# Neuroinflammation and Parkinson's Disease: Understanding the inflammatory process induced by chronic peripheral injection of LPS in LRRK2 G2019S KI mice in correlation with age

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A Thesis submitted to The Faculty of Graduate Studies and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science

in

Neuroscience

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# Abstract

Parkinson' disease is a progressive neurodegenerative disease arising from a collective effect of advancing age, genetic vulnerabilities and environmental toxins. The objective of the current study was to elucidate a synergistic effect of the advanced age, G2019S mutation and immunological stress (LPS) on neuroinflammation. In the present study, male mice were given five intraperitoneal injections of 250mg/kg of LPS (or saline) every alternate day across the two levels of the age and genotype (age; old vs young, genotype; WT vs G2019S). In line with our expectation, there was a significant loss of TH+ cells in old-G2019S mice that received LPS compared to the young-WT that received saline. There was a significant effect of age and genotype on baseline locomotor activity of these animals. Age and genotype predominantly affected other aspects like increased CX3CR1 expression and increased SiRT3 expression in SNc.

# Acknowledgement

I would like to take some time here at the beginning of my thesis to acknowledge the work and support of members of the Hayley lab in completion of this project.

A first thank-you goes to Teresa Fortin; she has been great support with her profound knowledge in behavioural and molecular techniques. Throughout this two year, she has been my to-go person, thank-you for being there and having all the answers to "Teresa, I can't find it?" questions. A special thank-you to Dr. Zachary Dwyer (Ph.D.) for working with me on the study design, helping me understand minute details in this project, and for the care and effort he put into teaching me the techniques and methodologies required for this project. I would also like to give special thanks to all the Ph.D. and masters students for their timely support and guidance.

And of course, my family, who have been there by me with their never-ending support and love which has helped me steer through difficult times. Living thousands of miles away from family can be difficult, but with my amazing friends, especially Manu, it has been easy-breezy. Without your constant support and encouragement, this would not have been what it is now.

Finally, I would very much like to thank my supervisor Dr. Shawn Hayley for his constant guidance that helped me throughout this project. His enthusiasm for research and his subject knowledge has inspired me to learn and develop as a researcher in pursuit of my thesis. Thank you all for the help, support and encouragement.

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# Abbreviations

| | |
|---|---|
| AD | Autosomal Dominant |
| ATP | Adenosine Triphosphate |
| BBB | Blood-Brain Barrier |
| CNS | Central Nervous System |
| COR | C terminal of ROC |
| DA | Dopamine |
| DAT | Dopamine Transporter |
| ERK | Extracellular signal-Regulated Kinase |
| ETC | Electron Transport Chain |
| FAT | Focal Adhesion Targeting |
| IL | Interleukin |
| JAK | Janus kinase |
| JNK | c-Jun N-terminal kinases |
| LPS | Lipopolysaccharides |
| LRRK2 | Leucine-Rich Repeat Kinase 2 |
| MAPK | Mitogen-Activated Protein Kinase |
| MDV | Mitochondrial-Derived Vesicles |
| MHC | Major Histocompatibility Complex |
| MitAP | Mitochondrial Antigen Presentation |
| MMP | Matrix Metalloproteinase-3 |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| NFκB | Nuclear Factor kappa-light-chain-enhancer of activated B cells |
| OCR | Oxygen Consumption Rate |
| PARK | Parkinson disease-associated genes |
| PD | Parkinson's Disease |
| PKC | Protein Kinase A |
| PQ | N, N′-dimethyl-4,4′-bipyridinium dichloride |
| ROC | Ras of complex proteins |
| ROS | Reactive Oxygen Species |
| SNc | Substantia Nigra pars compacta |
| SNX9 | Sorting-Nexin9 |
| VMAT | Vesicular Monoamine Transporter 2 |
| VTA | Ventral Tegmental Area |
| WD40 | Tryptophan-Aspartic (W-D) |
| Wnt | Wingless/Integrated |

# 1. Introduction

## 1.1 Parkinson's Disease

Parkinson's disease is one of the most common neurodegenerative disorder affecting 1 in 100 over the age of 65 [1] [2]. It is a chronic and progressive disorder that affects the motor system [1]. The four cardinal symptoms are bradykinesia (slowness of movement), resting tremor (unintentional, rhythmic muscle movement), rigidity (abnormal stiffness) and postural instability [2][3]. These symptoms become more pronounced over time, and individuals have difficulty in talking, walking and doing other simple tasks. Chance of developing Parkinson's disease increase exponentially with age and tends to the peak at the age of 80 [4]. While age remains one of the significant risk factors, gender and ethnicity are also risk factors in developing the disease [5]–[7]. The cross-sectional and longitudinal studies of sex differences in PD suggested a significantly higher incidence among men, with the relative risk being 1.5 times greater in men than women [8]. The more benign phenotype of the disease in women is thought to be likely due to estrogen [9]–[11]; however, as the disease advance, women are at higher risk of developing highly disabling treatment-related complications compared to men [9].

Basal ganglia are subcortical nuclei associated with various functions including control of the voluntary motor movements, behaviours and emotions [12]. The substantia nigra provides input that plays a central role in reward and movement [12], [13]. The substantia pars compacta serve mainly as an output to the basal ganglia circuit supplying the striatum with dopamine [14], depletion of which cause dysregulation of motor circuits resulting in the clinical manifestation of PD [15], [16]. Loss of dopamine-producing neurons vary in a different degree at different locations of the midbrain, and the most severe loss occurs at the SNc [12]– [14] reaching 80% at the end-stage of the disease [15], [16]. Besides depletion of 60-80% of dopamine-producing neurons, the presence

of Lewy bodies, accumulations of protein α-synuclein makes up the main pathology of PD [17]–[22]. Although the exact cause that accounts to the death of the dopaminergic neuron is unknown, several hypotheses suggest a combination of genetic and environmental causes rather than a strict genetic or strict environmental cause.[23]. While one of the causes consists of an excessive accumulation of α-synuclein in these cells [20], [21], [24]–[27], the other mechanism involves the mitochondrial dysfunctions due to increased oxidative stress [28]–[32] and ageing [30], [33]–[35], mutations in genes like LRRK2 [36]–[38], SNCA [27], [39], [40], PINK1 [41], [42], PARKIN[43] and their role in mitochondrial dysfunction [44]–[48]. Environment factors like infections/traumatic brain injuries[49]–[52], as well as prolonged exposure to insecticides and pesticides, plays a substantial role in PD [53]–[61]. PD is a result of a very complicated interaction between factors like age, genetic predisposition, environmental toxins that leads to dysfunction of multiple cellular systems.

Early diagnosis of a disease can be a crucial step in delaying or preventing the progression of a disease and in a very complicated disease like PD that has multiple risk factor and progression is quick, diagnosis becomes harder to achieve at an early stage of the disease. Diagnosis of PD occurs with the onset of motor symptom, especially bradykinesia and additionally, at least one other cardinal motor features (rigidity, postural instability or resting tremor) [62]. 50% of the nigral neuronal loss at the time of diagnosis suggests a long period of nigral degeneration before the appearance of clinical features/symptoms [63]. In the early stages of the disease, loss of DA neurons is restricted to the ventrolateral substantia nigra and becomes more widespread at the end stage of the disease [33]. One-fourth of patients diagnosed with PD during their lifetime have an alternative diagnosis at the time of postmortem. This misdiagnosis is attributable to relying on only initial diagnosis, as diagnostic accuracy improves by time and follow-up visits [64].

Levodopa, L-3,4-dihydroxyphenylalanine, is a precursor of dopamine and is the most potent drug for managing PD symptoms, particularly those related to the slowness of movement [65]. Levodopa is administered with a dopa carboxylase inhibitor to increase its bioavailability and tolerability. Although there are new insights on the effective administration of levodopa, questions are raised as to the limitations posed by the drug in reducing/stopping the ongoing neurodegeneration [65]. A neuroprotective disease-modifying agent that could reduce neurodegeneration and provide better management of the motor and non- motor symptoms is essential in existing disease therapy.

## 1.2 Ageing

Increasing research in age-related disease is due to the increasing ageing population and increasing average life expectancy in developed nations. While there are many age-related diseases, Parkinson's disease is the second most common disease in the aged population, after Alzheimer's disease [62]. It affects 1% of the population over the age of 60 and reaches up to 5% of the population above the age of 85, highlighting the impact of advancing age on the risk of developing the disease [66][62]. Thus, understanding the correlation between age and dopamine cell loss is an essential facet towards disease assimilation.

Most of the significant changes happen in the brain, like age-related volume reduction in the gray (especially prefrontal) matter, and shrinkage of sensory and entorhinal cortices and some regions, especially in the hippocampus and the prefrontal white matter, shrinkage increases with age [67]. Most of these changes account for reduced cellular activity, damage or decrease in the number of neurons due to disease conditions or natural ageing phenomenon. SNc DA neurons have a higher oxygen consumption rate (OCR) leading to higher basal oxidative phosphorylation which contributes to its increase reactive oxygen species (ROS) production [68]. SNc DA neurons are also

different in terms of their axonal arborization compared to their neighbour, VTA (Ventral Tegmental Area) neurons by 113% higher [87], [88]. With these correlated results, one can only draw a picture of the presence of highly active mitochondria or a high density of mitochondria, or both in these neurons [68]. Mitochondrial dysfunctions have been thought to be involved in PD in multiple ways: with advancing age, mtDNA deletions tend to get higher in SNc neurons compared to others [30], [70]–[72], these mutations can cause protein dysfunctions leading to disruptions in mitochondrial [73], [74]. Mitochondrial complex-I is more susceptible to environmental toxins like MPP+ [75]–[78]; this can create tremendous oxidative stress within the cell, disrupting its processes and accumulation of protein aggregates like α-synuclein [78].

To conclude, with the advancement of age, there is a significant decline in multiple processes like dopamine metabolism, protein degradation, and ATP production that can create oxidative stress in the SN neurons which can overburden them due to their vulnerability attributed by constant firing and high-density mitochondria.

## 1.3 Environmental Factor

Exposure to certain chemicals can be less harmful while others can be hazardous; some people might have inherited genetic makeup which can make them less or more susceptible to these toxins than others. With these regards, a neurodegenerative disease like PD, which is thought to be caused by multiple factors, can manifest uniquely for each person [23], [79]. Widely used pesticides, Rotenone and Paraquat, were discovered around the 19th century. The neurotoxic effects of these pesticides were unclear until the first paper published in 1983 that linked the MPTP poisoning with PD. Accidentally sold as synthetic heroin, MPTP was used intravenously by four young individuals who subsequently developed parkinsonism [58]. Paraquat (PQ), N, N′-dimethyl-4,4′-bipyridinium dichloride, is a quick-acting non-selective herbicide [80], [81]. The possibility that PQ might

contribute to the neuropathology of PD was due to its structural similarity with MPP+ [82]. Studies have proposed a mechanism by which PQ reduces to PQ+ in the presence of a reducing agent or NADPH oxidase on microglia, mediating the interaction with DAT [83], [84] and it induces α-synuclein upregulation and aggregation [84], [85]. Increase in intracellular PQ+ results in increased ROS production and cytotoxicity [86] resulting in cell death.

