THE PERSISTENT AND MULTIDIMENSIONAL MICROGLIAL RESPONSE TO PATHOLOGICAL ALPHA-SYNUCLEIN AGGREGATION

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ABSTRACT

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Parkinson's Disease, the second most common neurodegenerative disease, affects approximately 1 million people in the USA with 60,000 newly diagnosed people each year. Pathologically, PD is characterized by the presence of proteinaceous alpha-synuclein (α-syn) inclusions (Lewy bodies) and the progressive loss of the nigrostriatal dopamine (DA) neurons. While the exact cause of PD remains unknown, mounting evidence has suggested that neuroinflammation may play a significant role in PD progression. The pathological features of PD can be recapitulated *in vivo* using the α-syn preformed fibril (PFF) model of synucleinopathy in rats. Specifically, in association with accumulation of phosphorylated α-syn (pSyn) inclusions in the SNpc, microglia increase soma size and MHC-II expression. This microglial response parallels pSyn inclusion formation, peaking at 2 months following intrastriatal PFF injection, months prior to the SNpc degeneration observed in the model. The overarching question of this dissertation is: does the microglial response to pathological α-syn accumulation contribute to degeneration? In Aim 1 of this dissertation an inhibitor of colony stimulating factor 1 receptor (CSF1R) was used to partially deplete microglia within the context of the α-syn PFF rat model in order to determine whether degeneration of the nigrostriatal system can be attenuated. Despite significant microglial depletion, increased soma size and expression of major-histocompatibility complex-II (MHC-II) on microglia within the α-syn inclusion bearing substantia nigra pars compacta (SNpc) was maintained. Further, partial microglia depletion did

not impact degeneration of dopaminergic neurons in the SNpc. Paradoxically, long term partial microglial depletion increased the soma size of remaining microglia in both control and PFF rats was associated with widespread MHC-IIir expression in extranigral regions. These results suggest that partial microglial depletion is not a promising anti-inflammatory therapeutic strategy for PD and that this approach may induce a heightened proinflammatory state in remaining microglia. Aim 2 of this dissertation built on a previous study RNA-Seq dataset that identified multiple upregulated innate and adaptive immune transcripts in the inclusion bearing SNpc in the PFF model. Complementary approaches of fluorescent in situ hybridization (FISH) and droplet digital PCR (ddPCR) were used. FISH results identified an **a**-syn **a**ggregate associated **m**icroglial (a-SAM) phenotype that is characterized by upregulation of *CD74, CXCl10, RT1-A2, GRN, CSF1R, Tyrobp, C3, C1qa* and *Fcer1g*. ddPCR results identified additional neuroinflammatory genes, *Cd4, Stat1, Casp 1, Axl* and *IL18*, that are significantly upregulated in inclusion bearing nigral tissue. Collectively these findings implicate that the deposition of pathological α-syn inclusions in the SNpc is associated with perturbations in immune functions related to complement, inflammasome and T cell activation, phagocytosis, and interferon gamma signaling. Collectively, the findings of these dissertation experiments demonstrate that the microglial response to pathological α-syn aggregation is persistent and multifaceted. This comprehensive understanding of the multidimensional response of microglia to pathological α-syn aggregates may help to uncover novel therapeutic targets that could facilitate future anti-inflammatory, disease-modifying strategies for PD.

For those
who motivated me by saying I couldn’t
and for those
who supported me by saying I could.

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PREFACE

Upon completion of this dissertation, one chapter has been published and two chapters are currently being prepared for submission. Specifically, Chapter 2 was published as an open access article in Neurobiology of Disease in September 2022 (PMID: 35764290). Chapters 3 and 4 are in preparation for submission.

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KEY TO ABBREVIATIONS

6-OHDA	6-hydroxydopamine
a-SAM	Alpha-Synuclein aggregate Associated Microglia
α-syn	Alpha-synuclein
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AD	Alzheimer's disease
ANOVA	Analysis of variance
Axl	Axl receptor tyrosine kinase
BBB	Blood brain barrier
Bst1	Bone marrow stromal cell antigen 1
C1qa	Complement component 1q subcomponent alpha
C3	Complement component 3
C3d	Complement component 3d
Casp1	Caspase 1
Ccr3	G protein-coupled c-c chemokine receptor type 3
Cd4	T-cell surface glycoprotein cd4
Cd74	Cd74 antigen (invariant polypeptide of major histocompatibility complex, class ii antigen-associated)
CNS	Central nervous system
COMT	Catechol-o-methyltransferase
CSF	Cerebral spinal fluid

Csf1r	Colony stimulating factor 1 receptor
Cxcl10	C-X-C motif chemokine ligand 10
D1	Dopamine receptor 1
D2	Dopamine receptor 2
DA	Dopamine
DAB	3,3' diaminobenzidine
DAMPs	Disease associated molecular patterns
DAT	Dopamine transporter
DBS	Deep brain stimulation
ddPCR	Droplet digital polymerase chain reaction
DM	Diabetes mellitus
Fcer1g	Fc fragment of IgE receptor Ig
FISH	Fluorescence in situ hybridization
GBA	Glucocerebrosidase
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GPe	Globus Pallidus externa
GPi	Globus Pallidus interna
GPNMB	Glycoprotein NMB
Grn	Progranulin
GWAS	Genome wide association study
HLA-DR	Human leukocyte antigen-antigen D related

huPFFs	Human preformed fibrils
IACUC	Institutional Animal Care and Use Committee
Iba1	Ionized calcium binding adaptor 1
IF	Immunofluorescence
IHC	Immunohistochemistry
Il-18	Interleukin- 18
Il-1a	Interleukin-1-α
Il-1b	Interleukin-1-beta
Il-2	Interleukin-2
Il-4	Interleukin-4
Il-6	Interleukin-6
Il23a	Interleukin 23 subunit alpha
iLBD	Incidental Lewy body disease
ir	Immunoreactive
Irf3	Interferon regulatory factor 3
Kg	Kilogram
L-DOPA	Levodopa
LCM	Laser capture microdissection
Lpl	Lipoprotein lipase
LRRK2	Leucine-rich repeat kinase 2
Lyz2	Lysozyme 2
M1	Pro-inflammatory microglia

