td>
<td>1 Generation of second messenger molecules</td>
</tr>
<tr>
<td>2 Integrin alphaIIb beta3 signaling</td>
<td>1 Inflammasomes</td>
</tr>
<tr>
<td>2 NOTCH2 intracellular domain regulates transcription</td>
<td>1 p75NTR recruits signalling complexes</td>
</tr>
<tr>
<td>2 p75 NTR receptor-mediated signalling</td>
<td>1 Signaling by EGFR in Cancer</td>
</tr>
<tr>
<td>2 PI-3K cascade</td>
<td>1 Signalling by GPCR</td>
</tr>
<tr>
<td>2 PI3K events in ERBB2 signaling</td>
<td>1 Signaling by PDGF</td>
</tr>
<tr>
<td>2 PI3K events in ERBB4 signaling</td>
<td>1 The NLRP3 inflammasome</td>
</tr>
<tr>
<td>2 PI3K/AKT activation</td>
<td>2 PI3K/AKT Signaling in Cancer</td>
</tr>
<tr>
<td>2 PIP3 activates AKT signaling</td>
<td>2 PIP3 activates AKT signaling</td>
</tr>
<tr>
<td>WT Macrophages</td>
<td>LRRK2 KO Macrophages</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>2 Regulation of Lipid Metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)</td>
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<tr>
<td>2 Role of LAT2/NTAL/LAB on calcium mobilization</td>
<td></td>
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<tr>
<td>2 Signaling by PDGF</td>
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<tr>
<td>2 ZBP1(DAI) mediated induction of type I IFNs</td>
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</tr>
<tr>
<td>1 AKT phosphorylates targets in the nucleus</td>
<td></td>
</tr>
<tr>
<td>1 Cross-presentation of particulate exogenous antigens (phagosomes)</td>
<td></td>
</tr>
<tr>
<td>1 DAP12 interactions</td>
<td></td>
</tr>
<tr>
<td>1 DAP12 signaling</td>
<td></td>
</tr>
<tr>
<td>1 GPCR ligand binding</td>
<td></td>
</tr>
<tr>
<td>1 Hemostasis</td>
<td></td>
</tr>
<tr>
<td>1 Negative regulators of RIG-I/MDA5 signaling</td>
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</tr>
<tr>
<td>1 NGF signalling via TRKA from the plasma membrane</td>
<td></td>
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<tr>
<td>1 Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways</td>
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</tr>
<tr>
<td>1 PPARA Activates Gene Expression</td>
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<tr>
<td>1 Regulation of actin dynamics for phagocytic cup formation</td>
<td></td>
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<td>1 TRAF3-dependent IRF activation pathway</td>
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<td>1 Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds</td>
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It was noted that pathways could be enriched for both WT and LRRK2 KO macrophage datasets. This is as when applying different differential gene expression cut-off values, a different set of genes was analysed. Robustness of this analysis was observed from the fact that those pathways with the highest frequency of observation were far less likely to be enriched in both genotypes. By comparing LPS datasets, a range of areas of biology were identified (table 15). Notably some of the highest scoring pathways related to phospholipid metabolism and signal transduction, and phagocytosis. Other areas of biology such as protein biosynthesis and signal transduction were identified. Signal transduction pathways were diverse, including ‘G-protein coupled receptors’, ‘phospholipid signalling’, and ‘direct innate immune signalling pathways’ such as ‘cytosolic sensors of pathogen-associated DNA’, and ‘p38MAPK events’. There was some similarity in differentially regulated pathways upon MDP treatment of macrophages; including MAPK activation and phospholipid related signalling (table 16). A stronger representation of cytoskeleton associated pathways where reported as differentially regulated by LRRK2 upon MDP stimulation, with several levels of the Rho GTPase signalling pathway reported, as well as pathways directly relating to phagocytosis, including ‘FCGR activation’.
5.3.6 Metabolomics

Shotgun lipidomics was carried out in parallel to RNA sequencing. Macrophages were treated with 100 ng/ml of LPS or 10 μg/ml MDP for two hours, or were starved of nutrients for two hours by replacement of growth media with EBSS. Following treatment, methanol extraction of lipids and fatty acids was performed, and resulting metabolites analysed by mass spectroscopy. ‘Partial least squares discriminant analysis’ (PLS-DA) revealed dramatic differences in lipid composition between samples from nutrient starved macrophages and remaining samples (fig 52a). This reflects dramatic changes in lipid composition as lipids are used as an energy source. A difference between WT and LRRK2 KO macrophages was the next most prevalent factor in lipid composition, suggesting LRRK2 does have an effect on lipid metabolism. To assess the affect of innate immune treatment on lipid composition, PLS-DA analysis was performed on WT and LRRK2 KO datasets to generate separate models (fig 52b). As before, starvation accounted for the most significant change in lipid composition, however separation was also observed of MDP treated samples as compared to LPS treated, or untreated cells. This separation of MDP treated samples was present in both WT and LRRK2 KO models, but appeared to be stronger in WT macrophages, suggesting LRRK2 may be affecting lipid composition following MDP treatment.

To assess the contribution of ceramides to overall changes in lipid composition between treatments, loadings plots were generated for each genotype and the distribution of ceramides examined by PLS-DA analysis (fig 53a). In WT macrophages, clustering of ceramides was clearly visible separated from PLS-DA axis 1 and 2. In comparison, much weaker separation of ceramides was visible in LRRK2 KO macrophages, with no separation evident on PLS-DA axis 2 (fig 53b).
Figure 52: PLS-DA plots of lipid composition. a: Combined model of all samples. b: Separated models of WT and LRRK2 KO macrophages.
Figure 53: Loadings plots suggest an LRRK2 dependent effect of MDP treatment on ceramide metabolism. Greyed out symbols are non-ceramide metabolites. 

a: WT macrophages. b: LRRK2 KO macrophages.
To address ceramide metabolism more directly, the proportion of ceramide compared to total measured metabolite was analysed. In untreated macrophages, no statistically significant differences were observed between WT and LRRK2 KO genotypes (fig 54a). However, treatment with MDP resulted in a statistically significant increase in total ceramide compared to untreated cells in WT macrophages, while no difference was observed in LRRK2 KO macrophages. In comparison, no changes in total ceramide were observed upon LPS treatment of macrophages (fig 54b). This indicates an LRRK2 dependent increase in ceramide is observed in macrophages upon MDP stimulation.

In total, twelve different ceramide species could be detected by mass spectroscopy. These are identified by the total number of carbons in the molecules fatty acid acyl tails, followed by the number of double bonds in the acyl tails. For instance, ‘Ceramide (42:2)’ will contain fatty acid acyl groups with a total of 42 carbons, and 2 double bonds. It should be noted that this does not uniquely identify a ceramide, as it does not provide information of the distribution of carbons or double bonds across the two fatty acid acyl chains found on a ceramide. Of twelve detected ceramide species, eight revealed LRRK2 dependent increases upon MDP stimulation, with the four remaining ceramide species detected at relatively low levels (fig 55). In comparison, only the proportion of ceramide (36:1) was increased upon LPS stimulation (data not shown).
**Figure 54: Proportion of ceramides.** Error bars are S.E.M. of samples extracted from three mice. Stats are paired two-tailed t tests. 

a: Untreated macrophages. 
b: 10 g/ml MDP or 100ng/ml LPS treated macrophages as indicated.
Figure 55: Individual ceramide species respond to MDP treatment in an LRRK2 dependent manner. Error bars are S.E.M. of samples extracted from three mice. Stats are paired two-tailed t tests.
5.4 Discussion

The use of RNA sequencing technology allowed the role of LRRK2 to be examined across the macrophage transcriptome in response to inflammatory stimuli. Thus, the role of LRRK2 in the innate immune system could be probed very broadly. The transcriptome is dynamic in the innate immune response, changing over time. In line with previous experiments, a time-point of two hours after innate immune treatment was chosen for RNA extraction. This was deemed appropriate to detect early changes in gene expression profile, which may be modulated by LRRK2, without being complicated by secondary effects of cytokine signalling on the transcriptome.

Reads obtained from RNA sequencing were of high quality and sufficient depth for differential gene expression analysis with a mean read depth of 22.2 million reads/sample, of which 19.5 million reads could be uniquely mapped to gene encoding regions of the genome. As a guideline, a significant positive correlation has been described between the detection of all but highly-abundant genes, with increasing read depth from 1.6 million to 10 million reads. No significant improvement was then detected between 10 million and 20 million reads (Wang et al., 2011). This places the present study as sufficient for the description of differential gene expression of all but very poorly expressed genes. Read quality control was performed by ‘Cambridge Genomic Services’ using an automated pipeline, with manual oversight. Reads were judged to be of high quality by criteria including ‘per base sequence content’, ‘per sequence GC content’, ‘sequence length distribution’, and ‘sequence duplication levels’. Around 90 % of reads were uniquely mapped to genes, with only a small proportion of reads mapped to ribosomal, intronic or intergenic regions of the mouse genome. Ambiguity in read mapping due to sequence alignment with multiple genes could be minimised by the use of longer read lengths or paired-end sequencing, however 90 % of successfully mapped reads with the read depth described was sufficient to proceed with analysis using DEseq2.

It is interesting that only a low number of differentially expressed genes were observed between LRRK2 genotypes under resting conditions. This may indicate that LRRK2 only exerts an effect on the macrophage transcriptome under
stimulated conditions. This would agree with previous observations that LRRK2 expression can be induced upon Ifn-γ or LPS stimulation. This finding also reflects work by another group that identified no changes in gene expression in unstimulated human fibroblasts or brain tissue between G2019S LRRK2 carriers and controls (Devine et al., 2011). No obvious connection exists between the genes that are differentially expressed under unstimulated conditions, but previously explored themes of LRRK2 interactions with the cytoskeleton and innate immunity do arise. This is through the microtubule motor protein, KIF21A; and a regulator of the membrane attack complex, CD59a (Brooimans et al., 1992; Marszalek et al., 1999; Baalasubramanian et al., 2004). KIF21A expression is enriched in the brain, with relatively low expression in innate immune cells. The fact that NNT is a regulator of oxidative stress is interesting, as this is a pathway strongly linked to degeneration of dopaminergic neurons (Rydström, 2006; Bolam and Pissadaki, 2012). In macrophages, oxidative stress acts via the extracellular matrix to increase cell adhesion while also increasing inflammatory cytokine release via inhibition of ‘histone deacetylase 2’ (HDAC-2) (Kirkham, 2007).

By comparing bulk transcription data, it can be seen that compared to treatment with LPS, a smaller number of differentially expressed genes were detected upon MDP treatment. This is to be expected given the requirement for MDP to pass through the cell membrane for detection by the cytosolic NOD2 receptor (Girardin et al., 2003). MDP detection by NOD2 is also known to lead to an attenuated response compared to detection of PAMP molecules by TLRs (Moreira and Zamboni, 2012). Visualisation of data revealed that LRRK2 KO macrophages appeared to be more responsive to differential up-regulation in response to LPS than WT macrophages. This was confirmed by quantification of gene expression data, and also found to be true for differential down-regulation of gene transcription in response to LPS. It is possible that this observation could be a consequence of lower variability of gene expression in LRRK2 KO macrophages compared to WT macrophages; a methodological and statistical issue rather than an effect of LRRK2. Such a systematic error would be expected to manifest in the MDP treated dataset. However, in contrast to LPS datasets, treatment of macrophages with MDP resulted
in remarkably similar bulk gene responses between LRRK2 genotypes. This suggests that LRRK2 may indeed act to ‘dampen’ the overall LPS transcriptional response in macrophages.