Inflammation in the brain can also occur as a consequence of brain injury or infectious agents like bacteria and viruses. Some cases reported acute parkinsonism caused as a result of viral infection [87]–[89]. In an experiment, infecting neonatal rats with Japanese encephalitis virus, induced neuronal degeneration of SNc neurons at an older age, causing movement disorder that resembled PD. [90] Besides virus, a bacterial infection is also thought to play a role in PD pathology [91]. A study conducted to understand the prenatal infections such as bacterial vaginosis representing as a risk factor for PD; female rats were given LPS at embryonic day and pups were monitored for signs of dopamine loss at postnatal day 21. The prenatal exposure significantly reduced striatal DA and increased the presence of TNF-α in the CNS [91].

Apart from these exogenous toxins and infections, dopamine, to some extent, is also thought to be toxic for DA cells [92]. Dopamine metabolism causes a high number of oxidants like hydrogen peroxides. In a crisis of degeneration of the neurons in SNc, the remaining neurons become hyperactive to restore the dopamine in the system, which leads to an increased DA turnover creating more oxides and further damage [93]–[95]. Dopamine oxidation can play a role in neuronal degeneration as well. It can auto-oxidized to form o-quinone inducing mitochondrial dysfunction, formation and stabilization of neurotoxic protofibrils of alpha-synuclein, dysfunction in protein degradation systems and oxidative stress [92].

### 1.3.1 Lipopolysaccharide

LPS is found in the outer membrane of Gram-negative bacteria and acts as endotoxins. LPS can initiate acute inflammation responses inducing a diverse range of effects, including septic shock [96]. Lipid A moiety is the endotoxin component of LPS [97] and activates Toll-like receptors family in the innate immune system [98]–[100]. In CNS, LPS activates microglia by binding to TLR4 [101]. With the increasing evidence of the role of neuroinflammation in degeneration of nigrostriatal dopaminergic pathway in Parkinson's Disease [102]–[106], LPS has been extensively used to mimic the DA neuronal death and glial cell activation in the disease model [98], [107], [108]. In neuronal cultures as well as in vivo models, DA neurons are shown to be more sensitive to LPS than non-DA neurons attributable to the abundance of microglia [109].

Both single and repeated dose LPS models are widely studied to understand the inflammatory processed in CNS and to determine methods or novel compounds to intervene in the degeneration induced by inflammation. Intrastriatal injection of LPS has shown to significantly reduce DA neuron through increased pro-inflammatory factors, increase ROS and matrix metalloproteinase-3 (MMP-3) [107]. Another study showed that LPS injection into striatum caused accumulation of α-syn and increased defects in the mitochondrial respiratory chain [108]. In a study, to determine if LPS can induce permanent damage to different brain regions (SN, Medial Forebrain Bundle and Striatum), rats were injected with LPS into these regions and assessed one-year post-injection. Apart from seeing acute effects of LPS, DA neuronal loss persisted post one year in SN compared to other brain regions [110]. There was a significant loss of DA neurons accounting up to 50-60% with increased DA turnover pointing out to a compensatory mechanism rather than cellular recovery in this region [110]. In a study to test the involvement of dopamine in LPS induced neuronal degeneration, α-methyl-p-tyrosine, a tyrosine hydroxylase inhibitor was injected in the rats

[111]. The result showed that not only did inhibition of tyrosine hydroxylase produced considerable protection against the LPS insult, but systemic treatment with L-DOPA also reversed the protective effect of α-methyl-p-tyrosine [111]. In mice, striatal injection of LPS followed by the administration of methamphetamine, an inducer of dopamine efflux, exacerbated both neuroinflammation and motor impairment, further indicating a role for DA itself in LPS damage [112].

## 1.4 Genetic Factors

A paper published in 1996 identified the first mutation responsible for PD and mapped it to chromosome 4q21-q23 [113]. Further research the following year identified the α-synuclein mutation in an Italian family with hereditary PD [27]. There are so far six chromosomal regions that cause monogenic PD; mutation in a single gene enough to cause disease phenotype [114]. Eighteen chromosomal loci are cumulatively termed PARK and numbered chronologically based on time of discovery [114].

Majority of PD cases are sporadic and are poorly understood but has been speculated to be caused by both environmental and genetic factors. Although genetic defects are accountable for less than 10% of the PD cases [115], understanding the mechanisms of these genes can provide insight into the molecular mechanism of neuronal degeneration in PD. PARK1, PARK2, PARK6, PARK7, and PARK8 are some of the PD-linked genes [116], [117]. While mutations in SNCA (PARK1) and LRRK2 (PARK8) causes an autosomal-dominant form of PD, Parkin (PARK2), PINK1 (PARK6)and DJ-1 (PARK7) account for the autosomal-recessive mode of inheritance [115], [116].

### 1.4.1 Leucine-rich Repeat Kinase 2

PARK8 or LRRK2 (leucine-rich repeat kinase 2) located on the chromosome 12p11.2- q13.1 encodes for 280kDa, 2527 amino acid LRRK2 protein or Dardarin [36], [134], [135]. LRRK2 protein is present in the cellular membrane, including the outer membrane of mitochondria [136]. LRRK2 belongs to the ROCO superfamily of proteins, and it is structurally similar to LRRK1 [137], [138]. LRRK2 consist of an ankyrin repeat sequence at the N-terminal, a leucine-rich repeat (LRR) domain, a ROC (Ras of complex proteins) [138] domain followed by COR (C terminal of ROC) [138], a kinase domain and a C-terminal WD40 domain [139]. While ROC beholds the GTPase activity with sequences similar to the Rab-like proteins, the kinase domain of the protein belongs to the mitogen-activated protein kinase-kinase-kinase subfamily [140]. Leucine-rich repeats impart the protein-protein interaction motif to LRRK2 while WD40 domains interact with lipids associating it with intracellular membranes [140], [141]

The physiological functions of the protein are attributable to two of its enzymatic domains: kinase domain (catalyzing phosphorylation) and ROC-GTPase domain (GTP-GDP hydrolysis) [140]. Recent studies have shown that it might be involved in vesicle trafficking [142], [143], protein degradation [144], autophagy [145], [146] and immune system [145], [147]–[149]. LRRK2 is also shown to bind MAP2K4 which is an important enzyme in the MAPK pathway responsible for phosphorylation of MAPK inducing changes in transcription [150]–[153]. MAP2Ks are involved in the activation of p38 and JNK pathways that are known to control cell proliferation, apoptosis, inflammation and immune responses, production of cytokines and cell differentiation [154], [155]. Mutation in LRRK2, specifically kinase domain, have shown to hyper-phosphorylate MAP2K4 leading to a degeneration of resident neurons [153], [156]. β-catenin is a protein that regulates and coordinates cellular adhesion [157], [158] and is a transcription factor that is responsible for

regulation of up to 400 genes that are involved in multiple aspects of cellular functions like inflammation and immune response, cellular growth, apoptosis and synaptic maintenance [157]–[160]. It acts as an intracellular signal transducer in the Wnt signalling pathway [159]. Wnt signalling pathway carries out cell migration and neural patterning during embryonic development [161]–[163], formation and modulation of neuronal circuits [164], [165], maintaining tissue homeostasis and tissue regeneration [166], [167]. In Alzheimer's disease, loss of synapses is thought to be due to alterations in this pathway [168], [169]. The ROC-COR domain of LRRK2 has shown to interact with key components of the Wnt signalling pathway called disheveled proteins [170]–[172]. Researches have established that Wnt signalling is essential for the development of dopaminergic neurons and the negative effects of decreased Wnt signalling in neuronal survival are well understood [173]–[176]. Deregulation in Wnt signalling has shown to play an important role in impaired adult neurogenesis in PD [170]. Reactive microglia were shown to protect dopaminergic cells by activating the canonical Wnt pathway, thereby promoting neurogenesis [170]. LRRK2 mutations are thought to decrease Wnt signalling through disheveled proteins interactions [170]. LRRK2 is also thought to activate NAADP (Nicotinic acid adenine dinucleotide phosphate) which generates a Ca2+ influx calcium-dependent CaMKK1β (Calcium-dependent protein kinase-kinase β) eventually activating AMPK (adenosine monophosphate-activated protein kinase) which is followed by a persistent increase in autophagosome [177]–[180]. LRRK2 has a role to play in vesicle dynamics and retromer functions; it interacts with mitochondria and mitochondrial proteins and also interacts with the immune system and its components [140], [181].

Studies earlier proposed that within the immune system, LRRK2 might be involved in the activation and maturation of immune cells [182], [183]. Some studies reported an increased LRRK2 expression in microglia when induced with an inflammatory stimulus such as LPS [184], [185]. In

murine microglia-cell culture, down-regulated or mutated LRRK2 has shown to reduce pro-inflammatory cytokines, both mRNA and protein levels, upon induction with LPS [186]. NF- κB (nuclear factor kappa-light-chain-enhancer of activated B cells), a major transcription factor for innate and adaptive immune response, was significantly reduced in an LRRK2 Knock-down microglia when induced by LPS, promoting prolonged inflammation [187], [188]. Even though there is evidence of LRRK2 interacting with the immune system, a specific pathway or signalling cascade related to LRRK2/involving LRRK2 is not established yet.

Interaction of different pathways, specifically related to the immune system, apoptosis and similar suggest that mutation in LRRK2 can influence or even induce unsolicited cellular degeneration. LRRK2 mutations, especially the ones that affect its catalytic sites, kinase or GTPase, are found to be involved in different pathogenesis [189]– [193]. Some of the most commonly studied mutations in PD are R1441C, R1441G, R1441H, Y1699C, I2020T, N1437H and G2019S [194]. R1441 is a multi-amino acid substitution of the same residue in a highly conserved GTPase domain [195]–[199]. R1441C is the second most recurring mutation after G2019S in LRRK2 PD [200]. Y1699C mutation lies between the GTPase and Kinase domain and is one of the most common mutations in the United Kingdom, affecting 25 subjects over four generations [201]. Both the R1441C/H/G and Y1699C mutations together are thought to increase the kinase activity of the protein [196], [202].