M2	Anti-inflammatory microglia
MAO	Monoamine oxidase
Mg	Milligram
MHC-I	Major histocompatibility complex I
MHC-II	Major histocompatibility complex II
mPFFs	Mouse preformed fibrils
Mpp+	1-methyl-4-phenylpyridine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
NK	Natural killer
Nlrp3	Leucine-rich repeat-containing protein 3
NSAID	Non-steroidal anti-inflammatory drug
P2ry12	Purinergic receptor p2y, g-protein coupled 12
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PD	Parkinson's disease
PET	Positron emission tomography
PFA	Paraformaldehyde
PFFs	Preformed fibrils
Pink1	PTEN-induced kinase-1
PLX3397b	Pexidartinib
PRKN	Parkin

pSyn	Phosphorylated alpha-synuclein at ser 129
ROS	Reactive oxygen species
rPFFs	Rat preformed fibrils
Rt1-a2	Rt1 class 1a, locus a2
SN	Substantia nigra
SNCA	Alpha-synuclein gene
SNpc	Substantia nigra pars compacta
SNr	Substantia nigra reticulata
Stat1	Signal transducer and activator of transcription 1
STN	Subthalamic nucleus
STR	Striatum
Syt11	Synaptotagmin 11
TBS	Tris-buffered saline
TGF-b	Transforming growth factor- beta
TH	Tyrosine hydroxylase
TLR4	Toll like receptor 4
Tmem175	Transmembrane protein 175
TNF- α	Tumor necrosis factor-alpha
TPM	Transcripts per million
Tx-100	Triton x-100
Tyrobp	Transmembrane immune signaling adaptor TYROBP
Ug	Microgram

ul	Microliter
UPDRS	Unified Parkinson's disease rating scale
Vmat2	Vesicular monoamine transporter 2
Vps35	Vacuolar protein sorting associated protein 35
WT	Wild type
YOPD	Young Onset PD

Chapter 1: Introduction

History of Parkinson's Disease

When observing his own patients from a distance, as well as three strangers, the English surgeon Dr. James Parkinson took note of similar significant movement issues in all six individuals. In 1817 Dr. Parkinson published his work "An Essay on the Shaky Palsy" in which he described the neurodegenerative disease that he called *Paralysis Agitans*. He wrote:

> *"Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured".* (Parkinson, 2002)

About 50 years later, Jean-Martin Charcot in 1872 took the characterization further and described the fatigue and arthritic changes seen in patients, often, before the onset of the tremor. He coined the term Parkinson's disease to describe what he observed, discarding the name *Paralysis Agitans* or shaking palsy due to his observation that patients were not necessarily weak, nor always presenting with a tremor (Charcot, 1872).

Demographics of Parkinson's Disease

Parkinson's disease (PD) is currently the second most common neurodegenerative disease, second only to Alzheimer's Disease. The greatest risk factor is advanced age with the incidence of PD increasing with each decade of life. However, it is estimated that 4% of patients with Parkinson's are diagnosed before the age of 50, with men being diagnosed in a ratio of 2:1 as compared to women (Cerri et al., 2019). In the United States, nearly one million people are estimated to be living with PD, and 60,000 people are diagnosed each year. These numbers are

expected to reach 1.2 million by the year 2030 as the baby boomer generation ages (Marras et al., 2018). It is estimated that the yearly economic burden is 51.9 billion in the United States alone (Yang et al., 2020), including medical costs as well as non-medical costs related to missed work, early forced retirement, and family caregiver time (Boiles, 2019).

Clinical Presentation in Parkinson's Disease

Motor Symptoms

Whereas each patient with PD presents clinically with slightly different motor dysfunction, the clinical diagnosis of PD is based upon the identification of some combination of the cardinal motor signs of tremor, rigidity, akinesia/bradykinesia and postural instability (TRAP) (Frank et al., 2006). These cardinal motor symptoms have been associated with PD since Dr. James Parkinson described them in 1817 (Parkinson, 2002). These motor symptoms often emerge slowly and often an individual is not diagnosed with PD until the disease has progressed. The diagnostic criteria for PD require both akinesia/bradykinesia with at least one other of the three cardinal motor symptoms (Fahn, 2006; Jankovic, 2008). However, as these motor symptoms can be associated with other movement disorders, additional clinical evaluation may be required to confirm PD. This can include responsiveness to dopaminergic pharmacotherapy or single photon emission computed tomography (SPECT) to image dopamine transporters in the striatum (DATScan) (Seifert & Wiener, 2013). Symptom progression in PD can be monitored by the Unified Parkinson's Disease Rating Scale (UPDRS) (Postuma et al., 2015). To date there are currently no clinical biomarkers for PD, confirmation of PD diagnosis can only be made postmortem.

Non-Motor Symptoms

The motor symptoms of PD have been appreciated since Dr. Parkinson's initial observations in 1817, however our understanding of the disease has evolved to the realization that it is a complex multisystem disorder. The presence of non-motor symptoms is increasingly recognized as part of the symptomatic picture of PD. Indeed, research demonstrates that 20-90% of PD subjects have at least one major non motor symptom, non-motor symptoms that significantly impact quality of life (Pellicano et al., 2007; Tibar et al., 2018). These non-motor symptoms can include, but are not limited to, rapid eye movement (REM) sleep dysfunction, anosmia (loss of smell), constipation, anxiety, and depression. Often these non-motor symptoms can occur 5-10 years before the onset of motor symptoms (Pont-Sunyer et al., 2015). Furthermore, as the disease progresses, cognitive decline is estimated to affect about 80% of the population (Aarsland et al., 2005; Biundo et al., 2016).