With this in mind, it is interesting that following two-parameter analysis, all LPS differentially responding genes demonstrated enhanced transcription in LRRK2 KO macrophages compared to WT. A similar effect was previously seen in qRT-PCR experiments for TLR2. Manual inspection of RNA sequencing data confirmed enhanced TLR2 transcription in LRRK2 KO macrophages compared to WT macrophages upon either LPS or MDP stimulation (data not shown). By carrying out two-parameter analysis, WT and LRRK2 KO datasets could be combined to give a rigorous analysis of statistically significantly responding genes to a treatment and between LRRK2 genotype (Chen et al., 2008; Robinson et al., 2009). Only a small number of differentially responding genes could be identified this way, with none identified between MDP treatment datasets. The most striking finding of this analysis is that of 11 differentially responding genes to LPS stimulation, three are chemokines, and one is a chemokine receptor-like protein. CCL3 and CCL4 form isoforms of ‘macrophage inflammatory protein 1’; MIP-1α and MIP-1β respectively. CCL5 is also known as ‘regulated on activation, normal T cell expressed and secreted’ (RANTES). These chemokines are all members of the ‘CC Chemokine/Receptor family’ and share a common receptor in CCR5. CCL3 and CCL5 may also bind CCR1, while CCL5 binds a further receptor; CCR3 (Zlotnik and Yoshie, 2000). These chemokines are all classified as pro-inflammatory, meaning they are induced by inflammatory stimuli to recruit inflammatory cells to a site of inflammation. This is as opposed to homeostatic chemokines, which are constitutively expressed in certain tissues (Turner et al., 2014). CCRL2 is chemokine receptor-like protein, with over 40 % sequence identity to CCR1, CCR2, CCR3 and CCR5 (Migeotte et al., 2002), and highest amino acid sequence similarity to CCR1. Interestingly, CCRL2 has been reported as a non-canonical receptor for CCL5 (Yoshimura and Oppenheim, 2011); as well as CCL19 and chemerin (Akram et al., 2016). While there have been mixed reports as to the effect of LRRK2 on cytokine production, very little interest has been shown in the potential effect of LRRK2 on chemokine production; which makes these
findings particularly exciting. A microarray screen of unstimulated mouse microglia has shown that CX3CR1, a non-canonical chemokine receptor of fractalkine, expressed exclusively in microglia, is upregulated by knockout of LRRK2 (Ma et al., 2016). This reaffirms that chemokine responses may be involved in LRRK2 biology in diverse immunological contexts.

A number of transcription factors have been identified by two-parameter analysis of the LPS dataset. ATF3 is a negative regulator of pro-inflammatory TLR4 signalling, acting as part of the LPS induced negative-feedback loop (Gilchrist et al., 2006). Knockout of LRRK2 causes increased transcription of this regulator upon LPS stimulation, meaning that in the simplest scenario, it might be expected that the over-active kinase G2019S mutation of LRRK2 would lead to a decrease in transcription of Atf3; resulting in attenuated negative feedback of TLR4 signalling, enhanced inflammation, and greater neuronal stress. Two further transcription factors were identified by two-parameter analysis. MXD1 and CSRNP1. MXD1 acts in a network with MYC and MAX, forming the MYC/MAX/MXD1 axis (Cascón and Robledo, 2012). MXD1 is in competition with MYC for the binding of MAX; with balance of MXD1/MAX and MYC/MAX dimers controlling transcriptional output. Implications of the disruption of this regulatory axis are mainly studied in cancer, where overexpression of MYC is a common pathway to disease (Dang, 2012). As part of its disease causing function, MYC signalling has been shown to affect cell adhesion, cell shape, and reduce cell migration through modulation of the actin cytoskeleton (Liu et al., 2012); this in turn is thought to occur by inhibition of JNK (Ma et al., 2017). Functional relevance to LRRK2 exists here, as LRRK2 is a member of the MLK family of MAPKKK proteins. Indeed, the G2019S overactive kinase mutation of LRRK2 has been linked to activation of the MKK4-JNK pathway in neurons, resulting in disease (Chen et al., 2012). Finally, CSRNP1 is a transcription factor that is upregulated by Axin, as well as inflammatory stimuli in the form of IL-2 (Ishiguro et al., 2001; Gingras et al., 2007). Axin is a negative regulator of the Wnt signalling pathway, acting to sequester the transcription factor β-catenin to the cytoplasm (Nakamura et al., 1998). Relevance of CSRNP1 to LRRK2 comes not just from induction by inflammatory stimuli, but also through interaction with the Wnt
pathway. LRRK2 has been connected to three major components of canonical Wnt signalling: dishevelled (Sancho et al., 2009), ‘lipoprotein receptor-related protein 6’ (LRP6) (Berwick and Harvey, 2012), and ‘glycogen synthase kinase 3 beta’ (GSK3β) (Lin et al., 2010). Interestingly, studies have shown LRRK2 to affect Wnt signalling remarkably similarly to axin, with LRRK2 suggested to inhibit Wnt signalling in the absence of stimulation by participating in the β-catenin destruction complex, but enhancing formation of Wnt signalosomes at vesicular membranes following Wnt stimulation; increasing Wnt signalling activity (Berwick and Harvey, 2013). The significance of parallels between CSRNP1 and Axin is unknown, as little is known of the function of CSRNP1 other than that there is redundancy with other members of the Csrnp family (Gingras et al., 2007). However, a growing appreciation of Wnt signalling in modulating the macrophage inflammatory response is developing (Schaale et al., 2011). While these studies highlight the interaction of LRRK2 with canonical Wnt signalling, it should not be overlooked that LRRK2 has also been linked to other branches of Wnt signalling. For example, LRRK2 may play a role in Wnt ‘planar cell polarity’ (PCP) signalling after a connection was established by proteomics (Salašová et al., 2017). The PCP pathway involves the cytoskeletal adaptors Rac, Cdc42 and RhoA, as well as JNK; all of which have been discussed as interaction partners of LRRK2. Similarly, the Wnt-Ca^{2+} pathway results in nuclear translocation and activation of NFAT; a protein that has been shown to be maintained in the cytoplasm by a non-canonical pathway involving interaction with LRRK2 (Liu et al., 2011). The regulation of Csrnp transcription by LRRK2 reflects and strengthens the established role of LRRK2 in Wnt signalling.

Two genes identified by two-parameter analysis can be linked directly to Parkinson’s disease. Abtb2 encodes ‘Ankyrin-rich BTB/POZ domain containing protein-2’ (BPOZ-2). This protein is the subject of attention in Parkinson’s research, as it appears to cause inhibition of alpha-synuclein aggregation (Roy and Pahan, 2013). Lentiviral delivery of the BPOZ-2 gene appears to stimulate autophagic clearance of alpha-synuclein, resulting in reduced alpha-synuclein pathology in the basal ganglia (Roy et al., 2016). It is interesting that such a gene should be identified in macrophages, however the physiological significance is not clear, as alpha-
synuclein pathology is only abundant in a neuronal context. Another protein identified by two-parameter analysis is the G protein-coupled receptor, HCAR2, otherwise known as ‘niacin receptor 1’. Ketone bodies are thought to be the main substrate for HCAR2, however activation by niacin is also observed. Niacin has been touted by many as a treatment for Parkinson’s disease, although evidence is lacking (Wakade et al., 2014; Wakade and Chong, 2014). Activation of HCAR2 in macrophages has an anti-inflammatory effect. The precise mechanism of this effect is a matter of on-going research, however activation by niacin results in inhibition of CCL2 induced macrophage migration (Lukasova et al., 2011), as well as an inhibited response to LPS stimulation; as measured by Nf-κB activation (Digby et al., 2012) or inflammatory cytokine release (Zandi-Nejad et al., 2013). A recent report has suggested that inflammatory inhibition by HCAR2 activation is mediated via a Gβγ-PKC-ERK1/2 pathway (Shi et al., 2017). Unfortunately, activation of ERK1/2 by niacin in this pathway induces a strong flushing response in humans, limiting the use of niacin as a potential treatment for inflammatory disease (Richman et al., 2007). LRRK2 has been suggested to be phosphorylated by PKC zeta; a Ca2+ and diacylglycerol-independent kinase that functions in the PI3K pathway and MAPK cascade (Zach et al., 2010). HCAR2 activation also leads to G,-mediated inhibition of adenyl cyclase, resulting in reduced cAMP levels, and increased levels of prostaglandins via activation of cyclooxygenase (Offermanns and Schwaninger, 2015).

Rapgef3 is the strongest differentially responding gene to LPS, with an 11-fold difference in expression observed between LRRK2 KO and WT macrophages following LPS treatment. Rapgef3 was also identified following analysis of individual RNA sequencing datasets as the only gene to experience inhibited transcription in WT cells, and enhanced transcription following LRRK2 knockout. EPAC1 is the protein encoded by Rapgef3. While HCAR2 activation reduces intracellular cAMP, EPAC1 acts down-stream of cAMP. EPAC1 is a GEF that functions in a parallel branch of cell signalling to PKA; resulting in a wide range of cellular consequences, many of which are mediated through a direct interaction with Rap1 (de Rooij et al., 1998). Downstream of Rap1 signalling is Rac1 (Arthur et al., 2004). Rac1 controls many
areas of biology, but has a strong association with the cell cytoskeleton via Arp2/3 and Cofilin (Bid et al., 2013). It is therefore unsurprising that overexpression of EPAC1 leads to strong phenotypes in cell morphology. In primary monocytes, the EPAC1-RAP1 axis has been shown to affect cell adhesion as well as PKA-independent chemotaxis towards CCL2 (Lorennowicz et al., 2006). However, a later report suggests that EPAC1 only carries out immunological functions in mature macrophages, and in conjunction with PKA (Bryn et al., 2006). While the specific effect of cAMP and downstream signalling on migration appears to be highly context dependent (dose, time, space, cell type) (Howe, 2004), it seems indisputable that EPAC1 does play a role in chemotaxis of macrophages. In the light of RNA sequencing results, EPAC1 may underlie reported LRRK2 chemotatic phenotypes, and perhaps even explain contradictory results obtained by different groups (Choi et al., 2015; Moehle et al., 2015). It would be interesting to combine chemotaxis assays on WT and LRRK2 KO macrophages with cAMP or a EPAC1 specific analogue of cAMP: 8-CPT-2'-O-Me-cAMP (Kang et al., 2003). Linking EPAC1 to innate immune signalling more directly, it was shown that EPAC1 activation induces expression of ‘suppressor of cytokine signalling 3’ (SOCS-3) in response to cAMP, leading to potent anti-inflammatory effects (Sands et al., 2006), and may also modulate phagocytosis in neutrophils (Scott et al., 2016). Intriguingly, EPAC1 has been linked to Ca^{2+} release in myocytes, through activation of ‘calcium-calmodulin-dependent protein kinase II’ (CAMK2) (Pereira et al., 2007). CAMK2 is a holoenzyme consisting of a variable, but tissue specific mix of six CAMK2 subunits (Hanson and Schulman, 1992); of which the CAMK2β subunit was earlier identified as a differentially expressed gene between unstimulated WT and LRRK2 KO macrophages. EPAC1 and CAMK2 are further associated in myocardial hypertrophy, a process which involves functional modulation of cell morphology, cytoskeletal dynamics, and protein synthesis; all of which are functions also attributed to LRRK2 in different cellular contexts. In myocytes, EPAC1 induces hypertrophy via Ca^{2+} dependent activation of Rac, calcineurin and the downstream transcription factor NFAT (Morel et al., 2005). If LRRK2 is involved in this pathway through modulation of EPAC1 expression, then this Ca^{2+} dependent modulation of NFAT would complement the previously described Ca^{2+} independent LRRK2-NFAT pathway (Liu et al., 2011). Indeed, Ca^{2+} signalling is
emerging as playing a role in innate immune signalling (Feske, 2007). Finally, EPAC1 activation has been linked to enhanced neurite outgrowth in both PC12 and SH-SY5Y neuronal cell lines (Mains et al., 1990; Monaghan et al., 2008). It is remarkable that the strongest gene to be identified in this screen is tied to so many areas of LRRK2 biology. As mentioned, EPAC1 is a cAMP regulated gene, functioning in parallel to PKA. As such, untangling the respective contributions of EPAC1 and PKA to cAMP induced processes is an on-going process. Indeed, several connections exist between LRRK2 and PKA, with LRRK2 thought to be both a ligand of PKA (Li et al., 2011; Muda et al., 2014), and a negative regulator of PKA activity (Parisiadou et al., 2014). Therefore, it may be necessary to review where EPAC1 may be contributing to these observed effects.