### 1.4.2 G2019S mutation in LRRK2

G2019S, the substitution of Gly for Ser at the start of the kinase activation segment, is the most common LRRK2 mutation that causes a clinical and pathological phenotype similar to iPD [203], [204]. It is associated with 5-6% familial PD and 1-2% sporadic PD [203]. G20109S mutation affects the kinase function of the protein, exhibiting an increased kinase activity [205].

G2019 is part of a highly conserved region/motif which involves in kinase activities by cleaving γ-phosphate from ATP with the help of aspartate (D2017) [205]. In most kinase, there is an activation loop that steers the protein activation in such a way that a conformational change in the activation loop, often allowing the accessibility to the substrate, determines its state of activity or inactivity [206]. G2019S is part of such an important conformationally flexible region of an activation loop that steers the kinase activity of the protein [207]. Glycine is a small amino acid with a smaller side chain, makes the shifting of the loops in orientations relative to the two lobes of the enzyme, easy [207]. The Ser substitution, a bulkier and negatively charged amino acid might damage the flexibility of the activation loop and probably locking it in the active conformation, kinase domain highly accessible to its substrates [207]. Although the majority of LRRK2 G2019S substrates are unknown, it is thought to interact with components of DA neurotransmission [208], [209], JNK pathway [153], [210], α9-syn [211], and the immune cells like microglia [186], [212], [213]. G2019S mutation is thought to alter synaptic structures and functions during the developing stage to create abnormalities that might participate in the generation of PD symptoms in a later stage of life [214]. G2019S KI mice also show an age-related change in DAT and VMAT2 functions compared to the WT [215]. Neuronal loss in SN due to G2019S mutation is attributable to the activation of apoptotic pathways. LRRK2 transgenic mice show an elevated level of active p-MKK4ser257 [153]. An activated phospho-MKK4ser257 can activate downstream target, p-JNKThr183/Tyr185 and phospho-c-JunSer63 which leads to an upregulation of pro-apoptotic protein Bim and FasL and leads to the formation of caspase-9, caspase-8, and caspase-3, initiators of the apoptotic pathway [153]. Apart from neurons, LRRK2 is also expressed on microglia [216], [217]. Microglia are brain macrophages that scan through the brain for foreign substances or injuries. They isolate the lesion site by extending their processes, to prevent further damage [218]. FAK

(focal adhesion kinase) is a non-receptor tyrosine kinase that helps in cell migration, proliferation, and survival [219]. It consists of an N-terminal FERM domain, a tyrosine kinase domain and a C-terminal focal adhesion targeting (FAT) domain [213]. FAK is importantly phosphorylated at Y397 to regulate its adhesion and migration pathway, and LRRK2 G2019S-gain-of-function seems to inhibit FAK in a kinase-dependent manner and thus negatively regulating microglial motility [213].

## 1.5 Neuroinflammation

Chronic inflammation is a well-established hallmark of neurodegenerative diseases like PD [102]–[104]. Microglia are the brain macrophages that are considered to be a key player of neuroinflammation [104]. Due to the high sensitivity of microglia, it is possible that cytotoxicity and tissue damage occurred from an excessive and protracted inflammatory response, results in the neurodegeneration [102].

### 1.5.1 Microglia

During 1916-1927, an epidemic ravaged North America and Europe. It was influenza-like illness causing a 40% mortality rate in affected areas and survivors of the epidemic later developed PD in less than ten years [220]. In 1988, a study conducted on post-mortem brains of PD patients reported a presence of active neuropathological processes at the time of death [56]. A similar autopsy study showed the presence of highly reactive microglia and increased the level of cytokines such as TNF-α, interleukin-1β, IL-2, IL-4, IL-6 and TGF (transforming growth factor) in nigrostriatal regions in PD patients compared to their age-matched unaffected individuals [221]–[223]. Later, animal models based on the SNc injections of immunostimulants like LPS and thrombin have also shown similar induction of inflammatory process resulting in neuronal degeneration [109]–[111], [224]–[229]. These results suggest that the chronic inflammation in CNS

precipitated neurodegeneration in PD patients.

Microglia are glial cells located throughout the brain and spinal cord [230]. These macrophages in the brain are the first barriers of the innate immune response [103], [230]. They have branched processes that extent upon activation of microglia [231]. Even though highly active, the ramified or "resting" form of microglia has long branched processes, and smaller cellular body and are constantly surveying their environment for infections or lesions [231]. In the reactive state, microglia go through a non-phagocytic phase before entering the phagocytic phase, during which it secretes cytokines, pro-inflammatory molecules to attract more microglia to the injury site [231]. Active phagocytic microglia take up a large ameboid shape that allows it to engulf and phagocytose the antigen to present it to T-cells [231]. Very sensitive to their environment, imparted by ion channels, cytokines, chemokines, and Toll-like receptors, they can even react to changes in ion homeostasis [105]. Recent studies have suggested that the homeostasis of microglia is not a mere lack of insult or signal; Instead, neurons actively maintain and regulate microglia through various signals and pathways [232]–[234]. Neurons in slighter distress, the ones that can be rescued/recovered from, send more signals, mostly chemical of nature, to stimulate microglia to nurse the injured neurons [232].

On the other hand, when the injury sustained is severe and irreversible, the signal sent across stimulates microglia to turn neurotoxic [232]. There are two kinds of signals, one that activates the microglia- excitatory and the other that antagonizing-inhibitory and there are two subtypes for both the types- released signals and membrane-bound signals [232]. Examples of released inhibitory signals are CX3CL1, CD22, TGF-β, membrane-bound inhibitory are CD200, CD47, the one that consists of immunoglobin superfamily. CX3CL1 can also act as released excitatory signal depending on the amount released [235].

Additionally, extracellular matrix protein, MMP-3, is also found to regulate microglia [236]. Release by neurons, matrix metalloproteinase 3, are proteins that are involved in breaking down of extracellular matrix during development, wound repair [236]. In a study conducted mouse microglia cell line, BV-2 cells, MMP-3 mediated microglial activation by an extensive release of TNF-α from microglia and activated NF-κB pathways, and the activation mostly depended on ERK (extracellular signal-regulated kinase) [236]. Besides MMP-3, neuromelanin is also thought to activate microglia. Neuromelanin is believed to have a protective effect on neurons as we age, by mediating chelation of metals [106]. They are synthesized from L-DOPA and are thought to be a way of storing excess dopamine and L-DOPA [236]. Postmortem brains of PD patients had a large number of free extracellular neuromelanin [237].

Microglia can also function as antigen-presenting cells and express MHC class -II molecules on their surface [105]. When triggered by IFN-γ, a potent stimulant of MHC-II, it upregulates MHC II expression along with CD40, ICAM-1 triggering T cell proliferation and production of IL-4 by Th2 cells [238], [239]. IL-1 and TNF-α are the two important proinflammatory cytokines released by microglia upon activation by a pathogen or pathogen components like LPS [104]. IL-1 and TNF-α disrupt the BBB (blood-brain barrier) by inducing astrocytes to release chemokines and adhesion molecules, and this facilitates the penetration of white blood cells to the infected area creating a CNS inflammation [240], [241]. Persistent upregulation of these cytokines and related pathways can lead to prolonged inflammation causing excessive neurodegeneration as seen in PD patients.

### 1.5.2 Chemokines

Chemokines are small chemotactic cytokines that regulate a variety of leukocytes during inflammation [242]. They are smaller proteins weighing up to 8-10 kDa and have four cysteine amino acid residues conserved to form a three-dimensional structure [242], [243].

Fractalkine/Neurotactin/CX3CL1 is a unique chemokine that was discovered in 1997 [244] CX3CR1 is exclusive to microglia [245]. The uniqueness of this particular chemokine is its ability to exist in two different forms: membrane-bound protein tethered to the neurons and as a soluble factor released upon cleavage at N-terminal [246]. With this dual mode of existence, it acts as an adhesion molecule when membrane-bound and extracellular chemoattractant when in a diffusible form [246]. It plays a crucial role in various functions of microglia, notably migration and active surveillance [247]. It also plays a role in hippocampal neurogenesis, maturation of neurons, survival of developing neurons, learning and memory [247]. Studies have shown that deleting fractalkine or its receptor results in exacerbation of inflammation and neurodegeneration [248]. The pattern of activation cascades dependents upon the concentration and form of fractalkine. At a lower dose, it may serve as a microglial regulator, aiding in the process of microglial migration and at a higher dose, it can act as a pro-inflammatory cytokine [249]; it is neuroprotective in soluble form and neurotoxic in the membrane-bound form [248]–[252].

# 2. Research Proposal

Although existing researches revolve around ageing, genetic predisposition and immunological insult as causative factors of Parkinsonism, there is lesser probing into the existence of correlation or interaction between the three factors. LPS induced microglia in an environment with increased kinase activity of LRRK2 (G2019S) and complication of advancing age can aggravate the immunological reaction in the system to cause excessive loss of neurons. With this in mind, the focus is to explore such exacerbated immune response in older LRRK2 G2019S mice in response to immunological insult such as LPS.

The use of animal models to spawn a disease phenotype enables us to understand the

mechanisms of disease progression, and it also allows us to explore potential treatment methods. While existing models of PD does not precisely resemble the disease phenotype, some of the aspects of these models are of high value to our understanding of the disease. One such model that interrogates the immunological element of the disease is LPS induced inflammation. It recreates some traits of PD like substantial activation of microglia and selective loss of SNc neurons. Another animal model of PD is the genetic model. LRRK2 G2019S mutation is the most common genetic cause of PD [139]. LRRK2 G2019S KI shows an elevated kinase activity in the brain of both heterozygous and homozygous mice and impairments in dopamine release and mitochondrial functions is also shown to increase with age in these mice [253]. Although there is a resistance to motor decline associated with ageing in these mice [254], it lacks an understanding of repercussions caused by multiple hits like head trauma or infection/inflammation of the brain, which might be the case of the actual disease manifestation.

This multi-hit model of Parkinson's disease suggests that an individual with genetic susceptibility can increase his/her chance of developing disease phenotype when "hit" by an immunological insult and this can amplify with the complication of ageing.

We hypothesize that genetic, inflammation and ageing factor, apart from causing disease phenotype individually, can collaborate to amplify the effect of one another, causing a significant outcome. It is a valid argument that these factors, mainly the genetic and immunological, may not interact with one another to generate a higher result than what is already observed in other LRRK2 G2019S models or inflammation models of PD. In either case, induction of an inflammatory insult using LPS can alter/change multiple signaling processes/pathways, some of which are known to interact with LRRK2 [213], [255], [256], which will serve as an important data to expand our knowledge on LRRK2 functioning and neuroinflammation in PD.