Neuropathology of Parkinson's Disease

The neuroanatomical circuitry in which dysfunction contributes to the emergence and progression of PD is the basal ganglia. The basal ganglia are a group of interconnected subcortical nuclei that have been found to be responsible for controlling movement, motor learning, executive functions and emotions (Redgrave et al., 2010). This group of subcortical nuclei include the caudate and putamen (collectively termed the striatum (STR)), the globus pallidus pars interna (GPi), the globus pallidus pars externa (GPe), the subthalamic nucleus (STN) the substantia nigra pars compacta (SNpc) and the substantia nigra pars reticulata (SNr) (Fig 1.1A). The classical model utilized to understand the basal ganglia and its role in PD consists

of two parallel but opposing pathways, the direct and indirect pathways, that mediate information flow within and output from the basal ganglia. The SNpc sends dopaminergic projections to the striatum which binds to the dopamine receptor 1 (D1, direct pathway) or dopamine receptor 2 (D2, indirect pathway) receptors located on the medium spiny neurons within the striatum (Calabresi et al., 2014). While these pathways and their roles in disease are currently being refined, they remain a helpful framework for understanding the facilitation and impedance of movement (Simonyan, 2019).

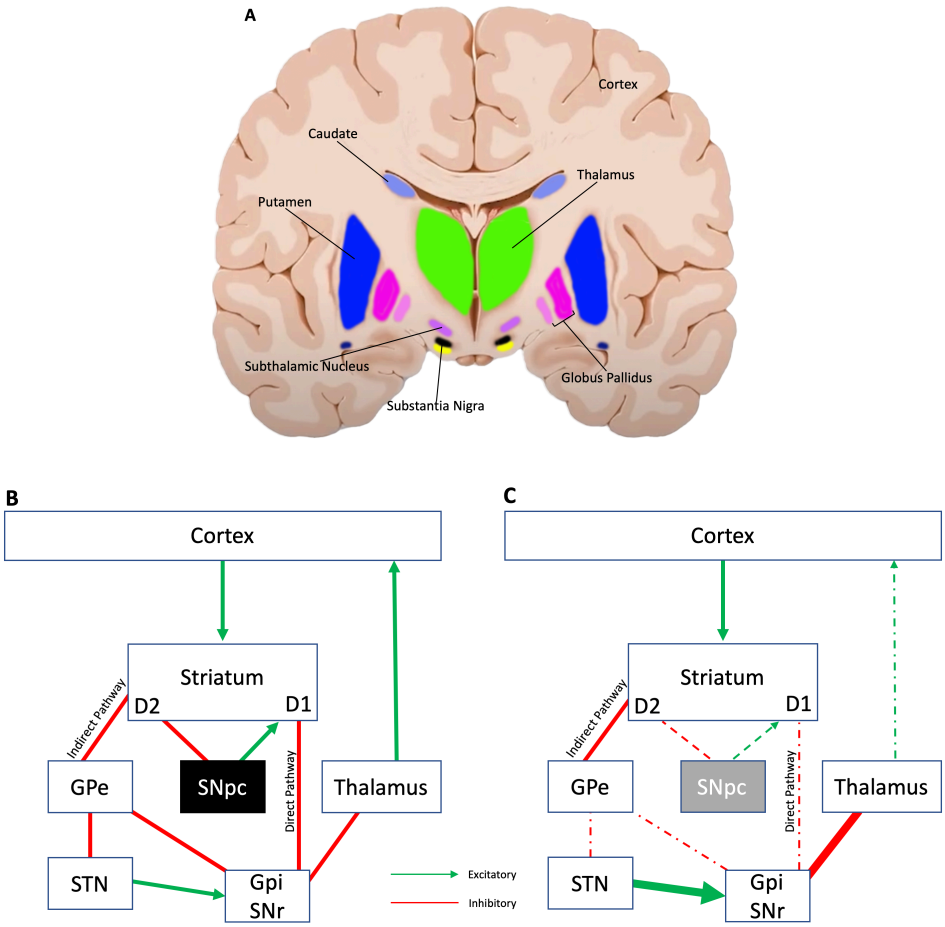

Figure 1.1. Basal Ganglia Circuitry.

A: Structures of the basal ganglia within the human brain. **B:** Basal ganglia circuit within a healthy brain allows for controlled balance between the direct and indirect pathways. **C:** Basal ganglia within a PD brain has an imbalance between the direct and indirect pathways due to the loss of neurons within the SNpc. Dotted lines indicate degeneration of the pathway. Thickness of lines indicates level of activity Abbreviations: SNpc= Substantia nigra pars compacta; D1= excitatory D1-like DA receptor; D2= inhibitory D2-like DA receptor; GPe= globus pallidus externa; GPi= globus pallidus interna; GPe=globus pallidus pars externa; STN= subthalamic nucleus; SNr= substantia nigra pars reticulata.

Direct Pathway of the Basal Ganglia

The direct pathway is named as such due to the monosynaptic circuit between the STR and either of the primary output structures, the GPi and the SNr. When the direct pathway is activated by DA binding to the D1 receptor, this results in the facilitation of movement through the inhibition of the inhibitory control the GPi has on the thalamus (Calabresi et al., 2014; Redgrave et al., 2010). Movement facilitation occurs when glutamatergic neurons of the motor cortex (corticostriatal pathway) activate the medium spiny neurons in the STR to release the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the GPi and the SNr, leading to the inhibition of activity within these regions. Normally the GPi and SNr are tonically inhibiting the thalamus, however corticostriatal activation lessens this inhibition of the thalamus, thus thalamic activity increases, exciting the motor cortex and effectively allowing for movement to occur (Fig. 1.1B).