TRIM25 is involved in the antiviral response by regulating the formation of the RIG-I signalosome, leading to fine-tuning of interferon production (Sanchez et al., 2016). The RIG-I signalosome is formed by interaction of the CARD domain of MAVS with a tetrameric assembly of CARD domains formed by RNA-bound RIG-I. RIG-I CARD domains are only available for MAVS binding when activated by RNA binding, and it is this active form of RIG-I that may be stabilised by TRIM25 activity. TRIM25 generates K63 polyubiquitin chains that bind around and stabilise active RIG-I, enhancing anti-viral responses and IRF3 activation (Blander, 2014).

To complement the specificity of genes identified by two-parameter analysis, pathway analysis against the reactome database identified broad areas of biology that appeared to be perturbed by LRRK2 KO in the context of LPS or MDP stimulation. Perhaps the most notable feature of these results was the strong representation of pathways relating to modulation of the cellular cytoskeleton, in both LPS and MDP datasets. These include relatively specific pathways such as ‘signalling by Rho GTPases’, ‘Rho GTPase cycle’, and events further upstream of Rho GTPases, such as ‘G alpha (12/13) signalling events’. Identification of these pathways corresponds with the idea that LRRK2 is modulating cytoskeletal dynamics in an inflammatory context. Indeed, phagocytosis related pathways are identified in pathway analysis. In response to LPS, ‘regulation of actin dynamics for phagocytic cup formation’ is identified, while in response to MDP stimulation, ‘FCGR activation’
is identified as modulated by LRRK2, as well as ‘role of phospholipids in phagocytosis’. Indeed phospholipid metabolism is another set of pathways that appear to be strongly identified in this analysis. Many aspects of PI3K signalling and metabolism are weakly identified as LRRK2 dependent, following stimulation with either LPS or MDP, as well as the ‘GAB1 signalosome’, required for activation of PI3K activity. Phospholipid metabolism is important in a wide range of cellular signalling, but is notable for its role in regulating actin dynamics and phagocytosis via Rho GTPases (Araki et al., 1996; Beemiller et al., 2010; Schlam et al., 2015). Innate immune signalling processes were identified as expected, however it is perhaps surprising that these were less prevalent than the pathways mentioned above, given that cells had been activated by PRR ligands. LPS stimulation resulted in differences in ‘p38MAPK events’, but also ‘cytosolic sensors of pathogen-associated DNA’, ‘Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways’ and ‘the NLRP3 inflammasome’. These latter pathways relate to cytosolic sensors and signalling molecules for the detection and immunological response to intracellular PAMPs (Rathinam et al., 2012). In comparison, stimulation with MDP led to differential expression of genes involved in ‘MAP kinase activation in TLR cascade’, and ‘p38MAPK events’; signalling events that generally occur downstream of cell surface PRRs (Akira and Takeda, 2004). This is intriguing given that LPS is primarily detected at the cell surface by TLR4, and MDP is detected by intracellular NOD2. Ultimately, pathway analysis provides a way to look broadly at RNA sequencing data and identify trends that may be missed by looking only at specific genes identified by two-parameter analysis. Pathways identified this way reaffirm the idea that LRRK2 is involved in regulation of the cytoskeleton of macrophages, and resulting immunological processes such as motility and phagocytosis. Perhaps in the light of identified differentially regulated genes such as Rapgef3 (encoding EPAC1), examination of phagocytosis and macrophage motility and chemotaxis should be revisited with a focus on cAMP induced signalling.

Isolation and analysis of metabolites from macrophages revealed no difference in ceramide levels between WT and LRRK2 KO unstimulated macrophages. This is in contrast to observations made in brain homogenate
(Ferrazza et al., 2016). In fact, a non-significant trend of decreasing ceramide was observed in LRRK2 KO macrophages. The metabolism of ceramides is not straightforward. Six ceramide synthase isoforms exist in mammalian cells, each with different tissue specificities and substrate preference for the generation of ceramides. On top of this, cell types display differences in the balance of de novo ceramide synthesis compared to ceramide salvage through the breakdown of more complex sphingolipids and glucosylceramide (Gillard et al., 1998; Mullen et al., 2012). Due to this, it is unsurprising that differences in ceramide levels in unstimulated cells do not replicate what is seen in the brain. Stimulation of macrophages with MDP did result in an increase in ceramide levels. This increase was dependent on the expression of LRRK2, suggesting LRRK2 does regulate pathways responsible for the generation of ceramide in macrophages as well as neurons. In contrast to MDP, stimulation with LPS did not result in an increase in ceramide, most likely due to the early time point at which metabolites were isolated after stimulation. Ceramide de novo synthesis is induced by lipid A only after approximately 12 hours (Sims et al., 2010). Whether ceramide increase is exacerbated by pathogenic LRRK2 mutants remains to be addressed. As discussed, ceramide accumulation is able to activate the NLRP3 inflammasome leading to inflammation (Vandanmagsar et al., 2011). As a counter-point to this, accumulation of glucosylceramide following GBA1 deficiency has also been shown to drive inflammation, via the formation of anti-glucosylceramide immune complexes (Pandey et al., 2017). In neurons, accumulation of glucosylceramide has even been shown to stabilise oligomeric forms of alpha-synuclein, driving neuroinflammation and degeneration (Mazzulli et al., 2011). Clearly, perturbation of ceramide metabolism can cause inflammatory dyshomeostasis by a range of mechanisms. Indeed, ceramide salvage pathways have been suggested to be more important to macrophage inflammasome driven inflammation than de novo ceramide synthesis (Camell et al., 2015). Therefore, an increase in ceramide may cause a corresponding decrease in other ceramide derivatives including other sphingolipids and sphingosine; with overall consequences on immunological signalling. A shotgun approach of lipids analysis using ESI-QTOF was applied in these experiments. This method lacks the resolution to identify specific ceramides. Given findings made
during this study, a future experiment should be designed to analyse not just ceramides, but also sphingolipids and sphingosine; molecules which may also be modulated during innate immune stimulated lipid metabolism. By applying a triple quadrupole approach, specific identification of these lipids can be achieved. Triple quadrupole mass spectrometry employs fragmentation of lipids of interest, allowing analysis of lipid fatty acid acyl chain masses; facilitating high-resolution detection and identification of lipids. Future experiments should also follow ceramide levels at later time points, to assess if LRRK2 is involved in TLR4 induced ceramide generation.

Summary

To summarise, the application of global analysis techniques has allowed the identification of roles of LRRK2 in the innate immune response. It is interesting to observe how some of these roles fit into areas of biology already described, but in a new context. For example, identification of Csrnp1 as an LRRK2-differentially responding gene to LPS stimulation ties into association of LRRK2 with the Wnt pathway, but now in an immunological context. Another example is the association of LRRK2 with cAMP signalling raised by EPAC1 differential regulation by LRRK2. The reciprocal regulation between LRRK2 and PKA has been described in the context of neurons, but an association of the parallel cAMP signalling regulated by EPAC1 with LRRK2 has never been described. Indeed that EPAC1 activation has been previously linked to neurite extension in neurons, a highly reproducible phenotype of LRRK2 knockout or mutation, reinforces the fundamental efficacy of exploring neuronal LRRK2 functions in an innate immune context.

The identification of EPAC1 and CAMK2β ties LRRK2 to Ca\(^{2+}\) signalling in macrophages, coinciding with previous reports of LRRK2 involvement in the regulation of NFAT signalling and the Wnt-Ca\(^{2+}\) pathway. These areas of LRRK2 involvement are broad areas of biology that are emerging as relevant to innate immune signalling. Genes and pathways identified here also tie LRRK2 to better understood areas of biology, as exemplified by the small network of chemokine proteins CCL3, CCL4, CCL5, the chemokine-like receptor CCRL2, EPAC1, and HCAR2 which appears to regulate inflammation via CCL2; and has been connected to a
cAMP dependent mechanism. There are conflicting reports on the effect of LRRK2 on macrophage cytokine release, but almost nothing published on the links between LRRK2 and chemokines. These results reflect previous work that was aimed at identifying immunological functionality of LRRK2 interactions with the cell cytoskeleton and vesicular trafficking machinery. Genes identified by RNA sequencing reaffirm this association, via Camk2b that associates directly with the cell cytoskeleton; as well as EPAC1, which regulates the Rho GTPase protein Rac1. Reactome pathway analysis places further emphasis on a role of LRRK2 in membrane dynamics and cytoskeletal processes such as phagocytosis, but also in the regulation of phosphatidylinositol processing. Lipid analysis revealed no differences between ceramide levels in resting macrophages, in contrast to findings made in brain tissue, but also revealed an LRRK2 dependent increase in ceramide levels in response to MDP stimulation.
6. Project discussion

6.1 Taking results into the future

The task of identifying the role of LRRK2 in innate immunity has been approached from multiple angles during this project. These include both structural and functional studies, and a focus that has ranged in scale from biophysical features of LRRK2, all the way up to genome wide analysis of the effect of LRRK2 knockout on the macrophage response to innate immune stimuli. As a result, these approaches have managed to answer specific questions that have been raised by contradictory reports within the LRRK2 literature, while also uncovering apparent new areas of LRRK2 biology that may prove important to our understanding of the underlying pathological mechanisms of Parkinson’s disease, and inflammatory disease more generally.

Structural studies were focused on the LRR domain of LRRK2, a non-enzymatic domain directly adjacent to S910 and S935 phosphorylation sites, important for 14-3-3 chaperone binding (Dzamko et al., 2010; Nichols et al., 2010). It is unfortunate that characterisation of this LRR domain was not successful during this project, however significant steps were taken towards this aim through the generation of multiple constructs for the expression of the LRR domain. Recent molecular modelling and electron microscopy studies have suggested that expression of individual domains such as that of the LRR, may prove difficult due to the tight packing of domains of LRRK2 within a larger globular structure (Guaitoli et al., 2016). In particular, the use of VLR hybridisation for the generation of VLR-capped constructs may overcome the need for such intramolecular contacts between domains, and provide a route towards successful LRR expression in the future.