## 2.1 Experimental Design

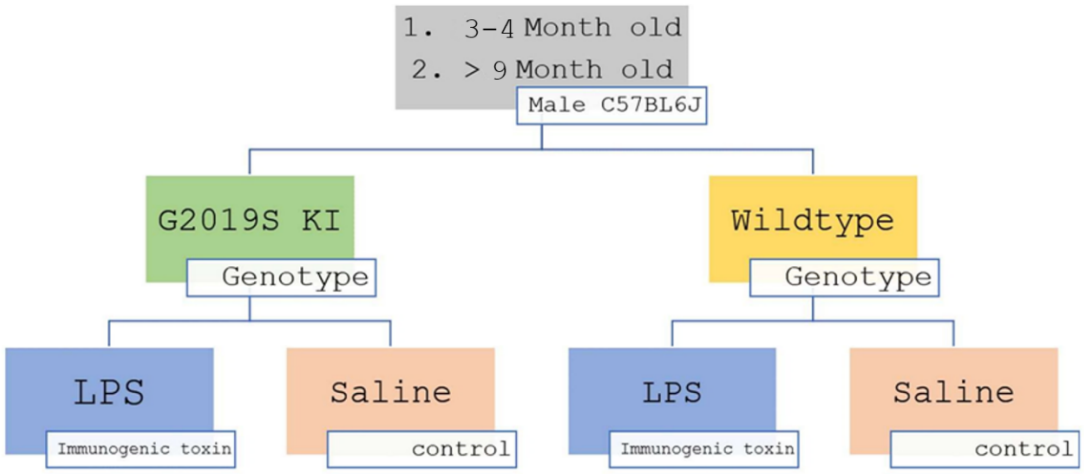


**Figure 2.1:** Experiment model: Diagram explaining animals used in the study. Male mice in 2-4 months of age and older than nine months of age from each genotype were given either saline (control) or LPS.

## 2.2 Experimental Timeline

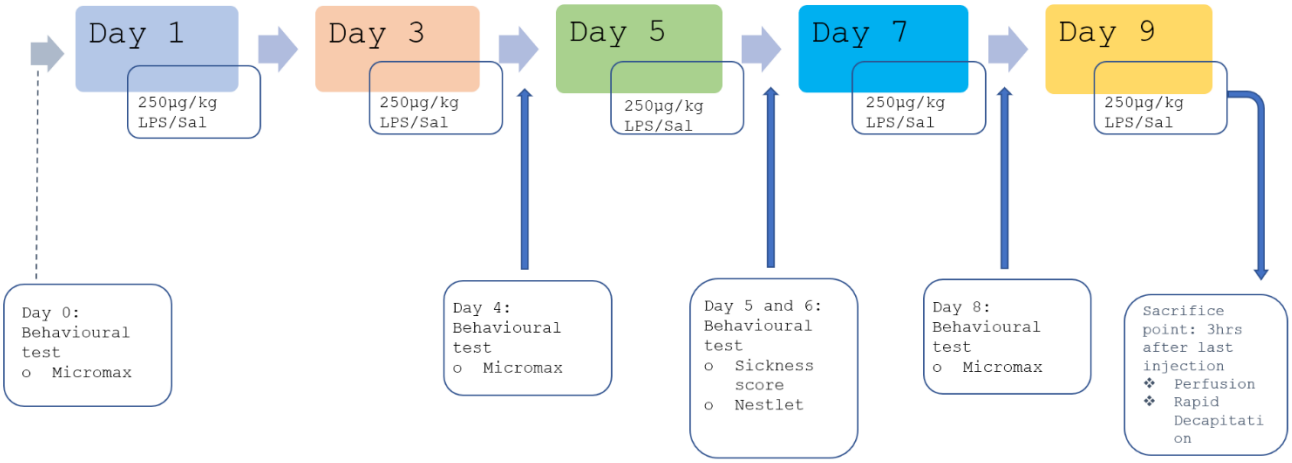


**Figure 2.2:** Experimental Timeline: 10 mice from each group (Genotype X Age) are given LPS (250µg/kg) or Saline every alternate day starting Day 1 via IP. Nestlet score, sickness and weight are measured every day 2 of the injection. Three hours post the last injection on Day 9, half of the animals were sacrificed by perfusion and the other half by rapid decapitation.

# 3. Methods

## 3.1 Animal

A total of 80 male G2019S KI mice on a C57BL6/J background used in this study, were born at Roger Guindon Hall's Barrier Facility. The desired genotype (LRRK2 G2019S KI and Wildtype) were generated through Het to Het breeding. They are group-housed with a maximum of 4 animals per cage and held in a room maintained under constant temperature and humidity condition with 12hr/12hr light/dark cycle. They have free access to food (Harlan Labs 2014 rodent chow) and water. They were individually housed with nestlet only, one week before the start of the experiment. All aspects of this experiment were approved by the Carleton University Committee for Animal Care and adhered to the CCAC ethical standards.

## 3.2 Injections

Animals were divided into two groups based on age, old group if more than nine months of age and young group if 3-4 months of age. Each group contained a total of 40 animals (20 LRRK2 G2019S and 20 wildtypes). This group was further divided into treatment groups, either receiving 250µg/kg of LPS or an equivalent volume of saline through IP injections. LPS was freshly made each morning of the injection days, which are Day 1, Day 3, Day 5, Day 7 and Day 9.

## 3.3 Behavior

### 3.3.1 Weights

Animals are weighed every morning of the injection days (Day1, Day 3, Day 5, Day 7 and Day 9) during the experiment.

### 3.3.2 Sickness Score

Animals were observed for sickness every day throughout the experiment, and they were scored for sickness 1-hour and 24-hours post 3rd injection (on Day 5). Sickness was scored based on Table 3.3.2.

| Score | Symptoms |
|---|---|
| 1 | Normal looking, healthy, active |
| 2 | Slight lethargy, ptosis (droopy eyes) or piloerection (puffy fur) |
| 3 | Very lethargic, ptosis and piloerection, curled body posture |
| 4 | Very sick appearance, ptosis, piloerection, curled body posture, difficulty breathing, and general nonresponsiveness |

**Table 3.3.2:** Sickness Score: scoring criteria for the assessment of sickness in mice.

### 3.3.3 Nestlet Scoring

Nests are important for small animals as it helps conserve heat, in reproduction and provides shelter from a possible predator. Nest building is a goal-directed behaviour that depends upon motivation, physical ability and orofacial/forelimb movements, therefore helping us understand the motor coordination skills in mice under different treatment group. Post 3rd injection (Day 5) mice were placed in a new standard polypropylene cage with a fully intact nestlet, and the ability to build nest was assessed by assigning scores based on the quality of nest after 1-, 3-, 5- and 24-hours post-injection (Table 3.3.3) [257].

| Score | Criteria |
|---|---|
| 1 | a nestlet had been shredded up to 10% |
| 2 | nestlet was shredded up to 25% and scattered |
| 3 | 50% of the nestlet had been shredded and somewhat gathered |
| 4 | 75% of nestlet was shredded, pieces are gathered and connected |
| 5 | 75–100% of nestlet is shredded into a localized and defined nest with walls |
| 6 | 100% of nestlet is shredded and built into a tight 3D nest that rises above the mouse and is localized in one corner |
| 7 | 0.5 points was given when all of the criteria was met for the lower whole number and some, but not all, of the criteria were met for the next number on the scale. |

**Table 3.3.3:** Nestlet Scoring Criteria

### 3.3.4 Micromax

Micromax is the most standardized general measure for locomotor activity and helps detect any abnormalities in motor behaviour. Measurements of horizontal motor activity were obtained for uninterrupted 12h on Day 0, Day 4 and Day 8. Nestlets were removed from the cages before placing them in the Micromax. Animals were given full access to water and food.

## 3.4 Sacrifice

### 3.4.1 Perfusion

Animals were administered with 0.15mL of sodium pentobarbital intraperitoneally. Blood was flushed using 5mL of saline through the left ventricle to the right atrium followed by 40mL of 4% paraformaldehyde to fix the tissue.

### 3.4.2 Rapid Decapitation

In the rapid decapitation group, animal's trunk blood was collected into tubes containing 10ul of 10% EDTA, to prevent clotting immediately following the decapitation. The blood was then spun in a pre-chilled centrifuge at 2,000g for 20 minutes, and the resulting plasma collected and flash-frozen to -80°C. The brains of these mice were extracted, sectioned and the striatum and SNc were punched within 3 minutes of decapitation for Western blotting.

## 3.5 Molecular Analysis

Brains harvested from perfusion were sectioned via cryostat at –20°C after flash freezing and sections were collected in 0.1M PB with 0.1% sodium azide (for tissues stained within two weeks) or collected in a (2ml) centrifugation tube with a 1.5ml cryoprotectant (for long term storage). Sections were stained for TH+ cells in SNc; Brain punches collected from the animals

sacrificed by rapid decapitation were extracted for western blotting to examine levels of CX3CR1, Wave2, SiRT3 and DAT.

### 3.5.1 Immunohistochemistry

To assess the dopamine cells, we will be using Tyrosine hydroxylase (TH). Brains were sliced into 40um thick sections on Shandon AS620 cryostat (Fisher Scientific), and sections were immediately placed in a 0.1M PB solution containing 0.1% sodium azide (pH 7.4). Every third section was selected for each stain (i.e. SNc TH). For TH staining, slices were washed in phosphate buffer saline (PBS) (pH 7.4) three times for 5 minutes each, followed by a 30-minute incubation in 0.3% hydrogen peroxide in PBS. Slices were then washed in PBS three times five minutes each and a 1-hour incubation in blocking solution containing 5% normal goat serum (NGS), 0.3% triton-X, with 0.1 M PBS (pH 7.2). Blocker was removed, and the slices were then incubated overnight in primary antibody solution (5% NGS, 0.3% Triton X, 0.3% bovine serum albumin (BSA) in 0.1 M PBS) with 1:2000 anti-mouse TH (Immunostar, Hudson, WI). The following day the primary antibody solution was removed, and sections were washed in PBS three times for 5 minutes each. Following the washes, antibodies in secondary solution (1.6% NGS, 0.16% Triton X, 0.3% BSA, in 0.1M PBS) were applied to the striatum (anti-mouse IgG; 1:500) for 2 hours, and to SNc (anti-mouse HRP; 1:200) for 4 hours. Following this, three times five- minute washes were applied to the striatum, which was then incubated again in secondary solution with HRP (1:1000) for an additional 2 hours. All sections were given three times five minutes PBS washes and sequentially exposed to a DAB reaction containing Tris-HCl, DAB and 0.6% hydrogen peroxide in dH20 for visualization. Sections were washed in PBS three times 5 minutes each after DAB exposure, and all sections were then slide mounted and set to dry overnight. Sections were dehydrated using a series of alcohol and clearene washes and subsequently cover-slipped using DPX. All incubations occurred at room

temperature. To determine the DA degeneration in the substantia nigra, MBF Bioscience Stereo Investigator was used for unbiased stereological count using the optical fractionator probe. The SNc was outlined under a 2.5x magnification, and TH-positive neurons were counted using a 60x oil immersion objective lens. Cells were quantified in 3-dimensional counting frames using a counting grid size of 90x90 um and a counting frame size of 60x60 um with a 15-um dissector height. Total SNc TH-positive cells were quantified. All analyses were conducted by the same individual blind to the experimental conditions.