Indirect Pathway of the Basal Ganglia

The indirect pathway is named as such due to the use of three synaptic connections between the STR and the GPi and the SNr. When the indirect pathway is activated by DA binding the D2 receptor, it leads to the inhibition of movement through the strengthening of the inhibitory hold the GPi has on the thalamus. Indirect pathway striatal neurons release GABA in the GPe resulting in the inhibition of the neurons in the GPe, which normally inhibit the STN. Decreased inhibition of the STN results in activation of the inhibitory GPi and SNpr (opposite effect of the direct pathway) which then increases the inhibition of the thalamus. Thus, when the cortex activates the indirect pathway the net effect is of inhibition of the thalamus and

therefore decreased excitation of the motor cortex and impedance of movement (Fig. 1.1B;(Calabresi et al., 2014; Simonyan, 2019).

Role of Dopamine in Modulating the Activity of the Direct and Indirect Pathway

The balance of movement control accomplished by the summed output of the direct and indirect pathways is modulated by the presence of dopamine. Dopamine (3, 4-Dihydroxytyramine, DA) is a monoaminergic neurotransmitter that plays a role in 4 main pathways:

1. Mesolimbic pathway: Pleasure and reward
2. Mesocortical Pathway: Cognition, working memory, and decision making
3. Tuberoinfundibular pathway: Prolactin secretion
4. Nigrostriatal pathway: Movement

(Bridges, 2019)

Within the nigrostriatal pathway, DA is released in the striatum from neurons that project from the SNpc. DA in the synapse has the ability to bind to D1 or D2 dopamine receptors leading to the activation of the direct and/or indirect pathways in the basal ganglia (as described above). However, in PD the inputs from the SNpc to the D1 and D2 receptors are changed due to the degeneration of the dopaminergic terminals that project from the SNpc to the STR leading to an imbalance between the direct and indirect pathways. This results in greater output from the indirect pathway leading to movement inhibition (Fig. 1.1C; Blandini et al., 2000; Redgrave et al., 2010).

Pathological Hallmarks of Parkinson's Disease

As previously stated, the confirmation of PD diagnosis occurs postmortem. The two main pathological hallmarks that are required for confirmation of PD diagnosis are:

1) Presence of proteinaceous/lipidaceous intraneuronal cytoplasmic inclusions (Lewy bodies)
2) Degeneration of the dopaminergic neurons in the substantia nigra pars compacta (SNpc).

(Kouli et al., 2018)

Lewy Bodies

Lewy bodies were first described by Friedrich Heinrich Lewy in 1912 after examining 85 patients both clinically, as well as postmortem. Lewy first described "*...spherical (globular), strandlike, and serpiginous (serpentine) forms are evident that stain bright red with Mann's technique [methyl blue-eosin mixture]*" within the dorsal nucleus of the vagus, Meynert's nucleus, and the thalamic paraventricular nucleus (Engelhardt & Gomes, 2017). It is now understood that Lewy bodies can be observed throughout the central and peripheral nervous system in patients with PD. In the brain, Lewy bodies are observed in structures ranging from the olfactory bulb, spinal cord, nucleus basalis of meynert, cortex, cerebellum, locus coeruleus, amygdala and most appreciated, in the SN (Giguère et al., 2018) . In the peripheral nervous system, Lewy bodies are observed in the enteric nervous system, sympathetic ganglia and the gastrointestinal system (Barrenschee et al., 2017). Although Lewy bodies were appreciated as a

major pathological hallmark in PD, it would take another 86 years before the major protein constituent, alpha-synuclein, was identified.

Lewy Bodies are Comprised of Alpha-Synuclein

Following the discovery that a point mutation in the SNCA gene (encodes for the protein alpha-synuclein) leads to an increased risk of PD (see genetic section for greater detail), Dr. Maria Spillantini, 1997, discovered that alpha-synuclein was a main component of Lewy Bodies (Spillantini et al., 1997, 1998). Alpha synuclein (α-syn) is a small 140 amino acid protein that is found abundantly in the brain, especially in the neocortex, hippocampus, substantia nigra, thalamus, and the cerebellum (Stefanis, 2012). The main intraneuronal localization of α-syn is in the nucleus and the synapse, hence the name. Recently α-syn has also been found in the periphery including the gut, appendix, tonsils, and red blood cells (Barbour et al., 2008; Killinger et al., 2018; Liu et al., 2020). α-syn is an intrinsically disordered protein in that its secondary structure can alter based on the environment and it requires an interaction with another molecule to form its tertiary structure (Stefanis, 2012). Whereas all the biological functions of soluble, monomeric α-syn remain to be revealed, it has been directly linked to the regulation of synaptic transmission, calcium regulation, mitochondrial homeostasis, gene expression, protein phosphorylation, and fatty acid binding (Bendor et al., 2013; Bernal-Conde et al., 2020; Calì et al., 2012; Kaplan et al., 2003) . In the process of forming aggregated Lewy bodies, α-syn transforms from a monomeric form to oligomeric and fibrillar forms and ultimately into the beta-sheet-rich amyloid fibrils that are seen in Lewy Bodies (Cremades et al., 2012; Marotta et al., 2021). While α-syn is appreciated as a major component, Lewy bodies also consist of many

other proteins including, but not limited to, structural proteins, neurofilaments, α-syn binding proteins, synphilin-1-binding proteins, cell cycle proteins, and ubiquitin-proteasome system proteins (Shahmoradian et al., 2019; Shults, 2006). Furthermore, it has been found that Lewy bodies also contain a large number of lipids and membrane fragments, lysosomes, misshapen organelles, vesicles, and mitochondria (Shahmoradian et al., 2019).