Much attention is focused on the enzymatic core of LRRK2. While this is where clustering of pathogenic mutations within LRRK2 have been identified, it is not the only source of LRRK2 functionality. For example, the WD40 domain of LRRK2 has been identified as required for the function of the adjacent kinase domain, yet has not been the focus of structural investigation (Jorgensen et al., 2009). Less is known about the interaction between the LRR domain of LRRK2 and functional activity, as the central location of the LRR domain within the LRRK2 gene means it cannot simply be truncated. The value of expressing individual
domains may also be questioned following the discovery of the tightly packed nature of the LRRK2 protein (Guaitoli et al., 2016; Sejwal et al., 2017). LRR expression may prove useful to ‘fish’ for binding ligands, and to our understanding of protein folding more generally. However, the LRR domain itself will always be ‘out of context’ without surrounding domains which will likely be required to constitute physiologically relevant LRRK2 binding interfaces. The ultimate way to overcome these limitations is through the generation of full-length proteins for structural and biochemical analysis (fig 56). As discussed previously, full-length LRRK2 expression has proven difficult by other groups, but represents a possible future direction for biophysical LRRK2 research (Sejwal et al., 2017). Establishment of specialist mammalian cell culture expression systems such as a WAVE bioreactor will facilitate a potential move towards full-length LRRK2 research in the future with the use of high quantities of non-adherent HEK293 cells for protein expression (fig 56).

**Figure 56: Experimental designs for protein expression: towards biophysical and biochemical LRRK2 analysis.** Development of mammalian protein expression systems may allow full-length LRRK2 expression in the future, thus overcoming the need to truncate domains. Globular LRRK2 from Guaitoli et al., 2016. ARM = Armadillo repeats, ANK = Ankyrin repeats, LRR = Leucine rich repeats, Roc = Ras of complex proteins, COR = C-terminal of Roc, KIN = Kinase.
Functional studies into the role of LRRK2 in macrophage immunology followed two routes: regulation of TLR trafficking, and control of phagocytosis. These have been described as dependent on vesicular trafficking and cytoskeleton dynamics respectively; but may be better considered as overlapping processes (Kamal and Goldstein, 2000). For example, the LRRK2 interaction partner Rab5 actively stimulates association of early-endosomes with microtubules, and the motility of those endosomes along microtubules (Nielsen et al., 1999). Investigation into the role of LRRK2 on TLR trafficking was focused on TLR4 and TLR2, two innate immune receptors with relatively well-characterised involvement in Parkinson’s disease through the detection of alpha-synuclein, and disease pathology in Parkinson’s patients; even in the absence of pathological LRRK2 involvement (Fellner et al., 2013; Kim et al., 2014; Daniele et al., 2015). Such analysis, while tailored to a Parkinson’s disease context, is still limited in scope as there are eight other TLRs expressed in humans (Barreiro et al., 2009), and a whole range of other families of PRR proteins involved in innate immunity. These include NLRs, but also ‘C-type lectin receptors’ (CLR), ‘RIG-I-like receptors’ (RLR), and ‘AIM2-like receptors’ (ALR) (Brubaker et al., 2015). It is due to these considerations that a later approach of RNA sequencing was applied. None-the-less, indications of a difference in TLR2 transcription between LRRK2 genotypes in response to MDP led to an interest in the potential for LRRK2 to modulate cytoplasmic PRR signalling. In contrast to vesicular trafficking, a role of LRRK2 in phagocytosis has previously been described, but has ultimately led to a conflicted understanding of the role of LRRK2 in this process through the use of different experimental setups (Marker et al., 2012; Schapansky et al., 2014; Moehle et al., 2015). The use of primary macrophages from LRRK2 KO mice represents a step forward in this particular area of research, allowing clarification that LRRK2 does not appear to be directly involved in phagocytosis in a range of different phagocytic, and immunological contexts. However, while phagocytosis can be measured in vitro, this may not be representative of physiological conditions. Indeed, pathway analysis of genes identified by RNA sequencing did identify potential involvement of LRRK2 in phagocytosis that may warrant future research. Identification of genes such Rapgef3, Csrnp1, and Camk2b by RNA sequencing, suggest that LRRK2 may be involved in broad areas of biology such as cAMP, Wnt, and Ca²⁺ signalling; the contributions of which are only recently being appreciated by innate immunologists (fig 57). With these considerations in mind, to assess the role of LRRK2 in particular processes such as phagocytosis faithfully, in vivo experiments where
such secondary messengers can be closely accounted for may yield more meaningful results than equivalent in vitro experiments. Such experiments would require the combination of genetic LRRK2 mouse models with a method such as lentiviral delivery of the alpha-synuclein encoding gene to induce alpha-synuclein pathology (Decressac et al., 2012). In contrast, the basic involvement of LRRK2 in modulating secondary messengers such as cAMP or Ca^{2+} may be better studied in vitro; where specific perturbations of homeostasis can be applied and secondary messenger responses assessed. For example, cultured macrophages may be stained with fluo-4, a Ca^{2+} responsive fluorescent dye to measure Ca^{2+} flux in response to stimulation of TLR4, and this response compared between LRRK2 genotypes (Paredes et al., 2008). A commercially available ‘enzyme-linked immunosorbent assay’ (ELISA) may be applied to the measurement of cAMP perturbations in macrophages, as more sophisticated ‘fluorescence resonance energy transfer’ (FRET) methods require macrophage transfection with fluorescent reporter constructs (Börner et al., 2011).

The finding that LRRK2 appears to modulate chemokine signalling in response to LPS stimulation is an exciting development in our understanding of the role of LRRK2 in innate immunity (fig 57). Perturbations to chemokine release by LRRK2 knockout or mutation may be expected to lead to a relatively subtle inflammatory phenotype as compared to direct perturbation of classical inflammatory cytokines. Indeed the uncovering of a chemokine related phenotype of LRRK2 knockout in response to LPS stimulation was unexpected, as the majority of similar experiments have focused on classical inflammatory cytokine signalling; which has resulted in conflicting reports (Gillardon et al., 2012; Kim et al., 2012; Moehle et al., 2015). In alignment with findings by RNA sequencing, one very recent report in microglia has uncovered an underlying difference in the microglia specific CX3CR1 receptor (Ma et al., 2016). Findings described using macrophages here, and by others using microglia, suggest that chemotactic signalling may be the focus of LRRK2 immunological research in the near future. Indeed, effects of LRRK2 knockout on chemotaxis have previously been described in both microglia and macrophages. These experiments were performed towards a gradient of ATP, but again led to conflicting results. Specifically, the LRRK2 G2019S mutation was suggested to enhance chemotactic responses in macrophages, while inhibition of chemotaxis was observed using microglia (Choi et al., 2015; Moehle et al., 2015). These conflicts may be attributed to differences to experimental setup or a cell-type specific effect, however overall they further demonstrate a role of LRRK2 in chemotaxis. It would be
particularly interesting to repeat such experiments using pBMDM cells from WT and LRRK2 KO mice, and to compare chemotaxis towards a range of chemoattractant agents. In particular, CCL2 and CCL5 should be trialled in such assays following the discovery of the differential regulation of Rapgef3 and Ccr12 transcription following LPS stimulation by RNA sequencing. This is as EPAC1 (encoded by Rapgef3) and Hcar2 have been linked to chemotaxis towards CCL2 (Lorenowicz et al., 2006; Lukasova et al., 2011), while CCR12 has been reported as a non-canonical receptor for CCL5 (Yoshimura and Oppenheim, 2011). Such experiments would however require the use of macrophages pre-treated with LPS to induce the LRRK2-regulated expression of chemokine receptors.
Figure 57: Developing an understanding of LRRK2 signalling in innate immunity. Transcriptomic analysis in response to innate immune stimuli highlights involvement of LRRK2 in Ca\(^{2+}\), Wnt, and cAMP associated processes. A particular regulation of chemokine signalling by LRRK2 was also highlighted suggesting a role for LRRK2 in cell migration and chemotaxis. Overall, these results indicate that LRRK2 involvement in phagocytosis and innate immune signalling may be best studied with a particular focus on developing areas of innate immune research, including Ca\(^{2+}\), Wnt and cAMP signalling. Future work could be taken into microglia in order to understand if such processes may be directly relevant to Parkinson’s disease and neuroinflammatory signalling.
6.2 LRRK2 as a research topic, and as a therapeutic target

Findings made using a transcriptomic approach illustrate perfectly how the study of LRRK2 may inform our understanding of Parkinson’s disease more generally. Each of the discussed signalling pathways of cAMP, Wnt, and Ca^{2+} have previously been independently linked to both innate immunity, and LRRK2. For instance, cAMP has been shown to inhibit inflammatory TNFα signalling in response to LPS, demonstrating a level of cross-talk between cAMP and TLR signalling (Wall et al., 2009). A later report has shown that cAMP sensitises macrophages to LPS signalling, and increases the production of pro-inflammatory IL-33 (Sato et al., 2016). A complex regulatory network has been described between Wnt and NF-κB signalling. The activation of Wnt signalling results in a context dependent modulation of the inflammatory output of cells, with either pro or anti-inflammatory effects mediated via β-catenin and GSK3 (Ma and Hottiger, 2016). Finally, involvement of Ca^{2+} signalling in innate immunology is exemplified by a very recent report showing that extracellular Ca^{2+} potentiates inflammatory signalling upon stimulation of TLR4, TLR3 and TLR9 via a Rap1 dependent mechanism (Tang et al., 2014; Tang et al., 2017). Rap1, as discussed extensively, is the canonical target of EPAC1 signalling. Taken together, the association provided by this work, and the work of others, between these pathways and LRRK2 really emphasise that cAMP, Wnt and Ca^{2+} signalling may not just be linked to inflammation, but also to neuroinflammation and Parkinson’s disease; even in cases of the truly-sporadic disease.

Inversely, much is made of the potential use of inhibition of LRRK2 kinase activity as a potential treatment for Parkinson’s disease (Atashrazm and Dzamko, 2016; Taymans and Greggio, 2016). While there is some mileage to this approach, it seems to be commonly overlooked that mutations to LRRK2 account for only a very small proportion of the overall Parkinson’s disease burden, with the most prevalent LRRK2 mutation of G2019S found in only up to 2% of sporadic cases of Parkinson’s disease in general western populations (Gilks et al., 2005). While modulation of LRRK2 enzymatic activity would inherently affect pathways relevant to disease pathology, the sheer number of pathways in which LRRK2 appears to be involved suggests that such modulation may lead to broad, un-targeted downstream effects; and so may lead to significant undesired outcomes in patients without underlying pathogenic mutations to LRRK2. These fears are substantiated by the finding that
LRRK2 kinase inhibitor treatment causes autophagy-related pathology in the lungs of non-human primates (Fuji et al., 2015). Furthermore, while the much focused upon G2019S mutation accounts for the majority of LRRK2 associated cases of Parkinson’s disease, it is not the only pathogenic mutation associated with pathology. It is unclear whether mutations to R1441 of the LRRK2 GTPase domain cause pathology by distinct or common mechanisms to that of G2019S mutation. In comparison however, it is well established that mutations to R1441 do not share the consistent three-fold enhancement of kinase activity caused by the G2019S mutation (Smith et al., 2006; Guo et al., 2007; Jaleel et al., 2007). Therefore, therapeutic inhibition of LRRK2 kinase activity may not be appropriate for all LRRK2 linked cases of disease.