### 3.5.2 Western Blotting

During sacrifice of non-perfused animals, tissue was collected either via manual dissection or punch-out from the ST, HIP, and SNc and flash-frozen at -80℃. These regions were removed from the freezer and placed on wet ice. Tissue was suspended in Radio Immuno Precipitation Assay (RIPA) buffer [50 mM Tris (pH 8.0), 150 mM sodium chloride, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate and 1% Triton X-100] mixed with 1 tablet of Complete Mini EDTA-free protease inhibitor (Roche Diagnostics, Laval, QC) per 10 mL of buffer and then sonicated for 10 seconds on ice to homogenize the tissue. The lysed cells were then centrifuged at 5000 RPM in a microcentrifuge (Montreal Biotech, Montreal, QC) for 10 minutes at 4˚C. The supernatant was collected, and the protein concentration determined using a bicinchoninic acid (BCA) kit (Thermo Scientific, Waltham, MA). Following protein concentration determination 5X loading buffer (containing (5% glycerol, 5% β-mercaptoethanol, 3% SDS and 0.05% bromophenol blue) was added to the supernatant in a 1 to 5 ratio. Samples were then placed into a heat bath at 105˚C for 5 minutes to denature the protein and cooled briefly on ice. Immediately afterwards, samples were placed into a -20˚C freezer until needed for Western blotting.

The following is the generalized procedure used to conduct all Western Blots, for a complete

list of regions, antibodies and deviations from this protocol please refer to Table 3.5.2. Proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). 10% SDS-PAGE gels containing separating buffer (370 mM Tris- base (pH 8.8), 3.5 mM SDS), and the stacking buffer (124 mM Tris-base (pH 6.8), 3.5 mM SDS), were placed in running buffer (25 mM Tris-base, 190 mM glycine, 3.5 mM SDS) in a mini-25 PROTEAN electrophoresis chamber (Bio-Rad, Hercules, CA). Samples at protein loads listed in Table 2 were loaded into the stacking gel along with the BLUeye Prestained Protein ladder (Froggabio, Toronto, ON) and run at 140 volts for 1 hour to separate the proteins based on weight. After electrophoresis, the gels were loaded into transfer cassettes and run at 100V for one hour at 4˚C in transfer buffer solution (25 mM Tris-base, 192 mM Glycine, 20% methanol). Proteins horizontally transferred onto Immobilon-FL PVDF membranes (Millipore-Sigma, Burlington, MA). The membranes were allowed to dry overnight and then reactivated with methanol, and total protein load concentration was determined.

To determine total protein load in each lane were incubated in a 0.001% Fast Green solution (0.001% fast green, 6.7% glacial acetic acid, 30% methanol, in water) for 5 minutes placed into wash solution (6.7% glacial acetic acid, 30% methanol, in water) twice for 2 minutes each. Membranes were imaged on a LI-COR Odyssey Fc imaging system on the 700 channels for 2 minutes. Membranes were then rinsed twice in Tris-buffered saline (TBS (pH 7.5)) blocked with 0.5% fish gelatin (Sigma) in TBS for 60 minutes. Membranes were incubated overnight on a rocker at room temperature in 0.05% fish gelatin in TBS with 0.1% Tween (TBS-T) at the concentrations listed in Table 2. The next day, unbound antibodies were removed using four five-minute washes with TBS-T at room temperature. Membranes were then incubated for one-hour in infrared conjugates (680 and 800, LI-COR) directed against the species the primary antibody was raised in at a concentration of 1:20 000 in 0.5% fish gelatin solution containing 0.2% Tween and 0.01%SDS.

Membranes were then washed in TBS-T (4 X 5 minutes) followed by 2 X 5 minutes washes in TBS and read on our LiCOR Odyssey Fc system at the appropriate wavelength for 6 minutes.

Western results were quantified using ImageStudio software (v5.0, LiCOR, Lincoln, NE). Briefly, total protein bands were outlined, and background subtracted from the left and right of each band. Each gel was then normalized to the strongest lane on the gel. Bands of interest for each antibody were outlined and background-subtracted based on the median values surrounding the band. Band values were divided with the normalized ratio to produce the raw numbers used in statistical analyses.

| Antibody | Region | Species | Block | Primary | Secondary | Gel | Protein |
|---|---|---|---|---|---|---|---|
| SiRT3 | SNc, ST | Rab | 1hour | 1:1000 | 1:20000 | 10% | 10ug |
| CX3CR1 | SNc, ST | Rab | 1hour | 1:2000 | 1:10000 | 10% | 2.5ug |
| Wave2 | SNc, ST | Rab | 1hour | 1:4000 | 1:10000 | 10% | 5ug |
| DAT | SNc | Rat | 1hour | 1:500 | 1:15000 | 7.5% | 10ug |

**Table 3.5.2**: Regions and Antibody for western blots.

## 3.6 Statistical Analysis

All the data were analyzed using a 2 (age; old vs young) X 2 (genotype; WT vs G2019S Knock-in) X 2(treatment; saline vs LPS) between-subjects analysis of variance (ANOVA) design. When there was an interaction, three-way ANOVA was followed by Fisher's post-hoc simple comparison. Data are presented in the form of mean +/- standard error mean (m+/-SEM). All data were evaluated using IBM SPSS Statistics Version 25 and differences were considered statistically significant when $p < 0.05$. All the graphs were generated using GraphPad Prism version 8.2.1.

# 4. Results

## 4.1 Behavior

### 4.1.1 Weight Change

A three-way ANOVA was run to access the effect of age, genotype and treatment on weight change. All the animals were weighed in the mornings of the injection days. Post 48 hours of the first injection, a significant ($F_{1,77}$ = 13.01, $p$=0.001) three-way interaction of age, genotype and treatment were found (Fig. 4.1.1). Indeed, older G2019S animals treated with LPS had significant weight loss compared to all the other groups ($p < 0.05$). Weight change post the 3rd injection also revealed a significant main effect of age ($F_{1,77}$=65.5, $p$<0.001) and treatment ($F_{1,77}$=7.827, $p$=0.007) but not genotype in the weight change (Fig. 4.1.1b). A similar effect was found on weight change post the 5th injection, the significant main effect of age ($F_{1,77}$=58.61, $p$<0.001) and treatment ($F_{1,77}$=6.39, $p$=0.014) were seen but not genotype (Fig. 4.1.1c).

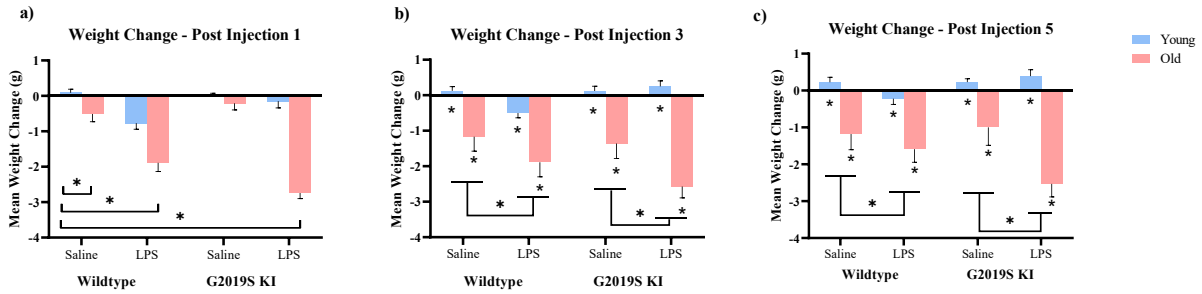


**Fig: 4.1.1**: Weight changes post 1st injection, 3rd injection and final injection. (a) there was significant three-way interaction between age, genotype and treatment on weight loss post 1st injection ($F_{1,77}$ = 13.01, $p$=0.001). (b). Age ($F_{1,77}$=65.5, p<0.001) and treatment ($F_{1,77}$=7.827, $p$=0.007) affected weight loss, while there was no significant effect of genotype post 3rd injection. (c) Age ($F_{1,77}$=58.61, $p$<0.001) and treatment ($F_{1,77}$=6.39, $p$=0.014) continued to affect weight lost post final injection.

### 4.1.2 Micromax

A three-way ANOVA was run to access the effect of age, genotype and treatment on mean beam break. There was a significant effect of age on beam break per animal over one 12-hour dark ($F_{1,77}$=6.97, $p$=0.01) on the baseline (prior to and irrespective of LPS treatment, i.e. Day 0) with 15.8% reduction in older animal compared to younger ones. Additionally, there was a modest effect of genotype ($F_{1,77}$=3.77, $p$=0.05) on mean beam break with G2019S animals showing 11.9% lesser beam break compared of their WT littermates. While the main effect of age was consistent at Day 4 ($F_{1,77}$=29.49, $p$<0.001) and at Day 8 ($F_{1,77}$=12.59, $p$=0.001), the main effect of genotype ($F_{1,77}$=6.68, $p$=0.012) was evident by Day 4. Indeed, a 13.6% reduction was evident in G2019S animals ($p < 0.05$). Further, an interaction between age and genotype ($F_{1,77}$=4.03, $p$=0.049) was significant on Day 4. The main effect of treatment was not significant until Day 8 ($F_{1,77}$=5.68, $p$=0.02), a 12.6% decrease in mean beam break in LPS animals compared to saline.