Under electron microscopy the ultrastructure of Lewy bodies can be characterized into two groups: mixed granular-fibrillar type and fibrillar type. The first type, a mix of granular and fibrillar components, the more common type, shows a tight granular core with a radiating fibrillar formation on the periphery. The second type, is composed of a mix of granular and mixed granular fibrillar type, has curved circular fibrils that shows "uniform density under light microscopy" (Roy & Wolman, 1969; Shults, 2006)

The mechanism whereby α-syn is transformed from a soluble, functional protein into the aggregated Lewy body inclusions observed in PD is still unknown. However, there are some factors that are thought to play a role in this α-syn transition. These include point mutations to the SNCA gene that increase the propensity of α-syn to aggregate, elevated levels of α-syn (duplication or triplication mutations) and oxidative stress (Lashuel et al., 2013). Furthermore, regulation of the intracellular level of α-syn in the system relies on proper degradation, suggesting that dysfunction in the ubiquitin proteasome pathway may also lead to aggregation (Ebrahimi-Fakhari et al., 2012; Lashuel et al., 2013). It also has been speculated that the aged brain environment contributes to α-syn aggregation (Hindle, 2010). In fact, it has been found that many people over the age of 60 exhibit Lewy body pathology in the absence of PD symptoms, a condition known as Incidental Lewy Body Disease (Adler et al., 2010; Markesbery

et al., 2009). Whereas Lewy bodies are considered a pathological hallmark of PD, it remains controversial whether Lewy bodies are in fact toxic, protective, or merely a surrogate marker of intraneuronal pathogenic mechanisms (Chartier & Duyckaerts, 2018; Engelhardt & Gomes, 2017; Mahul-Mellier et al., 2019; Markesbery et al., 2009). In the process of forming aggregated Lewy bodies, α-syn transforms from a monomeric form to oligomeric to fibrillar and then Lewy body (Cremades et al., 2012). However, whether the oligomeric or fibrillar form is toxic is still under investigation with studies associating multiple forms with cellular toxicity (Froula et al., 2019; Pemberton et al., 2011; Pieri et al., 2012; Roberts et al., 2015)

Braak Staging of Lewy Pathology

The identification of α-syn as a main component of Lewy bodies allowed for more detailed observations of Lewy pathology (Lewy bodies and Lewy neurites) in the parkinsonian brain. In 2003 Braak et al. put forth a framework for understanding the progression of Lewy pathology in PD based on observations in a series of PD brains collected at varying times after PD diagnosis (Braak et al., 2003). 6 stages were proposed:

Stage 1: Pathology observed in the dorsal motor nucleus and/or intermediate reticular zone along with the olfactory bulbs.

Stage 2: Pathology of stage 1 as well as pathology ascending into the caudate raphe nuclei, gigantocellular reticular nucleus, and the coeruleus-subcoeruleus complex.

Stage 3: Pathology of stages 1 and 2 as well as pathology ascension into the midbrain, including the central subnucleus of the amygdala, the SNpc, and the basal forebrain.

Stage 4: Pathology of stages 1-3 as well as pathology observed in the temporal mesocortex

Stage 5/6: Pathology of stages 1-4 as well as pathology observed in the neocortex and premotor areas.

Whereas Braak staging has been and remains the gold standard for pathological staging in PD, one major caveat related to this tissue set should be considered. Specifically, brains in which pathology in the dorsal motor nucleus was not observed were excluded from analysis (Burke et al., 2008; Jellinger, 2008; Kalaitzakis et al., 2008). This exclusion leads to the failure to classify around 50% of PD cases due to the lack of pathology in the dorsal motor nucleus (Jellinger, 2008).

Braak Prion Hypothesis

It has been proposed that sporadic PD can be caused by a pathogen entering the body via two routes: the nose to the olfactory bulb or through the gastrointestinal system to the brain via the vagus nerve. Initiation of Lewy pathology within the gastrointestinal (GI) tract may explain the early onset of the nonmotor gastrointestinal symptoms in PD patients. Furthermore, according to Braak, the cells in the GI tract share key characteristics with the vulnerable cells seen in the brain (specifically long, thin, and unmyelinated projections) (Braak & del Tredici, 2004). Recently it has been proposed that aggregated α-syn may be able to

propagate from cell-to-cell in a prion like fashion leading to further aggregation of endogenous α-syn and spread of the pathology. Considerable research currently focuses on whether this phenomenon occurs in the PD brain and raises the possibility that targeting prion-like spread could be an important therapeutic approach for slowing PD progression.

Nigrostriatal Pathway Degeneration

The second pathological hallmark of PD is degeneration of the nigrostriatal pathway, specifically the loss of melanized dopamine neurons in the SNpc and dopaminergic innervation to the caudate and putamen. The degeneration of the nigrostriatal system is the cause of the dopamine deficiency in the striatum, resulting in disruption of basal ganglia circuit activation that drives the motor symptoms of PD (Blandini et al., 2000). At the time of PD diagnosis patients are believed to have lost approximately 70% striatal dopamine and 50-60% loss of SNpc neurons (DeMaagd & Philip, 2015; Kordower et al., 2013; Kurowska et al., 2016). Studies evaluating early-stage PD brains suggest that axonopathy of the striatal dopaminergic terminals precedes the degeneration of the soma in the SNpc (Kordower et al., 2013).

Neurodegeneration within the pedunculopontine nucleus, locus coeruleus, dorsal motor nucleus of the vagus, and the nucleus basalis of Meynert also has been observed (Giguère et al., 2018). While not all neurons are affected in PD, the nigrostriatal dopaminergic neurons are particularly vulnerable. The exact reason for their vulnerability is still unclear, however, it is hypothesized that the high metabolic demand of dopaminergic neurons, the high level of reactive oxygen species that are made with the production of DA, iron and neuromelanin content, and proximity to high density of microglia in the SNpr (González-Hernández, 2010,

2010b; Hirsch et al., 1988; Kamath et al., 2022; Surmeier et al., 2017) may contribute to their sensitivity. Most recent studies indicate that particular subpopulations of SNpc neurons in the ventral tier that express the AGTR1 gene are selectively vulnerable in PD (Kamath et al., 2022).