Finally, work presented here has highlighted differences in LRRK2 function between cell types. For example, while an increase in ceramide levels has been described in brain tissue upon knockout of LRRK2, this effect was not observed in primary macrophages (Ferrazza et al., 2016). However, MDP treated macrophages did display an LRRK2 dependent increase in ceramide levels upon stimulation. It would be interesting to assess ceramide responses to MDP treatment in neuronal or microglial cultures in order make a comparison between neuronal and immunological cell ceramide responses. Transcriptome analysis revealed few differences in gene expression between LRRK2 KO and equivalent WT resting macrophages. This reflects findings made using a similar approach with G2019S LRRK2 brain tissue (Devine et al., 2011). The finding of differential gene responses to LPS in macrophages highlights that LRRK2 function varies not just by cell type, but also in a cell signalling context dependent manner. That EPAC1 relates directly to neurite extension in a neuronal context (Mains et al., 1990; Monaghan et al., 2008), and is also the gene identified as the most statistically significant differentially responding gene to LPS stimulation between LRRK2 macrophage genotypes, reaffirms that LRRK2 phenotypes and interactions identified in a neuronal context are able to inform our understanding of LRRK2 in innate immunity. Overall, many cell-type specific effects of LRRK2 can be observed: from the cytoskeletonally dependent morphological phenotypes which have been the indirect focus of this work, to effects on lysozyme sorting in gut Paneth cells (Zhang et al., 2015), and autophagy associated phenotypes in the kidneys and lungs (Herzig et al., 2011; Tong et al., 2012). Accordingly, it should not be forgotten that the targeting of LRRK2 enzymatic activity to
modulate neuronal or inflammatory LRRK2 phenotypes would likely cause a broad range of undesired effects in many areas of biology.

6.3 Moving beyond LRRK2 knockout in murine macrophages

The use of primary macrophages derived from LRRK2 KO mice has enabled the clarification of the role of LRRK2 where non-specific LRRK2 knockdown or chemical inhibition has led to uncertainty. Indeed, benefits associated with the use of genetic knockout systems towards uncovering the underlying biochemistry of proteins is well established. However, in the case of LRRK2 associated Parkinson’s disease, the best characterised pathological LRRK2 mutation, G2019S, appears to pertain to a gain-of-function mechanism rather than loss-of-function (West et al., 2005; MacLeod et al., 2006; Jaleel et al., 2007; Greggio and Cookson, 2009). The use of mice expressing G2019S mutant LRRK2 would therefore complement the use of LRRK2 KO mice for the specific study of G2019S LRRK2 associated pathobiology of Parkinson’s disease. That said, and as mentioned previously, simply studying the G2019S mutation of LRRK2 would result in functions of LRRK2 that may be mediated outside of the kinase domain, or that are involved in LRRK2 biology but are not necessarily associated with disease, being missed. Many non-kinase domain associated LRRK2 functions are likely to be relevant to Parkinson’s disease pathology given the existence of three different pathological mutations at position R1441 of the LRRK2 Roc domain alone (Simón-Sánchez et al., 2006; Haugarvoll et al., 2008; Ross et al., 2009). While the use of LRRK2 KO mice does provide a great insight to fundamental LRRK2 biology, the use of such mice does still have limitations. Again, this comes down to the fact that LRRK2 knockout does not result in the loss of functional activity alone, but actually results in the loss of both functional activity and a major protein scaffold that other enzymes may require for protein complex formation. To explain, LRRK2 contains a huge array of repeat structures either side of the catalytic core, any of which may act alone or in combination to bind other proteins and ligands (Mills et al., 2014). Knockout of LRRK2 will affect the formation of such complexes even if LRRK2 does not enzymatically modify the complex itself. Therefore, while LRRK2-associated immunological phenotypes may be identified by comparison of WT and LRRK2 KO macrophages, the use of ‘kinase dead’ D1994A LRRK2 could help to identify and separate the role of LRRK2 as a kinase, as compared to a molecular scaffold (West et al., 2007).
A further feature of this study is the use of bone marrow derived macrophages as compared to microglia. Bone marrow derived macrophages are a powerful tool to study macrophage function, with many advantages over the use of cell lines that have undergone an immortalisation process. In comparison to murine macrophages generated from bone marrow, classical isolation of primary microglia requires neonatal mice, is extremely time consuming, and is costly in terms of animals required. From three neonatal mouse brains, only around 900,000 microglia would be isolated, after two weeks of culture (Giulian and Baker, 1986; Floden and Combs, 2007). This compares to around 30 million pBMDM cells from a single adult mouse, after a single week of culture. It is also easy to forget that LRRK2 is functional in non-pathogenic contexts, as well as in conditions other than Parkinson’s disease and neuroinflammation; such as Crohn’s disease and Leprosy (Barrett et al., 2009; Zhang et al., 2009; Franke et al., 2010; Wang et al., 2014). Indeed, LRRK2 has a broad expression pattern involving many tissues outside of the brain; and is enriched in immune cells (Gardet et al., 2010; Maekawa et al., 2010). The use of microglia therefore comes at considerable cost, and is perhaps a less suitable model than that of primary macrophages for the study of LRRK2 in innate immunity as a whole. While differences between macrophages and microglia exist (Hickman et al., 2013), so too do differences between innate immune responses in mice and humans (Mestas and Hughes, 2004). Again, maintaining a focus on Parkinson’s disease in particular exacerbates species differences, as mice do not naturally develop neurodegenerative disorders. Ultimately, mouse models are limited, but provide the most efficient basis upon which to identify a role for LRRK2 in innate immunity due to the availability and identical genetic backgrounds of mice. An ideal model for future research would be the continued use of primary murine macrophages for the identification of immunological phenotypes of LRRK2 knockout, then the subsequent utilisation of primary murine microglia and perhaps human ‘induced pluripotent stem cell’ (iPSC) derived macrophages or microglia from patients suffering from LRRK2 associated Parkinson’s disease (Muffat et al., 2016; Pandya et al., 2017). This would allow the relevance of phenotypes identified in murine macrophages to be confirmed in the disease-relevant cell type of primary microglia, and human innate immune cells.

An alternate approach would negate the need for the routine use of primary macrophages at all. This would be the knockout or mutation of LRRK2 in cell lines using CRISPR/Cas9 technology (Ran et al., 2013; Hsu et al., 2014). Murine RAW264.7 macrophages
or BV-2 microglia both express LRRK2, and could be modified this way to (Schapansky et al., 2014). The human THP-1 cell line also expresses LRRK2, and could be similarly modified (Gardet et al., 2010), although a widely available human microglial cell line is lacking to complement this. Resulting cell lines would be extremely convenient, and would dramatically reduce the need for animals in LRRK2 research, as primary cells and tissues would be limited to follow-up experiments. This would improve the compliance of LRRK2 research with the policy of replacement, reduction and refinement of animal testing in research.

6.4 The future of LRRK2 research

Innate immunity has re-emerged over the past few years as a topic of interest to the LRRK2 research community. This research effort has been aided by the development of tools such as the LRRK2 mouse models as used in work described here, but also new methods such as the aforementioned CRISPR/Cas9 technology which facilitate in vitro experiments that had previously not been possible. The specific nature of such tools will render the development of LRRK2 specific kinase inhibitors obsolete for basic research purposes, and will vastly improve the quality of LRRK2 research. LRRK2 is clearly involved in a large number of cellular processes, as is fitting of a protein with not one, but two enzymatically active domains, and a whole range of potential protein-protein interaction surfaces. The LRRK2 interaction network that results is highly elaborate, and has proved difficult to unpick (Manzoni et al., 2015; Porras et al., 2015). Efforts to establish meaningful LRRK2 functions and relevance to pathology have not been aided by the use of the particularly misleading L2in1 inhibitor; which is thankfully becoming less-and-less prevalent in LRRK2 research. It is perhaps telling that over the past few years, many of the most interesting discoveries relating to LRRK2 function have come from groups without a previous interest in LRRK2 itself; and so have been able to view LRRK2 with a fresh perspective. This is exemplified by the discovery of roles of LRRK2 in NFAT signalling (Liu et al., 2011), lysozyme sorting (Zhang et al., 2015), and protein translation (Imai et al., 2008). The debunking of erroneous LRRK2 functions that has been occurring, in part through work such as that described here, should lead to the development of a greater consensus on the cellular roles of LRRK2 and their relative importance to pathogenicity.
The dichotomous nature of LRRK2 in neurons and immune cells will always exist, and it will be interesting to see how much overlap truly exists between LRRK2 function in these two specialised cell types. Ultimately, significant pathological phenotypes such as neurodegeneration may develop from the accumulation of many small changes on a molecular scale; this is the basis of modern systems biology based approaches to research (Chuang et al., 2010). Therefore, effects of LRRK2 in neurons, microglia, macrophages and even astrocytes most likely act in combination to cause conditions such as Parkinson’s disease, with no one factor truly acting in isolation. While studying these systems individually is fundamental to developing our understanding of LRRK2 function, it should not be forgotten that immunology and neuroscience are intimately linked all the way from neurogenesis, through neuroplasticity, to neurodegeneration. That LRRK2 is expressed in the archetypal cell type of both systems only acts to strengthen this link.
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8. Acknowledgements

I’d like to thank Nick Gay and Clare Bryant for hosting this project over the past four years. Both labs have afforded me the space, the resources, and the time, to go about my work. Similarly, the members and alumni of each lab have provided me with help when and where I’ve needed it. For structural work, Monique Gangloff has provided oversight, while Martin Moncrieffe and Samer Halabi have helped to provide day-to-day troubleshooting advice, and made the lab a fun place to work. Ardi, Miranda, Sophie, Joe, Michael, Julia, and Johannes have each served as examples of how to succeed as a PhD student – and will continue as role models into the future.

Clare and the members of her lab made taking this project in a functional direction possible. Managing a project as complex as LRRK2 has proved more challenging than I felt capable of dealing with at times. Clare’s lab in particular has helped me to overcome many of my difficulties – both with my project, and in myself, during my time with them. Lee Hopkins, Alessandra Bittante, and Clare, have each taken the time to support me during these more demanding periods. While encouraging me personally, each has also provided invaluable assistance in the lab – with Lee in particular making coming into work a delight. I’d like to thank Pani for managing LRRK2 mice; while he, Lee, John, Heather, Jessica, and Craig have also always been available to provide specialist insight when and where I’ve needed it. Clare’s lab would not be complete without her PhD students; Milton, Alessandra, Charlotte, Sam, Zsophie, Jiro, and Hannah, who have each contributed to the friendly and supportive environment within the lab. Perhaps above all, I’d like to thank Martyn Symmons for his continued interest and encouragement across my time with both Nick and Clare’s labs. Our chats have helped to keep me engaged with LRRK2 and research as a whole, while also being a continued source of inspiration for experiments and new lines of investigation – but no Martyn, I still refuse to set foot on your boat..!