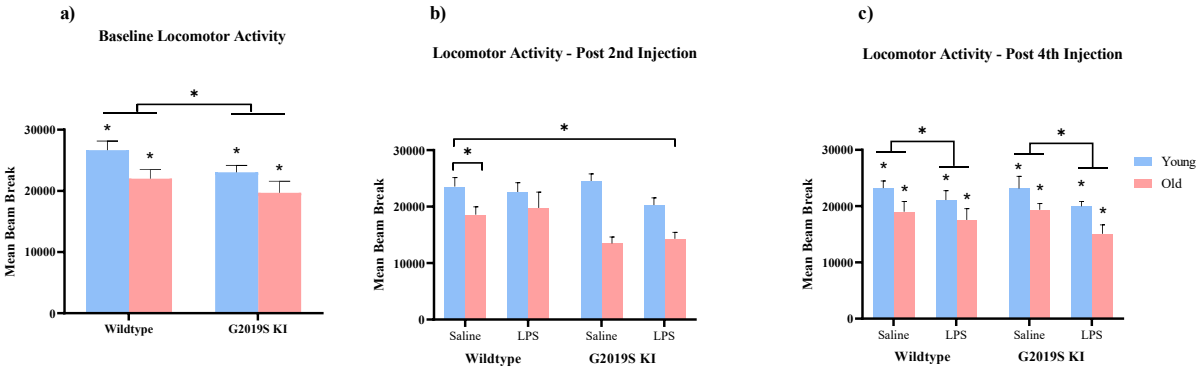


**Fig 4.1.2:** Mean beam breaks per animal over one 12-hour dark cycle at Day 0, Day 4 (post 2nd injection) and Day 8 (post 4th injection). (a) significant effect of age ($F_{1,77}$=6.97, $p$=0.01) and genotype ($F_{1,77}$=3.77, $p$=0.05) on locomotor activity at baseline. (b) interaction between age and genotype ($F$1,77=4.03, $p$=0.049) post 2nd injection, (c) effect of treatment ($F_{1,77}$=5.68, $p$=0.02) (LPS; Saline) was not significant until Day 8.

### 4.1.3 Sickness Scoring

A three-way ANOVA was run to access the effect of age, genotype and treatment on sickness behaviour. Sickness behaviour after 1-hour and 24-hours of 3rd injection was considered for assessment. One hour post the 3rd injection, in the absence of three-way interaction, a statistically significant age by treatment ($F_{1,77}$=20.13, $p$<0.001) interaction was found, although there was no genotype by treatment or age by genotype effect, on the sickness behaviour (Fig. 4.1.3a).

In particular, LPS induced robust sickness symptoms and the older mice displayed greater sickness scores in response to the LPS injection, compared to the younger animals ($p$< 0.05). 24-hours post the 3rd injection there was no significant three-way, the two-way or main effect of age, genotype and treatment on the sickness behaviour (Fig. 4.1.3b).

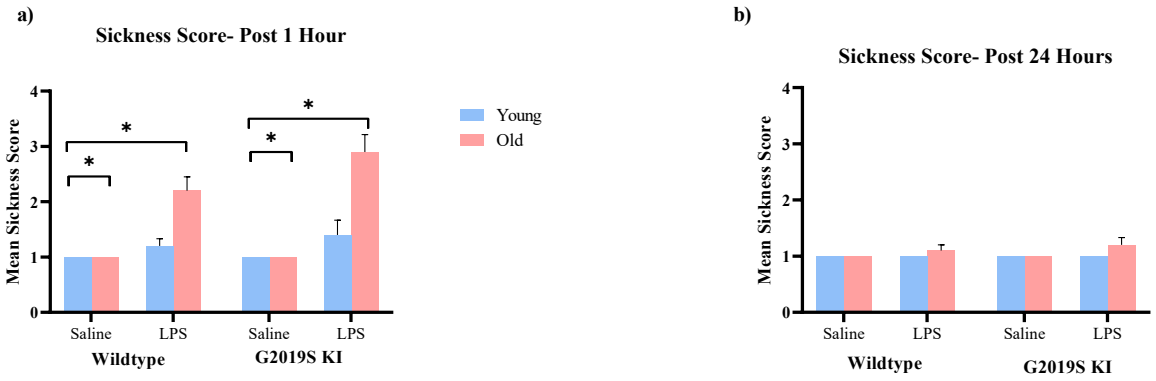


**Fig. 4.1.3:** Scores were taken 1-hour and 24-hours post 3rd injection, control animals, exhibited no sickness signs but (a) 1 hour post-injection, older animals had significantly higher sickness rating compare to younger ($F_{1,77}$=20.13, $p$<0.001); (b) 24 hours post the injection none of the animals exhibited a significant sickness, regardless of age, treatment or genotype.

### 4.1.4 Nestlet Scoring

A three-way ANOVA was run to access the effect of age, genotype and treatment on nesting behaviour. Nesting behaviour after 1 hour, 3-, 5- hours and 24 hours of the 3rd injection was considered for assessment.

1-hour post-injection, there was a significant two-way interaction between genotype by treatment ($F_{1,77}$=7.515, $p$=0.008) and a main effect of age ($F_{1,77}$=9.624, $p$<0.003) on nesting behaviour. Older mice displayed deficits in nesting, as did the LPS treated animals in which the marked effects were evident in the G2019S mutants ($p < 0.05$). Similarly, three hours post-injection, again, there was a significant two-way interaction between genotype by treatment ($F_{1,77}$=5.11, $p$=0.027) and main effects of age ($F_{1,77}$=8.73, $p$=0.004) and treatment ($F_{1,77}$=6.23, $p$=0.015) on nesting behaviour (Fig. 4.1.4a).

At five hours post-injection, there was no significant 3-way or 2-way interactions, but there was a main effect of age ($F_{1,77}$=17.68, $p$<0.001), treatment ($F_{1,77}$=15.79, $p$<0.001) and borderline effect of genotype ($F_{1,77}$=3.954, $p$=0.05) on the nesting behaviour. These same effects were still evident at 24 hours post-injection, with the main effect of age ($F_{1,77}$=7.27, $p$=0.009), genotype ($F_{1,77}$=5.24, $p$=0.025) and treatment ($F_{1,77}$=8.14, $p$=0.006) were found but no interactions (Fig. 4.1.4b).

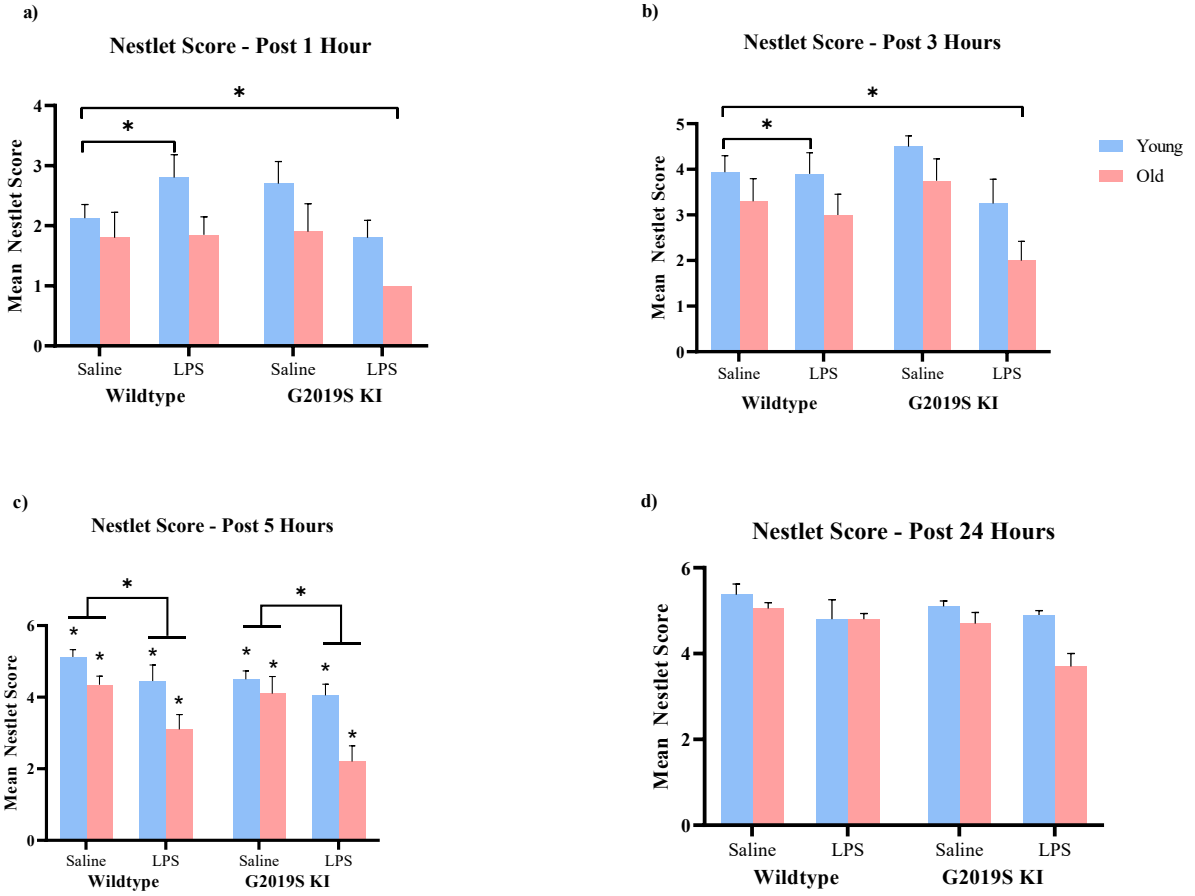


**Fig. 4.1.4:** Nesting behaviour after 1 hour, 3-, 5- hours and 24 hours of the 3rd injection was considered for assessment. G2019S-LPS showed significant effect of nesting post (a) 1 hour ($F_{1,77}$=5.11, $p$=0.027) and (b) 3 hours of injection ($F_{1,77}$=5.11, $p$=0.027). (c) a main effect of age ($F_{1,77}$=17.68, $p$<0.001), treatment ($F_{1,77}$=15.79, $p$<0.001) was consistent after 5 hours of injection. (d) all the three factors showed significant main effect post 24 hours of the injection.

## 4.2 Immunohistochemical Analysis

### 4.2.1 TH count

A three-way ANOVA was run on access the effect of age, genotype and treatment on the amount of TH positive cells within the SNc. Stereological quantification revealed a significant three-way interaction between age, genotype and treatment ($F_{1,39}$=11.75, $p$=0.002). In particular, the old-G2019-LPS mice had a 43.1% decrease in TH+ cells compared to young-WT-Saline and were significantly

reduced compared to all other groups ($p < 0.05$).