Current Symptomatic Treatments for PD

Therapeutic strategies for PD can aim to either mitigate the motor symptoms of the disease or attempt to slow the progression of Lewy body pathology and/or nigrostriatal degeneration. The most common symptomatic therapies used in the treatment of PD symptoms are discussed below.

Dopaminergic Pharmacotherapies

Levodopa/Carbidopa

The pharmacotherapies used for the treatment of the motor dysfunction in PD share a common strategy of increasing or prolonging the levels of DA at the synapse, in order to counteract the imbalance to the basal ganglia circuit, leading to better movement abilities and control. The most common dopaminergic medication currently used for the treatment of PD motor symptoms is levodopa (L-dihydroxyphenylalanine; (Dorszewska et al., 2014; Zahoor et al., 2018). Levodopa is the precursor to DA within the DA synthesis pathway and readily crosses the blood brain barrier (BBB). Levodopa can increase the production and release of DA from nigrostriatal DA neurons. In 1967, oral administration of levodopa became the standard PD treatment. A few years later, in the early 1970s, a dopa decarboxylase inhibitor (carbidopa) was added to the formulation to prevent metabolism of levodopa in the periphery by dopa

decarboxylase. As such, in 1975, the first levodopa combination, levodopa/carbidopa (Sinemet®), became readily available and it remains the gold standard of treatment for patients across many stages of PD (Tolosa et al., 1998).

Dopamine Receptor Agonists

Another pharmacotherapeutic approach for the treatment of PD motor symptoms is the use of DA receptor agonists. DA receptor agonists bind to the D1 and D2 receptors in the STR, mimicking DA. Unknown to those who first used this approach, DA receptor agonists were the first dopaminergic pharmacotherapy used to treat PD motor symptoms. In 1884, Dr. Edmond Weil treated a patient with Sydenham's chorea with apomorphine, a derivative of morphine, and observed a decrease in the patient's motor symptoms (Auffret et al., 2018; Tolosa et al., 1998). However, it wasn't until 1951 that Dr. Robert Schwab administered apomorphine to PD patients and observed motor symptom improvement, although the improvement was transient (Auffret et al., 2018; Tolosa et al., 1998). However, up to this point in time the dopaminergic properties of apomorphine were not appreciated. Later, in 1965 the structural similarity of apomorphine to DA was revealed followed shortly by identification of its presumed action at DA receptors (Ernst, 1965; Tolosa et al., 1998). This led to the development of novel DA receptor agonists with longer half-lives and receptor specificity. Current DA receptor agonists in use clinically for treatment of PD motor symptoms include bromocriptine, pramipexole, ropinirole, and cabergoline (Borovac, 2016). Often DA agonists are used in the early years after PD diagnosis, either prior to levodopa/carbidopa or in a polypharmacy approach with levodopa/carbidopa to promote the efficacy of lower doses. The rationale for this strategy is to

prolong the efficacy of levodopa/carbidopa and limit motor fluctuations (decline or shortened benefit of therapy) or prevent the development of levodopa-induced dyskinesias (Borovac, 2016).

Dopamine Metabolism Inhibitors

The third category of dopaminergic medications used to treat PD motor symptoms are metabolism inhibitors, specifically inhibition of catechol-o-methyltransferase (COMT) or monoamine oxidase (MAO) both of which metabolize DA, limiting its duration of action in the synapse (Bonifácio et al., 2007). COMT inhibitors were first FDA-approved for PD treatment in 1997 whereas MAO inhibitors were approved in 2006 (Smith et al., 2012; Zahoor et al., 2018). MAO-B inhibitors are used both as a monotherapy and used in a polypharmacy approach with levodopa/carbidopa. In contrast, COMT inhibitors are only prescribed in combination with levodopa/carbidopa as they do not provide symptomatic relief when used as a monotherapy (Armstrong & Okun, 2020). Similar to the use of DA receptor agonists, some movement disorder clinicians favor early use of MAO-B inhibitor monotherapy or COMT inhibitor polytherapy with lower levodopa/carbidopa to limit motor fluctuations and/or prevent levodopa-induced dyskinesias (Armstrong & Okun, 2020; Krishna et al., 2014).

Surgical Therapies

Deep brain stimulation (DBS), first approved by the FDA in 2002, has become the most common surgical interventions for PD. DBS for the treatment of PD symptoms involves the surgical implantation of an electrode into the basal ganglia circuitry (Gpi or STN) in order to

modify pathological circuit activity resulting from DA depletion in the striatum. STN or Gpi DBS is particularly effective at treating symptoms of tremor and akinesia/bradykinesia (Limousin & Martinez-Torres, 2008). In current clinical practice, PD patients are encouraged to consider DBS years after PD diagnosis when standard pharmacotherapy approaches are no longer providing sustained symptomatic relief or levodopa-induced dyskinesias are severe (Limousin & Martinez-Torres, 2008; Neimat et al., 2006)

Etiology of Neurodegeneration in PD

While the exact cause of PD is still unknown it is now accepted that PD is a heterogeneous and multifaceted neurodegenerative disease that involves interactions between genetic susceptibility and environmental exposures. While the previously mentioned therapies are used to treat the motor symptoms of PD, no therapies exist to slow the progression of Lewy body pathology and neurodegeneration in PD. Efforts to develop disease modifying PD therapeutics have been hindered by our limited understanding of PD etiology. The following section summarizes the factors and pathogenic mechanisms believed to contribute to neurodegeneration in PD

Contributing Factors

Age and Biological Sex

Age and biological sex may and can play a role in a person's susceptibility to developing PD. With PD being considered a disease of the aging, with the majority of cases developing between the age of 60-65, aging is considered a main risk factor for PD development (Collier et

al., 2011, 2017). The one caveat is young onset PD (YOPD), seen in 2% of the world's PD population, where PD is developed before the age of 40. Normal aging leads to cellular changes including mitochondrial dysfunction, less efficient ubiquitin proteasome protein degradation, and mild gliosis (Kanaan et al., 2010; Ross et al., 2015). These age-related cellular changes combined with a failure of compensatory mechanisms may lead to an increased susceptibility to PD (Hindle, 2010).