Finally, I’d like to show my appreciation to those outside the lab. Sergei, Naomi, Helene, Helena, Martin, Josie, Nanase, Olivia, Christine, Alex, Latika, Amy, and also my parents – all of whom have had to put up with me over the last four years. In particular, thank you to Daisy Luff, who I met in Nick’s lab on the first day of my PhD, and has gone on to become a closer friend than I could have imagined.
9. Appendix

Appendix table 1: Chromatographic conditions for the adapted Waters Corporation lipid method.

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Appendix table 2: Mass spectrometry conditions for the adapted Waters Corporation lipid method.

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Appendix table 3: Enriched pathways shared between WT and LRRK2 KO macrophages after LPS treatment. 100 ng/ml LPS. Differential gene expression padj < 0.01, pathway enrichment padj < 0.01.

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<td>Toll Like Receptor 4 (TLR4) Cascade</td>
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<td>Toll Like Receptor 7/8 (TLR7/8) Cascade</td>
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<td>Toll Like Receptor 9 (TLR9) Cascade</td>
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</tr>
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</tr>
<tr>
<td>Toll-Like Receptors Cascades</td>
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<tr>
<td>TRAF6 mediated induction of NFKB and MAP kinases upon TLR7/8 or 9 activation</td>
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</tr>
<tr>
<td>TRAF6 Mediated Induction of proinflammatory cytokines</td>
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<tr>
<td>TRIF-mediated TLR3/TLR4 signaling</td>
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### Appendix table 4: Enriched pathways shared between WT and LRRK2 KO macrophages after MDP treatment.
Differential gene expression padj < 0.01, pathway enrichment padj < 0.01.

<table>
<thead>
<tr>
<th>Reactome Pathway</th>
<th>Pathway enrichment</th>
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<tbody>
<tr>
<td>Activated TAK1 mediates p38 MAPK activation</td>
<td>0.004  0.006</td>
</tr>
<tr>
<td>Activated TLR4 signalling</td>
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<tr>
<td>Chemokine receptors bind chemokines</td>
<td>0.000  0.000</td>
</tr>
<tr>
<td>Class A/1 (Rhodopsin-like receptors)</td>
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</tr>
<tr>
<td>Cytokine Signaling in Immune system</td>
<td>0.000  0.000</td>
</tr>
<tr>
<td>Fcgamma receptor (FCGR) dependent phagocytosis</td>
<td>0.002  0.004</td>
</tr>
<tr>
<td>GPCR downstream signaling</td>
<td>0.009  0.010</td>
</tr>
<tr>
<td>Immune System</td>
<td>0.000  0.000</td>
</tr>
<tr>
<td>Innate Immune System</td>
<td>0.000  0.000</td>
</tr>
<tr>
<td>Interferon gamma signaling</td>
<td>0.000  0.000</td>
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<tr>
<td>Interferon Signaling</td>
<td>0.000  0.001</td>
</tr>
<tr>
<td>Interleukin-1 signaling</td>
<td>0.001  0.001</td>
</tr>
<tr>
<td>MyD88 cascade initiated on plasma membrane</td>
<td>0.001  0.000</td>
</tr>
<tr>
<td>MyD88 dependent cascade initiated on endosome</td>
<td>0.001  0.000</td>
</tr>
<tr>
<td>MyD88-independent cascade</td>
<td>0.000  0.000</td>
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<tr>
<td>MyD88:Mal cascade initiated on plasma membrane</td>
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<tr>
<td>p75NTR signals via NF-kB</td>
<td>0.002  0.003</td>
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<td>RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways</td>
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<td>Signal Transduction</td>
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<tr>
<td>Signaling by GPCR</td>
<td>0.003  0.002</td>
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<tr>
<td>Signaling by Interleukins</td>
<td>0.000  0.002</td>
</tr>
<tr>
<td>Signaling by the B Cell Receptor (BCR)</td>
<td>0.008  0.010</td>
</tr>
<tr>
<td>TAK1 activates NFkB by phosphorylation and activation of IKKs complex</td>
<td>0.000  0.000</td>
</tr>
<tr>
<td>TCR signaling</td>
<td>0.000  0.001</td>
</tr>
<tr>
<td>Toll Like Receptor 10 (TLR10) Cascade</td>
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<tr>
<td>Toll Like Receptor 2 (TLR2) Cascade</td>
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</tr>
<tr>
<td>Toll Like Receptor 3 (TLR3) Cascade</td>
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</tr>
<tr>
<td>Toll Like Receptor 4 (TLR4) Cascade</td>
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</tr>
<tr>
<td>Toll Like Receptor 5 (TLR5) Cascade</td>
<td>0.001  0.000</td>
</tr>
<tr>
<td>Toll Like Receptor 7/8 (TLR7/8) Cascade</td>
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<tr>
<td>Toll Like Receptor 9 (TLR9) Cascade</td>
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</tr>
<tr>
<td>Toll Like Receptor TLR1:TLR2 Cascade</td>
<td>0.001  0.000</td>
</tr>
<tr>
<td>Toll Like Receptor TLR6:TLR2 Cascade</td>
<td>0.000  0.000</td>
</tr>
<tr>
<td>Toll Like Receptors Cascades</td>
<td>0.001  0.000</td>
</tr>
<tr>
<td>TRAF6 mediated induction of NFkB and MAP kinases upon TLR7/8 or 9 activation</td>
<td>0.001  0.000</td>
</tr>
<tr>
<td>TRAF6 Mediated Induction of proinflammatory cytokines</td>
<td>0.001  0.000</td>
</tr>
<tr>
<td>TRAF6 mediated NF-kB activation</td>
<td>0.000  0.000</td>
</tr>
<tr>
<td>TRIF-mediated TLR3/TLR4 signaling</td>
<td>0.000  0.000</td>
</tr>
</tbody>
</table>
### Appendix table 5: Pathways uniquely enriched in WT macrophages after LPS treatment

100 ng/ml LPS. Pathways uniquely highlighted at a single differential gene expression p adjusted value (DGEpadj) or pathway analysis padj are shown in grey.

<table>
<thead>
<tr>
<th>Pathway enrichment padj &lt; 0.05</th>
<th>LRRK2 KO</th>
<th>WT</th>
<th>Reactome Pathway</th>
<th>LRRK2 KO</th>
<th>WT</th>
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<tbody>
<tr>
<td>Association of TriC/CCT with target proteins during biosynthesis</td>
<td>0.439</td>
<td>0.020</td>
<td>Folding of actin by CCT/TriC</td>
<td>0.694</td>
<td>0.066</td>
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<tr>
<td>Regulation of IFNG signaling</td>
<td>0.223</td>
<td>0.044</td>
<td>Association of TriC/CCT with target proteins during biosynthesis</td>
<td>0.439</td>
<td>0.020</td>
</tr>
<tr>
<td>Regulation of actin dynamics for phagocytic cup formation</td>
<td>0.207</td>
<td>0.034</td>
<td>Signaling by EGFR in Cancer</td>
<td>0.351</td>
<td>0.089</td>
</tr>
<tr>
<td>PI3K/AKT activation</td>
<td>0.199</td>
<td>0.035</td>
<td>Interleukin-7 signaling</td>
<td>0.344</td>
<td>0.095</td>
</tr>
<tr>
<td>Hemostasis</td>
<td>0.184</td>
<td>0.032</td>
<td>Regulation of IFNG signaling</td>
<td>0.223</td>
<td>0.044</td>
</tr>
<tr>
<td>Interleukin-6 signaling</td>
<td>0.122</td>
<td>0.016</td>
<td>Regulation of actin dynamics for phagocytic cup formation</td>
<td>0.207</td>
<td>0.034</td>
</tr>
<tr>
<td>Constitutive PI3K/AKT Signaling in Cancer</td>
<td>0.144</td>
<td>0.041</td>
<td>Signaling by FGFR in disease</td>
<td>0.253</td>
<td>0.081</td>
</tr>
<tr>
<td>PI-3K cascade</td>
<td>0.130</td>
<td>0.035</td>
<td>PI3K/AKT activation</td>
<td>0.199</td>
<td>0.035</td>
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<tr>
<td>PI3K events in ERBB2 signaling</td>
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<td>0.035</td>
<td>Hemostasis</td>
<td>0.184</td>
<td>0.032</td>
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<tr>
<td>PI3K events in ERBB4 signaling</td>
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<td>0.035</td>
<td>Downstream TCR signaling</td>
<td>0.223</td>
<td>0.074</td>
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<tr>
<td>PI3K/AKT Signaling in Cancer</td>
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<td>0.035</td>
<td>Signaling by PDGF</td>
<td>0.194</td>
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<tr>
<td>PIP3 activates AKT signaling</td>
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<td>0.035</td>
<td>Interleukin-6 signaling</td>
<td>0.122</td>
<td>0.016</td>
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<tr>
<td>TCR signaling</td>
<td>0.119</td>
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<td>Constitutive PI3K/AKT Signaling in Cancer</td>
<td>0.144</td>
<td>0.041</td>
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<tr>
<td>Downstream signal transduction</td>
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<td>0.026</td>
<td>PI-3K cascade</td>
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<td>Fc epsilon receptor (FCERI) signaling</td>
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<td>Role of LAT2/NTAL/LAB on calcium mobilization</td>
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<td>GAB1 signalosome</td>
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<td>PIP3 activates AKT signaling</td>
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<td>Role of phospholipids in phagocytosis</td>
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<td><strong>TAK1 activates NFkB by phosphorylation and activation of IKKs complex</strong></td>
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<td>0.035</td>
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<td>Downstream signal transduction</td>
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<td>Role of LAT2/NTAL/LAB on calcium mobilization</td>
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<td>0.026</td>
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<td>Regulation of actin dynamics for phagocytic cup formation</td>
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<tr>
<td>Association of TriC/CCT with target proteins during biosynthesis</td>
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<td>0.021</td>
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<tr>
<td>Association of TriC/CCT with target proteins during biosynthesis</td>
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<td>PI Metabolism</td>
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<td>Association of TriC/CCT with target proteins during biosynthesis</td>
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<td>Regulation of IFNA signaling</td>
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<td>PI Metabolism</td>
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<td>Interleukin-2 signaling</td>
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<td>The NLRP3 inflammasome</td>
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<td>0.018</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>The NLRP3 inflammasome</td>
<td>0.204</td>
<td>0.018</td>
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</tr>
<tr>
<td>Regulation of IFNG signaling</td>
<td>0.398</td>
<td>0.083</td>
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</tr>
<tr>
<td>Regulation of IFNA signaling</td>
<td>0.398</td>
<td>0.083</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The NLRP3 inflammasome</td>
<td>0.204</td>
<td>0.018</td>
<td></td>
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<td>Negative regulators of RIG-I/MDA5 signaling</td>
<td>0.170</td>
<td>0.089</td>
<td></td>
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</tbody>
</table>
### Appendix table 6: Pathways uniquely enriched in LRRK2 KO macrophages after LPS treatment.

100 ng/ml LPS. Pathways uniquely highlighted at a single differential gene expression p adjusted value (DGEpadj) or pathway analysis p adjusted value are shown in grey.