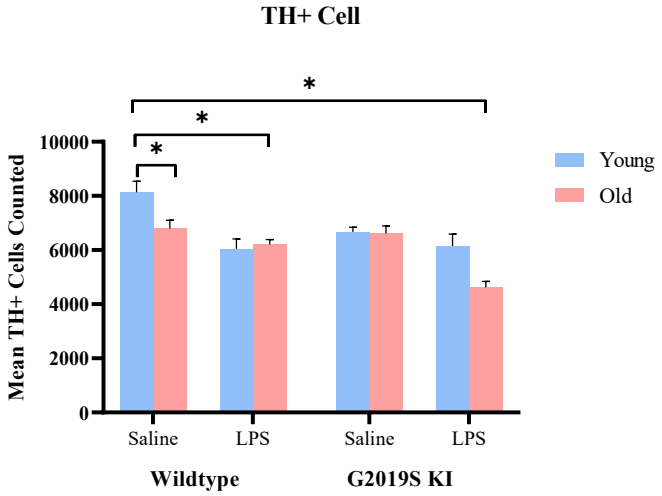


**Fig. 4.2.1:** Graph representing the average number of TH+ cells found in the SNc of saline and LPS injected animals in both genotypes. There was a significant three-way interaction in mean TH+ cell loss ($F_{1,39}$=11.75, $p$=0.002) with older G2019S animals injected with LPS showing 43% cell loss compared to younger WT saline.

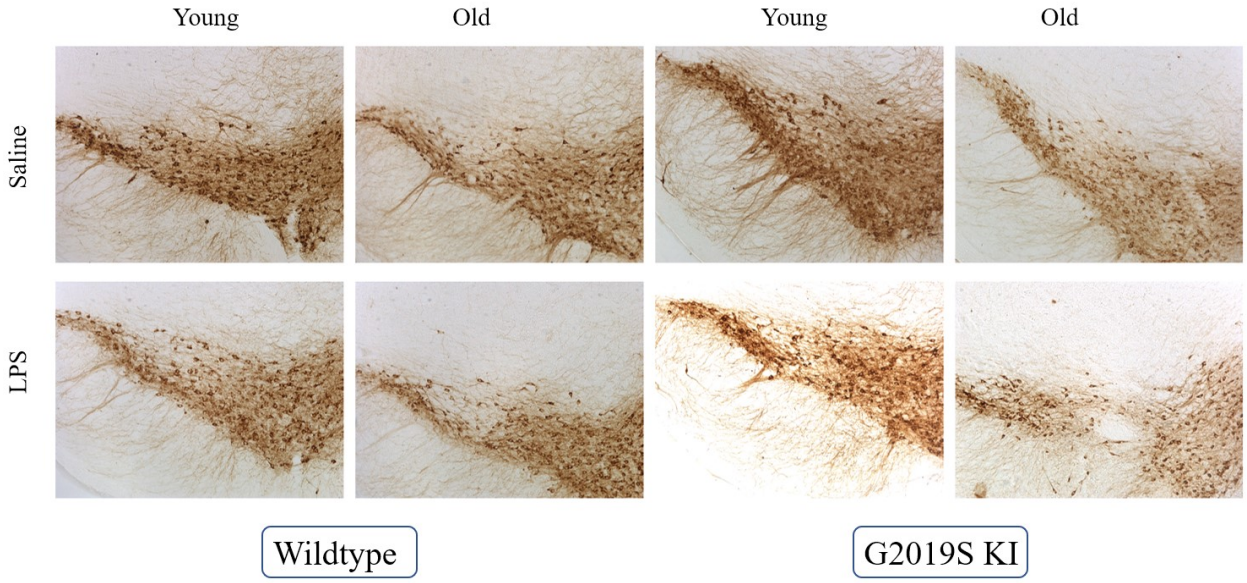


**Fig. 4.2.2:** Photomicrographs at 10x magnification on a light microscope showing tyrosine hydroxylase positive neuronal projections in the SNc of all the treatment groups. There was a significant interaction between the three factors on the TH+ cell loss in SNc ($F_{1,39}$=11.75, $p$=0.002).

# 4.3 Western Blotting

## 4.3.1 Striatum

A three-way ANOVA was run to access the effect of age, genotype and treatment on SiRT3, Wave2 and CX3CR1 protein levels. Western blot analyses of SiRT3, Wave2 and CX3CR1 in AST did not show a significant three-way or two-way interaction. A main effect of age ($F_{1,38}$=7.40, $p$=0.01) on SiRT3 protein levels was found, increasing 21.6% in older animals compared to younger but there was no significant genotype or treatment effect (Fig 4.3.1a). There was a modest but significant main effect of treatment ($F_{1,38}$=4.20, $p$=0.05) on Wave2 protein levels, but not age or genotype affected (Fig 4.3.1b). There was no significant interaction or main effect of age, genotype or treatment on the CX3CR1 protein levels (Fig 4.3.1c).

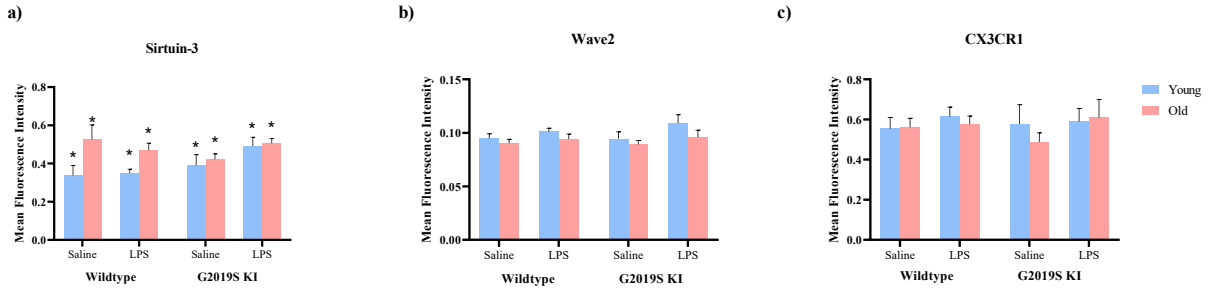


**Fig. 4.3.1:** There was no significant interaction between age, genotype and treatment on SiRT3, Wave2 and CX3CR1 protein expression in anterior striatum, however, (a) older animals showed a significant increase in SiRT3 and (b) Wave2 expression was modestly affected by treatment, LPS animals showing an increase in Wave2 compared to saline animals. (c) CX3CR1 did not change regardless of age, genotype or treatment.

## 4.3.2 Substantia nigra pars compacta

A three-way ANOVA was run on to access the effect of age, genotype and treatment on DAT, SiRT3, Wave2 and CX3CR1 protein levels. There was a significant three-way interaction ($F_{1,38}$=4.421, $p$=0.044) on DAT protein levels (Fig. 4.3.2d). In particular, Old-G2019-LPS had, and tremendous 218% increased expression of DAT compared to young-WT-saline animals ($p < 0.05$).

There was a main effect of age ($F_{1,38}$=5.97, $p$=0.02) and genotype ($F_{1,38}$=4.37, $p$=0.045) on SiRT3. Again, age ($F_{1,38}$=7.75, $p$=0.01) and genotype ($F_{1,38}$=10.8, $p$=0.003) had a significant contribution to mean CX3CR1 expression in SNc. Only age ($F_{1,38}$=10.88, $p$=0.003) had a significant effect on mean Wave2 expression in SNc.

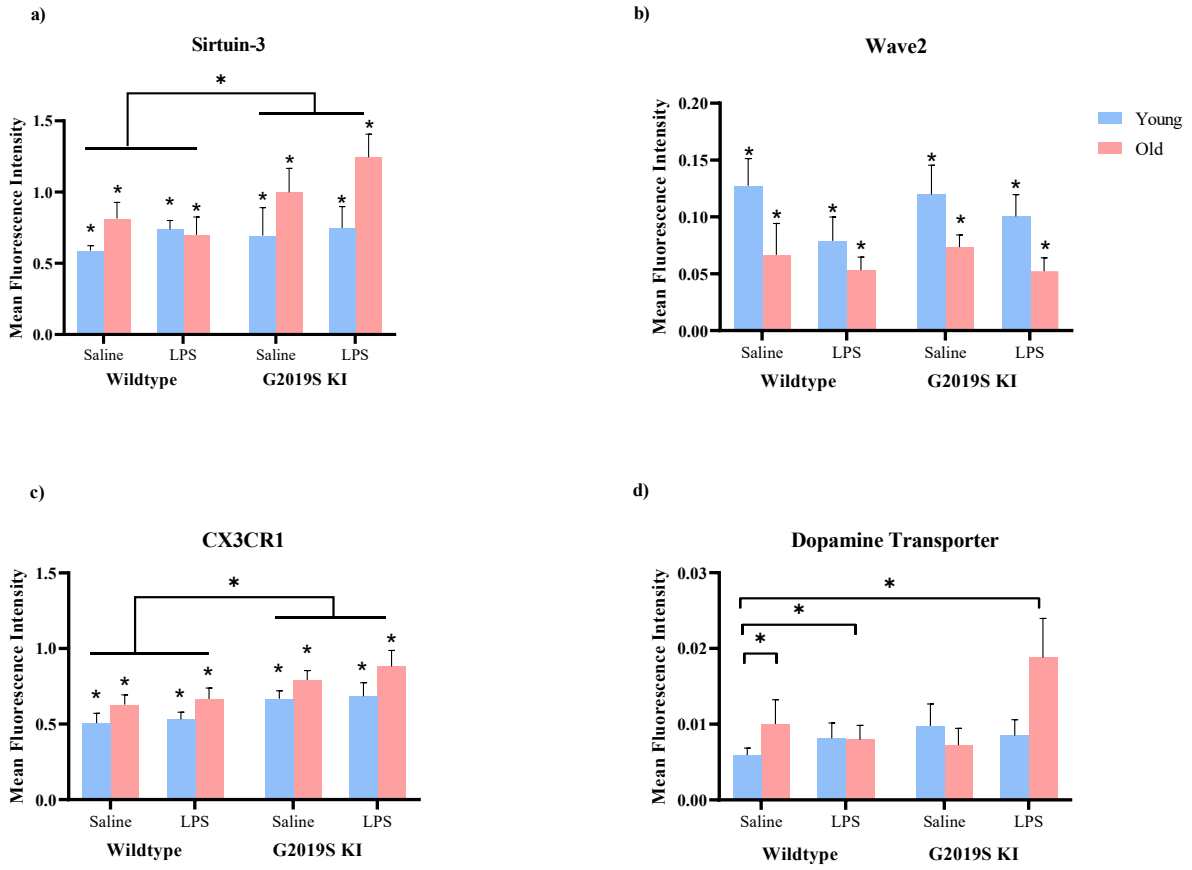


**Fig. 4.3.2:** No significant three-way interaction on SiRT3, Wave2 and CX3CR1. (a) Age ($F_{1,38}$=5.97, $p$=0.02) and genotype ($F_{1,38}$=4.37, $p$=0.045) had significant effect on SiRT3 protein expression in SN; (b) similarly, age ($F_{1,38}$=7.75, $p$=0.01) and genotype ($F_{1,38}$=10.8, $p$=0.003) had effect on CX3CR1 expression; (c) only, age effected Wave2 expression ($F_{1,38}$=10.88, $p$=0.003) and (d) there was a three-way interaction on the expression on DAT ($F_{1,38}$=4.421, $p$=0.044) in SN with 218% increase in older-G2019S animals treated with LPS compared to young-WT-saline animals.