Biological sex, specifically people assigned male at birth, have a 2x risk of developing PD than females (Baldereschi et al., 2000; Solla et al., 2012). However, it has been seen that while females have a lower risk their disease progression is more rapid, leading to higher mortality rates than male (Cerri et al., 2019). While the reason for this disparity is not known, some studies show an association between increased number of child births and the length of a female's reproductive lifespan (menarche until start of menopause) and lower susceptibility/later onset of PD (Frentzel et al., 2017). Whether this delay is due to prolonged estrogen exposure, which has been seen to play a neuroprotective role in the aging brain (Zárate et al., 2017) or something else, remains to be determined.

Ethnicity and Race

Certain genetic forms of PD have shown prevalence in different ethnic communities (e.g. the LRRK2, G2019S, mutations have high prevalence in the North African Berber and Ashkenazi Jewish population (Marder et al., 2015) and studies have been aimed to analyze whether ethnicity and geographical location can play a role in PD risk. Multiple studies have shown that there is an increased prevalence of PD in Hispanics and Caucasian populations as

compared to Asians and Blacks (Ben-Joseph et al., 2020; Wright Willis et al., 2010). Interestingly, the prevalence of PD in people of African descent living in the USA is higher than Black-aficans living in sub-saharan Africa (Ben-Joseph et al., 2020; Dotchin et al., 2008) leading to the understanding that geographical location may play a role potentially due be due to the exposure to differing environmental factors (Abbas et al., 2018) as well as diet (Jackson et al., 2019; Solch et al., 2022). In fact, there is an overall lower occurrence of PD in Asia when compared to North America, Europe, and Australia (Pringsheim et al., 2014). While the exact reason behind the aforementioned disparity in PD occurrence is not known, the socioeconomic differences between groups may play a role (F. Yang et al., 2016).

Pathogenic Mechanisms

Genetics

In 1997, a point mutation in the SNCA gene (an A30P missense mutation on the gene that codes for α-syn) was found on chromosome 4 of an Italian family with multiple familial cases of PD, as well as three different Greek families (Polymeropoulos et al., 1997). Following the A30P SNP finding, further mutations (A53T, G51D, E46K) and multiplications (duplications, triplications) of the SNCA gene were discovered in association with familial PD cases. While the mechanism leading to increased risk of developing PD is not known, it is hypothesized that the change in the amino acid sequence conferred by the SNCA point mutations alters the kinetics of α-syn folding and aggregation, whereas SNCA duplication and triplication leads to increased levels of endogenous α-syn, allowing for greater aggregation to occur.

Since the association between SNCA and PD was revealed, other autosomal recessive gene mutations have come been implicated in increased risk of PD, including PRKN, PINK1, and DJ-1, which lead to changes in the ubiquitin proteasome pathway, mitochondrial function, and oxidative stress activity, respectively. All the aforementioned gene mutations are quite rare. In 2004, an autosomal dominant mutation of LRRK2 (Leucine-rich repeat kinase 2) gene was identified (originally mapped in a Japanese family in 2002) and has quickly become recognized as one of the most common monogenic mutations seen in classical familial cases of PD (Billingsley et al., 2018). This was then followed, in 2011, by the discovery of another autosomal dominant mutation in VPS35 (vacuolar protein sorting associated protein 35) gene in a Swiss family (Williams et al., 2017). These two autosomal dominant mutations LRRK2 and VPS35 lead to toxic gain-of-function or toxic loss-of-function phenotypes, respectively, and classical onset of PD. The aforementioned genes are just a few of the genes that have been found to be implicated in PD risk, further research is still needed as not all familial PD cases can be linked to one of the known genes of risk.

Environment

In the early 1980s observations showed a correlation between childhood exposure in a rural environment (and drinking well water) and development of idiopathic PD. Further studies also have implicated pesticide and herbicide exposure with an increased risk of PD. The large umbrella epidemiological study conducted by Bellou et al. (2016) provided evidence suggesting that farming, rural living, welding, outdoor work, organic solvents, pesticides can all increase an individual's risk of developing PD (Bellou et al., 2016). While the mechanisms of induced risk are

still under investigation, preclinical studies provide evidence that pesticides/herbicides can lead to the death of dopaminergic neurons through different pathways including mitochondrial dysfunction, changes in microglial phagocytosis, and changes in reactive oxygen production. The knowledge that pesticides increase individual PD risk and induce parkinsonism pathology led to some of the first preclinical animal PD models, discussed later in this chapter.

Glucose Metabolism in Neurodegeneration

Meta-analysis has recently shown that Diabetes Mellitus (DM) leads to an increased risk of PD development (Yue et al., 2016). DM is considered one of the most common chronic diseases globally and is defined by the inability of cells to take in glucose for fuel. Neurons and astrocytes are known to require glucose metabolism to function correctly. Glucose metabolism is a highly regulated process that, when altered whether due to disease or aging, leads to a host of complications and neurodegeneration. Glucose metabolism dysfunction has been found to be an early event in sporadic PD (Dunn et al., 2014) and is proposed to be a possible mechanism underlying neurodegeneration. While the exact mechanism whereby glucose metabolism dysfunction leads to neurodegeneration is unknown, clinical trials have begun to test antidiabetic drugs in patients with PD. Antidiabetic drugs are hypothesized to elicit multiple pro-survival effects for neurons including enhanced mitochondrial function, decreased alpha-synuclein aggregation, and anti-inflammatory effects (Labandeira et al., 2022).