<table>
<thead>
<tr>
<th>Pathway enrichment padj &lt; 0.05</th>
<th>LRRK2 KO</th>
<th>WT</th>
<th>Pathway enrichment padj &lt; 0.1</th>
<th>LRRK2 KO</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammasomes</td>
<td>0.050</td>
<td>0.540</td>
<td>Antigen Presentation: Folding, assembly and peptide loading of class I MHC</td>
<td>0.100</td>
<td>0.914</td>
</tr>
<tr>
<td>The NLRP3 inflammasome</td>
<td>0.023</td>
<td>0.259</td>
<td>Extrinsic Pathway for Apoptosis</td>
<td>0.080</td>
<td>0.713</td>
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<tr>
<td>Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways</td>
<td>0.034</td>
<td>0.257</td>
<td>Inflammasomes</td>
<td>0.050</td>
<td>0.540</td>
</tr>
<tr>
<td>ERKs are inactivated</td>
<td>0.034</td>
<td>0.095</td>
<td>Cytosolic sensors of pathogen-associated DNA</td>
<td>0.072</td>
<td>0.378</td>
</tr>
<tr>
<td>Pre-NOTCH Processing in Golgi</td>
<td>0.035</td>
<td>0.086</td>
<td>The NLRP3 inflammasome</td>
<td>0.023</td>
<td>0.259</td>
</tr>
<tr>
<td>Interleukin-3, 5 and GM-CSF signaling</td>
<td>0.035</td>
<td>0.086</td>
<td>Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways</td>
<td>0.034</td>
<td>0.257</td>
</tr>
<tr>
<td>Interleukin-2 signaling</td>
<td>0.023</td>
<td>0.062</td>
<td>Signaling by NOTCH</td>
<td>0.036</td>
<td>0.243</td>
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<tr>
<td>p38MAPK events</td>
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<td>0.052</td>
<td>DEx/H-box helicases activate type I IFN and inflammatory cytokines production</td>
<td>0.056</td>
<td>0.256</td>
</tr>
<tr>
<td>TRAF3-dependent IRF activation pathway</td>
<td>0.018</td>
<td>0.052</td>
<td>TRAF6 mediated IRF7 activation</td>
<td>0.005</td>
<td>0.088</td>
</tr>
<tr>
<td>TRAF6-mediated IRF7 activation</td>
<td>0.031</td>
<td>0.536</td>
<td>G alpha (12/13) signalling events</td>
<td>0.030</td>
<td>0.600</td>
</tr>
<tr>
<td>Cytosolic sensors of pathogen-associated DNA</td>
<td>0.037</td>
<td>0.221</td>
<td>Loss of Nlp from mitotic centrosomes</td>
<td>0.051</td>
<td>0.728</td>
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<tr>
<td>TRAF3-dependent IRF activation pathway</td>
<td>0.037</td>
<td>0.221</td>
<td>TRAF3-dependent IRF activation pathway</td>
<td>0.017</td>
<td>0.208</td>
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<tr>
<td>Signaling by NOTCH</td>
<td>0.037</td>
<td>0.221</td>
<td>Loss of proteins required for interphase microtubule organization from the centrosome</td>
<td>0.051</td>
<td>0.728</td>
</tr>
<tr>
<td>Cytosolic sensors of pathogen-associated DNA</td>
<td>0.031</td>
<td>0.536</td>
<td>TRAF6-mediated IRF7 activation</td>
<td>0.005</td>
<td>0.088</td>
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</tbody>
</table>

DGEpadj < 0.05

290
<table>
<thead>
<tr>
<th>Pathway</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signaling by the B Cell Receptor (BCR)</td>
<td>0.017</td>
<td>0.079</td>
</tr>
<tr>
<td>Interleukin-6 signaling</td>
<td>0.013</td>
<td>0.073</td>
</tr>
<tr>
<td>p38MAPK events</td>
<td>0.017</td>
<td>0.065</td>
</tr>
<tr>
<td>Pre-NOTCH Expression and Processing</td>
<td>0.017</td>
<td>0.053</td>
</tr>
<tr>
<td>Mitotic G2-G2/M phases</td>
<td>0.018</td>
<td>0.418</td>
</tr>
<tr>
<td>Loss of Nlp from mitotic centrosomes</td>
<td>0.022</td>
<td>0.378</td>
</tr>
<tr>
<td>Loss of proteins required for interphase microtubule organization from the centrosome</td>
<td>0.022</td>
<td>0.378</td>
</tr>
<tr>
<td>G2/M Transition</td>
<td>0.027</td>
<td>0.330</td>
</tr>
<tr>
<td>Regulation of PLK1 Activity at G2/M Transition</td>
<td>0.025</td>
<td>0.276</td>
</tr>
<tr>
<td>p38MAPK events</td>
<td>0.047</td>
<td>0.130</td>
</tr>
<tr>
<td>TRAF3-dependent IRF activation pathway</td>
<td>0.047</td>
<td>0.130</td>
</tr>
<tr>
<td>Cell Cycle, Mitotic</td>
<td>0.080</td>
<td>0.347</td>
</tr>
<tr>
<td>Interleukin-2 signaling</td>
<td>0.081</td>
<td>0.292</td>
</tr>
<tr>
<td>TRAF3-dependent IRF activation pathway</td>
<td>0.017</td>
<td>0.208</td>
</tr>
<tr>
<td>Regulation of PLK1 Activity at G2/M Transition</td>
<td>0.055</td>
<td>0.516</td>
</tr>
<tr>
<td>Mitotic G2-G2/M phases</td>
<td>0.018</td>
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</tr>
<tr>
<td>Lysosphingolipid and LPA receptors</td>
<td>0.091</td>
<td>0.798</td>
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<tr>
<td>Mitotic G2-G2/M phases</td>
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<td>0.418</td>
</tr>
<tr>
<td>Loss of Nlp from mitotic centrosomes</td>
<td>0.022</td>
<td>0.378</td>
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<td>0.378</td>
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<td>0.378</td>
</tr>
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<td>0.378</td>
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<tr>
<td>Loss of Nlp from mitotic centrosomes</td>
<td>0.022</td>
<td>0.378</td>
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<tr>
<td>G2/M Transition</td>
<td>0.027</td>
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<td>Regulation of PLK1 Activity at G2/M Transition</td>
<td>0.025</td>
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<tr>
<td>p38MAPK events</td>
<td>0.047</td>
<td>0.130</td>
</tr>
<tr>
<td>TRAF3-dependent IRF activation pathway</td>
<td>0.047</td>
<td>0.130</td>
</tr>
<tr>
<td>Pre-NOTCH Expression and Processing</td>
<td>0.055</td>
<td>0.124</td>
</tr>
<tr>
<td>p38MAPK events</td>
<td>0.047</td>
<td>0.130</td>
</tr>
<tr>
<td>TRAF3-dependent IRF activation pathway</td>
<td>0.047</td>
<td>0.130</td>
</tr>
<tr>
<td>Pre-NOTCH Expression and Processing</td>
<td>0.055</td>
<td>0.124</td>
</tr>
<tr>
<td>p38MAPK events</td>
<td>0.047</td>
<td>0.130</td>
</tr>
<tr>
<td>Downstream signal transduction</td>
<td>0.069</td>
<td>0.130</td>
</tr>
<tr>
<td>Cytosolic sensors of pathogen-associated DNA</td>
<td>0.083</td>
<td>0.130</td>
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### Appendix table 7: Pathways uniquely enriched in WT macrophages after MDP treatment.

10 ug/ml MDP. Pathways uniquely highlighted at a single differential gene expression p adjusted value (DGEpadj) or pathway analysis p adjusted value are shown in grey.

<table>
<thead>
<tr>
<th>Pathway enrichment padj &lt; 0.05</th>
<th>Reactome Pathway</th>
<th>LRRK2 KO</th>
<th>WT</th>
<th>LRRK2 KO</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGEpadj &lt; 0.01</td>
<td>Dissolution of Fibrin Clot</td>
<td>0.729</td>
<td>0.026</td>
<td>Dissolution of Fibrin Clot</td>
<td>0.694</td>
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<tr>
<td></td>
<td>Integrin alphabeta3 signaling</td>
<td>0.35</td>
<td>0.038</td>
<td>Inflammasomes</td>
<td>0.439</td>
</tr>
<tr>
<td></td>
<td>MAP kinase activation in TLR cascade</td>
<td>0.065</td>
<td>0.014</td>
<td>p75 NTR receptor-mediated signalling</td>
<td>0.344</td>
</tr>
<tr>
<td></td>
<td>Rho GTPase cycle</td>
<td>0.094</td>
<td>0.049</td>
<td>Regulation of Lipid Metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)</td>
<td>0.223</td>
</tr>
<tr>
<td></td>
<td>Signaling by Rho GTPases</td>
<td>0.094</td>
<td>0.049</td>
<td>The NLRP3 inflammasome</td>
<td>0.207</td>
</tr>
<tr>
<td></td>
<td>TRAF6 mediated IRF7 activation</td>
<td>0.092</td>
<td>0.005</td>
<td>TRAF3-dependent IRF activation pathway</td>
<td>0.253</td>
</tr>
<tr>
<td>Adaptive Immune System</td>
<td>Cross-presentation of particulate exogenous antigens (phagosomes)</td>
<td>0.203</td>
<td>0.046</td>
<td>Adaptive Immune System</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>Downstream Signaling Events Of B Cell Receptor (BCR)</td>
<td>0.074</td>
<td>0.012</td>
<td>Dissolution of Fibrin Clot</td>
<td>0.061</td>
</tr>
<tr>
<td></td>
<td>GAB1 signalosome</td>
<td>0.123</td>
<td>0.044</td>
<td>GAB1 signalosome</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>NOTCH2 intracellular domain regulates transcription</td>
<td>0.204</td>
<td>0.044</td>
<td>Integrin alphabeta3 signaling</td>
<td>0.053</td>
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<td></td>
<td>Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways</td>
<td>0.077</td>
<td>0.006</td>
<td>MAP kinase activation in TLR cascade</td>
<td>0.059</td>
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<td></td>
<td>p38MAPK events</td>
<td>0.153</td>
<td>0.047</td>
<td>Negative regulators of RIG-I/MDA5 signaling</td>
<td>0.087</td>
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<tr>
<td></td>
<td>Signaling by the B Cell Receptor (BCR)</td>
<td>0.061</td>
<td>0.038</td>
<td>NOTCH2 intracellular domain regulates transcription</td>
<td>0.044</td>
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<tr>
<td></td>
<td>ZBP1(DAI) mediated induction of type I IFNs</td>
<td>0.111</td>
<td>0.029</td>
<td>p38MAPK events</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PPARA Activates Gene Expression</td>
<td>0.061</td>
</tr>
</tbody>
</table>