# 5. Discussions and Conclusions

## 5.1 Ageing and Oxidative stress in PD

Overall "clinical" state of the animal was accessed using sickness scores and weight change. Although general sickness ratings increased in animals that were old and received LPS, the mutation did not elicit an effect on these animals (Fig.4.1.3). However, G2019S mutation did have a significant effect on weight loss when combined with the age of these animals post the 1st injection of LPS (Fig.4.1.1). The fact that vehicle-treated older animals also showed a significant weight loss suggests how age might affect basal stress-levels (induced by handling and injections) in these animals (Fig.4.1.1).

Age is the most significant risk factor for the development of PD, and some of these risks stem from the increase in inflammatory and oxidative stress that occur in the brain with advanced age [68]. Indeed, age-related increases in oxidative stress stem from the disrupted mitochondrial functioning along with the accumulation of pathological protein aggregates, such as the Lewy bodies that occur in PD [78]. Environmental factors like head trauma or toxins can add to the cycle [87]-[90], so can immunological stressors, which can lead to accumulation of misfolded proteins and cause defects in mitochondrial respiratory chain inducing increased ROS production [108].

Several studies have demonstrated the role of reactive oxygen species (ROS) in neurodegeneration and cognitive decline in both human patients and animal models of diseases [267] - [271]. Reactive oxygen species (ROS) are byproducts of cellular respiration and cellular redox reactions such as the production of growth factors and inflammatory cytokines, and at optimal levels, ROS are thought to be a critical component of cellular homeostasis [272]. For examples, ROS can directly interact with p66SHC, a redox enzyme essential for stress-induced apoptosis

[273]. Failure in the regulation of these reactive oxygen species leads to oxidative stress in the cell. Oxidative stress can increase the chances of spontaneous mutations in cellular DNA. Previous studies have reported the presence of oxidized DNA, lipids and proteins in the brain tissues of both sporadic and familial PD patients, caused by increased oxidative stress [274], [275]. Interestingly, the concentration of oxidized protein, in the SN of healthy individuals, was found to be twice that of caudate, putamen, and the frontal cortex [276].

Normal somatic cells entering an irreversible growth arrest after a finite number of cell divisions, called senescence, contributes to the process of ageing. These cells are associated with high intracellular ROS and accumulated oxidative damage to DNA and proteins [277], [278]. Also, p53, a critical regulator of senescence, is activated in response to DNA damage elicited by ROS [279]. It is interesting to speculate whether increased ROS production regulates senescence pathway resulting in increased ROS generation that would maintain a steady progression of ageing.

Although the definitive mechanism leading to selective degeneration of SNc neurons are unknown, the ageing brain is often considered to be located on the preclinical continuum of Parkinson's disease. Precisely, multiple components of the dopamine system, like various dopamine receptors [280] and dopamine transporters [280], [281], [282] were diminished in older adults when compared to young adults. Additionally, studies have shown that dopamine neurons exhibited a progressive loss of autophagic function with ageing [283], [284]. Indeed, LRRK2 G2019S mutation accelerated this age-related loss of function [284]. These findings suggest that ageing and accumulating oxidative stress may actually "prime" the pathway for the neurodegeneration.

## 5.2 LRRK2 G2019S Mutation and PD

Increasing evidence suggests a role for LRRK2 in modulating inflammation; several studies have implicated LRRK2 in infection, particularly of bacterial. Besides PD, polymorphism in LRRK2 is linked to increased risk of developing Crohn's inflammatory bowel disease and increased susceptibility to leprosy [286]. While mechanisms through which LRRK2 may affect its substrates are still enigmatic, dysregulation of inflammatory processes is one of the major alterations in the LRRK2 mutation carriers. One such study demonstrated that mice lacking LRRK2 were more susceptible to peritoneal inflammation caused by salmonella and had increased mortality to infection [287]. Also, considering the association between gut microbiota and inflammation, reports of disparities in gut microbiome in PD patients and mouse models of PD provides an additional link between LRRK2 and inflammation [288], [289]. Besides immune cells that express LRRK2 at variable and relatively high levels, it is also expressed in dendritic cells and neutrophils [290]. Studies in both, cellular and animal models of LRRK2, support its role in regulation and control of autophagic pathways in the cell; for instance, G2019S KI mice were shown to facilitate a-syn neuropathy in older mice when compared to the younger [291]. Similarly, pharmacological inhibition of increased kinase activity, altered (normalized) the mitophagy rates in G2019S cells [292].

The effect of the G2019S mutation appears to be strongly correlated with age. Indeed, phenotypic differences in these mice are not prominent before six months of age and differences in motor behaviour, and oxidative stress continues to accumulate [284], [285]. We found that our G2019S animals exhibited decreased locomotor activity at baseline, i.e. prior to and irrespective of LPS treatment (Fig 4.1.2). Similarly, aged animals exhibited decreased locomotor activity at baseline (Fig. 4.1.2).

## 5.3 Neuroinflammation and Neurodegeneration in PD

Activated microglia and infiltration of circulating factors and cells into the CNS underlies the most fundamental aspects of a neuroinflammatory response, as observed in PD [102]-[106]. Neuroinflammation can further provoke oxidative stress and other pro-death mechanisms [293]. Studies in various animal models have demonstrated prolonged neuroinflammation after exposure to the endotoxin, LPS [266], and in particular, Haneul Noh et al. showed the regional specific neuroinflammation induced by LPS in mouse brain [293]. Indeed, it was demonstrated that SNc was relatively (when compared with thalamus, cortex and hippocampus) more vulnerable to the neurodegenerative effect of LPS [258].

LPS is a molecule present in the outer membrane of Gram-negative bacteria. A single injection of LPS can induce several cellular and molecular alterations, like elevated brain TNF-alpha that can last up to 10 months [295]. Chronic peripheral injection of LPS is shown to elicit extensive neuronal loss, a decline in dopamine levels, deficits in locomotor behaviour, altered cytokine profile on SN, and glial activation [296].

Neuroinflammation and oxidative stress are key players of numerous brain diseases that induce tissue damage during disease and ageing [267], [297]. Microglia, the resident immune cells of the brain, are major regulators of the inflammatory signals in the central nervous system [298]. When activated, microglia can become phagocytotic and can secrete cytokines, chemokines, and oxygen radicals [293]. Interestingly, phagocytic microglia can physically engulf axon terminals after induced by LPS [299]. Apart from neurotropic functions, activated microglia can also perform neurotrophic functions by producing anti-inflammatory factors and can unsheathe neurons under damaging or regenerating conditions [300]. Dystrophic morphology, cytoplasmic fragmentation, and shortened cellular processes are some of the marked features of aged microglia

[301]. Additionally, aged microglia exhibit an elevated inflammatory profile which is thought to induce vulnerability to a secondary insult such as infection or psychological stress [300]-[304].

One crucial factor in regulating neuro-immune communication and hence, neuroinflammatory pathology is the chemokine, fractalkine [248], [252]. Fractalkine, also known as CX3CL1, normally has chemoattractant immune properties, which means that it directs the migration and action of immune cells throughout the body [249]. CX3CL1 also is known to be produced by neurons and its receptor, CX3CR1, is expressed by microglia. Hence, microglia-neuron interactions are at least in part, mediated by CX3CR1/CX3CL1 signalling. Increase in CX3CR1 can be induced by inflammatory stimulations [260], it has also been shown to increase in Alzheimer model when stimulated with Aß [305]. In essence, it is thought that neurons in distress release CX3CL1, which then activates and recruits neighbouring microglia [306]. The microglia can either assist the neurons by engaging pro-survival signals or kill neurons through either phagocytosis or the engagement of pro-apoptotic signalling [300], [306].

Several studies are implicating the role of the CX3CL1-CX3CR1 axis in the aged brain. With reduced CX3CL1 expression, aged rodents showed a reduction of ramified microglia and an increased level of neuroinflammatory markers [308]. Moreover, as discussed above, an introduction of a toxin like LPS can amplify the microglial response in aged mice [299], [307]. The present study used a multi-hit model of PD to elucidate the mechanism through which age, the mutation in the LRRK2 gene and immunological stress can contribute to PD pathology. We hypothesized that in response to peripheral LPS, old G2019S animals would show an exacerbated activation of resident immune cells in the brain compared to their young and wildtype littermates. Our findings did indeed support a significant role of age and genotype in increasing neuroinflammation and oxidative stress leading to neurodegeneration in SNc. We presently

observed an increased TH+ cell loss in G2019S old animals treated with peripheral LPS compared to its counterpart. This result was significant with increased DAT, suggesting a compensatory mechanism following neurodegeneration, rather than restorative. We observed an elevated expression of SiRT3, which is thought to proportionately increase with ROS level, in SNc of aged and G2019S mice which did not alter with LPS injection, suggesting of a pre-existing oxidative stress in these animals; we also observed increased level of CX3CR1 in the SNc of aged and G2019S mice, but the LPS treatment did not further modify this and hence, did not appear to be involved in the neurogenerative effects.

## 5.4 Conclusions

Within this study, we examined the effect of immune-toxin, LPS on age and genotype. Briefly, we found that old and G2019S knock-in animals exhibited differential locomotor activity at the baseline compared to their littermates. The loss of TH+ neurons in SNc and elevated level of DAT suggested that increasing age and G2019S mutation did modify the neurodegenerative impact of peripheral LPS in these animals. In addition to this, an increase in SiRT3 and CX3CR1 expression in aged and mutant animals, support our theory that age, and genotype might prime the effect of immunological insult to induce a more significant impact. Our limited evidence of treatment having an added effect to this might arise from the fact that the animals were sacrificed post five injections and probing through their sickness behaviour and weight loss data post 3rd injection, it seems like that there might have been a compensatory mechanism that interfered with treatment effect.

Future studies should focus on following areas related to the involvement of age, sex and genotype effect on neuroinflammatory processes. Firstly, (a) sex differences are often driven by gonadal steroid hormones, and with regards to PD, estrogen is thought to have a neuroprotective effect. However, the efficacy of external administration of estrogen in PD is highly debated. In this

context, a better understanding of the role of estrogen and age in PD is much required. Secondly, given that there is increasing evidence suggesting early life infection affecting the disease manifestation in older age, a similar paradigm with G2019S mutation might help us understand the process of ageing in these mutant mice.

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