292
<table>
<thead>
<tr>
<th>DGEpadj &lt; 0.1</th>
<th>Regulation of Lipid Metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)</th>
<th>0.097</th>
<th>0.051</th>
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<tbody>
<tr>
<td></td>
<td>Rho GTPase cycle</td>
<td>0.051</td>
<td>0.115</td>
</tr>
<tr>
<td></td>
<td>Signaling by Rho GTPases</td>
<td>0.051</td>
<td>0.115</td>
</tr>
<tr>
<td></td>
<td>ZBP1(DAI) mediated induction of type I IFNs</td>
<td>0.029</td>
<td>0.082</td>
</tr>
<tr>
<td>Constitutive PI3K/AKT Signaling in Cancer</td>
<td>0.133</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>DAP12 interactions</td>
<td>0.066</td>
<td>0.022</td>
<td></td>
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<tr>
<td>Downstream Signaling Events Of B Cell Receptor (BCR)</td>
<td>0.115</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>GAB1 signalosome</td>
<td>0.123</td>
<td>0.006</td>
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<tr>
<td>Hemostasis</td>
<td>0.054</td>
<td>0.011</td>
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<tr>
<td>MAP kinase activation in TLR cascade</td>
<td>0.074</td>
<td>0.043</td>
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<tr>
<td>NGF signalling via TRKA from the plasma membrane</td>
<td>0.054</td>
<td>0.022</td>
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<tr>
<td>PI-3K cascade</td>
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<td>0.022</td>
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<tr>
<td>PI3K events in ERBB2 signaling</td>
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<td>0.022</td>
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<tr>
<td>PI3K events in ERBB4 signaling</td>
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<td>0.022</td>
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<tr>
<td>PI3K/AKT activation</td>
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<tr>
<td>PI3K/AKT Signaling in Cancer</td>
<td>0.229</td>
<td>0.022</td>
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<tr>
<td>PIP3 activates AKT signaling</td>
<td>0.229</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>Regulation of actin dynamics for phagocytic cup formation</td>
<td>0.074</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>Rho GTPase cycle</td>
<td>0.074</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>Role of LAT2/NTAL/LAB on calcium mobilization</td>
<td>0.316</td>
<td>0.047</td>
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<tr>
<td>Signaling by PDGF</td>
<td>0.15</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td>Signaling by Rho GTPases</td>
<td>0.074</td>
<td>0.022</td>
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<tr>
<td>Signaling by the B Cell Receptor (BCR)</td>
<td>0.117</td>
<td>0.012</td>
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</tr>
<tr>
<td>Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds</td>
<td>0.236</td>
<td>0.087</td>
<td></td>
</tr>
</tbody>
</table>
### Appendix table 8: Pathways uniquely enriched in LRRK2 KO macrophages after MDP treatment. 10 μg/ml MDP. Pathways uniquely highlighted at a single differential gene expression p adjusted value (DGEpadj) or pathway analysis p adjusted value are shown in grey.

<table>
<thead>
<tr>
<th>Reactome Pathway</th>
<th>LRRK2 KO</th>
<th>WT</th>
<th>Reactome Pathway</th>
<th>LRRK2 KO</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc epsilon receptor (FCERI) signaling</td>
<td>0.050</td>
<td>0.540</td>
<td>Cell junction organization</td>
<td>0.094</td>
<td>0.13</td>
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<tr>
<td>FCGR activation</td>
<td>0.023</td>
<td>0.259</td>
<td>DAP12 interactions</td>
<td>0.065</td>
<td>0.107</td>
</tr>
<tr>
<td>Signaling by SCF-KIT</td>
<td>0.034</td>
<td>0.257</td>
<td>FCERI mediated NF-kB activation</td>
<td>0.085</td>
<td>0.104</td>
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<tr>
<td>Imaging regulatory interactions between a Lymphoid and a non-Lymphoid cell</td>
<td>0.094</td>
<td>0.258</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAP12 recruits signaling complexes</td>
<td>0.094</td>
<td>0.111</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Signaling by EGFR in Cancer</td>
<td>0.094</td>
<td>0.239</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Signaling by PDGF</td>
<td>0.08</td>
<td>0.129</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell surface interactions at the vascular wall</td>
<td>0.036</td>
<td>0.156</td>
<td>Cell surface interactions at the vascular wall</td>
<td>0.036</td>
<td>0.156</td>
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<tr>
<td>FCGR activation</td>
<td>0.011</td>
<td>0.149</td>
<td>FCGR activation</td>
<td>0.011</td>
<td>0.149</td>
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<td>Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell</td>
<td>0.098</td>
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<td></td>
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<tr>
<td>Role of phospholipids in phagocytosis</td>
<td>0.012</td>
<td>0.137</td>
<td>Role of phospholipids in phagocytosis</td>
<td>0.012</td>
<td>0.137</td>
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<tr>
<td>Signaling by SCF-KIT</td>
<td>0.075</td>
<td>0.108</td>
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</tr>
<tr>
<td>Signaling by NOTCH</td>
<td>0.184</td>
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<td></td>
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</tr>
<tr>
<td>FCGR activation</td>
<td>0.019</td>
<td>0.258</td>
<td>Cell surface interactions at the vascular wall</td>
<td>0.089</td>
<td>0.112</td>
</tr>
<tr>
<td>Generation of second messenger molecules</td>
<td>0.028</td>
<td>0.086</td>
<td>FCGR activation</td>
<td>0.019</td>
<td>0.258</td>
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<tr>
<td>Inflammasomes</td>
<td>0.02</td>
<td>0.077</td>
<td>Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell</td>
<td>0.086</td>
<td>0.103</td>
</tr>
<tr>
<td>Role of phospholipids in phagocytosis</td>
<td>0.008</td>
<td>0.257</td>
<td>Role of phospholipids in phagocytosis</td>
<td>0.008</td>
<td>0.257</td>
</tr>
<tr>
<td>The NLRP3 inflammasome</td>
<td>0.015</td>
<td>0.077</td>
<td>Imaging regulatory interactions between a Lymphoid and a non-Lymphoid cell</td>
<td>0.072</td>
<td>0.125</td>
</tr>
</tbody>
</table>
Expression and Purification Trials of the Leucine Rich Repeat Domain of LRRK2

Introduction

Parkinson’s disease

Parkinson’s disease is the second most common neurodegenerative disorder affecting approximately 1% of people over the age of 65 years. With an ageing population, this severely debilitating, chronic disease will become of increasing prevalence, and represent a large burden on healthcare systems. Thus, a greater understanding of the pathological mechanisms underlying the condition is urgently required. Parkinson’s disease is characterised by the degeneration of dopaminergic neurons within the substantia nigra pars compacta (fig 2), resulting in loss of motor control as well as other cognitive and psychiatric problems from damage to surrounding neuronal tissue.

Interplay with innate immunity

Post-mortem analysis of Parkinson’s sufferers has shown increased levels of activated microglia and pro-inflammatory cytokines within the affected region of the brain. Microglia are the resident immune cells of the brain, expressing a wide range of innate immune Toll Like Receptors (TLRs). It is theorised that hyper activation of microglia causes inflammation and cellular damage, creating a feed-forward cycle of further cellular damage and immune cell activation.

LRRK2

Leucine Rich Repeat Kinase 2 (LRRK2) is a large multi domain protein (fig 1) of unknown function expressed in microglia as well as peripheral immune cells. Mutations in LRRK2 have been strongly associated with genome wide association studies to the familial form of Parkinson’s disease as well as other inflammatory diseases such as Crohn’s Disease. There are a large number of such mutations across different domains, which have been shown to affect kinase activity differentially. Cellular studies have revealed possible effects of different mutations to LRRK2, however no clear primary function or pathological mechanism in Parkinson’s has become clear. This work focussed on biophysical analysis of the potential ligand binding Leucine Rich Repeat (LRR) domain of LRRK2.

Modelling of LRRK2 LRR domain reveals homology to TL3

There is no known ligand for LRRK2, so the discovery that the potential ligand binding LRR domain has homology to TL3 suggests that LRRK2 could have a role in innate immune signalling. TL3 is a receptor for RNA, suggesting LRRK2 could potentially bind nucleic acids via discrete charged surfaces (fig 3).

Table 1: Expression systems and tags tried

<table>
<thead>
<tr>
<th>Expression System</th>
<th>Tag</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)</td>
<td>None</td>
<td>Poor expression, Insoluble</td>
</tr>
<tr>
<td>Roseda(DE3)pLysS</td>
<td>None</td>
<td>Poor expression, Insoluble</td>
</tr>
<tr>
<td>Roseda(DE3)pLysS</td>
<td>MBP</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Roseda(DE3)pLysS</td>
<td>HIS</td>
<td>Soluble</td>
</tr>
<tr>
<td>Roseda(DE3)pLysS</td>
<td>N518I-4 (high Fuses)</td>
<td>MBP, solubile, toxicous</td>
</tr>
</tbody>
</table>

LRR domain boundaries and construct design

Even in SF9 cells, protein precipitation was found to be a major limiting factor in the purification process, despite the increased solubility brought about by inclusion of an MBP tag in the construct.

To optimise purification of the LRR domain, six constructs were designed with varying staggered N termini around the predicted terminus of the LRR domain (fig 5a). The C-terminus was fixed at a position known to crystallise the Roc domain.

Each construct: LRR-6, was N-terminally tagged with a TEV cleavable His6-MBP tag (fig 5b).

Figure 3: Model of LRR domain of LRK2 based on TL3.

(A) Cartoon representation showing leucine rich repeats of LRK2. (B,C) Coulombic surface colouring of LRR6 domain. Point mutations are highlighted in yellow. Point mutations are highlighted in yellow. Of note is that LRRs are by definition similar motifs across different proteins; so much further work is needed to validate the significance and accuracy of this homology model.

Figure 5: LRR construct design. [A] Constructs designed with varying N termini, named LRR-1-6. [B] LRR constructs were tagged with His6 and Malto-binding Protein. LRR: Leucine rich repeat Roc: C-terminal of ROC.

Figure 6: Predicted Domain structure of LRRK2. ANK: Ankyrin repeats, SRC: Leucine rich repeats Roc: C-terminal of ROC.

Optimised purification of MBP-LRR6

Using constructs LRRK, LRRK3 and LRRK6 various purification strategies and conditions were trialled allowing comparison of the effectiveness of different:

- Purification techniques
- Purification buffer conditions
- LRR constructs

1. Ni Affinity purification was performed using a HitTrap Chelating column pre-equilibrated with Ni. Protein was eluted by increasing imidazole concentration to 250 mM over 20 column volumes (CV) of buffer.
2. The eluted protein fraction was dialysed to 150 mM NaCl, 20 mM Tris pH 8 overnight, and loaded onto a HiTrap Q column at 50 mM NaCl for anion exchange. Elution was performed by increasing NaCl concentration to 0.5 M over 20 CV.
3. MBP-LRR6 was concentrated and cleaved to LR6 by incubation with TEV protease overnight at 4°C.
4. Cleaved product was re-purified using a Ni HitTrap Chelating column but with now untagged LR6 present in the flow through (FT), indicated by a red arrow (LR6 = *43Da). This approach established a general protocol for purification of quantities of LR6 amenable for basic biophysical analysis, although further optimisation is required. Interestingly, use of MBP for affinity purification was found to be ineffective due to large amounts of protein precipitation.

LR6 binds heparin

Protein purified by the previously described optimised protocol (fig 6) was found, at neutral pH, to bind heparin; a highly negatively charged glycosaminoglycan known for binding to DNA binding proteins - thus ‘mimicking’ negatively charged DNA. This validates modelling performed showing homology to TL3 (fig 3).

Future work

- Generate final MBP-LRR6 baculovirus to improve LR6 yields, or attempt to improve solubility of protein expressed by bacterial expression systems.
- Continue optimisation of purification including incorporation of heparin affinity as a ‘polishing’ step.
- Trial LR6 capacity to bind heparin at high pH in order to eliminate TEV protease binding.
- Investigate the ability of LR6 to bind different nucleic acids by ‘electrophoretic mobility shift assay’.
- Continue screening crystallisation conditions and LR6 concentrations to obtain crystals for x-ray crystalllographic analysis.