Neuroinflammation

The belief that the brain was “immune privileged” was held for many years. This belief was based upon the presence of the blood brain barrier, as well as studies conducted in 1923 showing that tumors implanted in peripheral tissue were quickly rejected by the host's immune system, whereas tumors implanted into the CNS were able to thrive (Murphy & Sturm, 1923). It was believed that the immune system of the CNS was more “tolerant” than the peripheral immune system to help protect neurons from irreparable damage that can come from exaggerated and chronic inflammatory responses (Brioschi & Colonna, 2019). However, the revelation that CSF drainage into cervical lymph nodes occurs (Kida et al., 1993) and that cross talk exists between the CNS and peripheral immune system through the meningeal lymphatic vasculature (Aspelund et al., 2015) demonstrated that the brain was not as immune privileged as originally thought. Research into the interaction between the innate and adaptive immune systems and their responses in neurodegenerative diseases has been ongoing to better understand the role the immune system may play in the progression of disease.

Historically, PD was considered simply a movement disorder, however over the past decade it has come to light that PD can be classified as a multisystem disorder, including dysfunction within the immune system. Analysis of biofluids from patients, along with postmortem PD tissue analysis, have consistently shown a disturbed inflammatory environment (X. Chen et al., 2018; Lindqvist et al., 2013; Mogi et al., 1994; Nagatsu’ et al., 2000; Qin et al., 2016) . Cerebral spinal fluid (CSF) and plasma have revealed increases in inflammatory cytokines and positron emission tomography (PET) imaging have shown elevated and sustained

microglial responses as compared to aged, matched controls (Gerhard et al., 2006; Ouchi et al., 2005).

The evidence supporting the role that inflammation and the immune system may play in PD progression is continuously accumulating. To date, there is genetic, immunological, epidemiological, and pharmacological evidence that suggest and support an association between PD progression, neurodegeneration and inflammation. Some of that evidence is described below.

Activated Immune System

Innate Immunity

The seminal study showing increased reactive microglia within the parenchyma of postmortem PD tissue within the vicinity of PD pathology, specifically Lewy pathology and degeneration within the SNpc and STR (Mcgeer et al., 1988; McGeer et al., 1988) helped to establish the connection between neuroinflammation and PD. Microglia, the resident macrophages in the CNS, originate from yolk sac progenitors and are thought to colonize the cerebrum between the 4th and 24th weeks of gestation (Menassa & Gomez-Nicola, 2018) Microglia have many roles in helping to keep the healthy homeostasis of the brain including synaptic pruning, neurogenesis, and neuronal surveillance. Microglia show regional heterogeneity (Y.-L. Tan et al., 2020) as well as heterogeneous responses to insults (Tansey et al., 2022). Historically, microglia have been considered either in a state of "resting" (general surveillance state) or activated (M1: pro-inflammatory or M2: anti-inflammatory). However, increasing evidence suggests that microglial activity is more multidimensional rather than

linear. The spectrum of microglial "activation" is based on three basic microglial functions- Host defense, wound healing, and immune regulations - with each function able to overlap with the others (Mosser & Edwards, 2008).

Activated microglia are thought to play a role in the production and secretion of a multitude of inflammatory markers including interleukin-1-beta I (IL-1ß), tumor necrosis factor-alpha (TNF-α), interleukin-4 (IL-4), reactive oxygen species (ROS), nitric oxide species and other pro-apoptotic markers within the substantia nigra and CSF of patient with PD (Harms et al., 2021; Nagatsu' et al., 2000). It also has been shown that microglia can convert astrocytes to a toxic proinflammatory A1 astrocyte, which has been observed in the SN of PD patients (Liddelow et al., 2017). Additionally, genetic forms of PD have been shown to involve genes expressed by astrocytes with implications in astrocyte biology (e.g. *GBA*, *LRRK2, PINK1*) (Booth et al., 2017)

Analysis of microglia within the parenchyma of postmortem PD tissue identified increased levels of the human leukocyte antigen related D (HLA-DR, Human analog for major histocompatibility complex-II, MHC-II) within the vicinity of PD pathology, specifically Lewy pathology, within the SNpc and STR (Mcgeer et al., 1988; McGeer et al., 1988). HLA-DR is a cell surface protein found on antigen presenting cells (i.e. microglia) that interact with T-cells and the adaptive immune system (Schetters et al., 2018).

Adaptive Immunity

The presence of MHC-II within the vicinity of PD pathology, along with immunoglobulin G on dopaminergic neurons, suggests that the adaptive immune system may also play a role in

PD. Brochard et al. showed an increase in both CD4+ and CD8+ T cells within the midbrain of patients with PD as compared to controls (Brochard et al., 2009). Interestingly, some studies have shown a decrease in peripheral circulating CD4+ T cells in patients (Jiang et al., 2017; Saunders et al., 2012). Furthermore, it was found that changes in the dopamine receptor expression on T cells correlates with the severity of disease (Elgueta et al., 2019; Kustrimovic et al., 2016) suggesting that dopamine receptors on immune cells may play a role in PD progression (Tansey et al., 2022). Functional changes in T cells have been seen with increased expression of TNF receptors (Bongioanni et al., 1997) along with an increase in interferon gamma and TNF production by circulating effector T cells (Kustrimovic et al., 2018). Additionally, T cells from PD subjects, and not control subjects, are able to recognize different forms of α-syn peptides (Lindestam Arlehamn et al., 2020; Sulzer et al., 2017)

GWAS Studies Show Inflammatory Loci and Increased PD Risk

Several variants of genes have been identified to modulate the risk of developing sporadic PD including LRRK2 (encodes dardarin), SNCA (encodes α-syn), GBA (encodes glucocerebrosidase), PRKN (encodes E3 ubiquitin-protein ligase parkin), PINK1 (encodes PTEN-induced kinase 1), and VPS35 (encodes vacuolar protein sorting associated protein 35). Additionally, genome-wide association studies (GWAS) have linked ~90 loci to sporadic PD with some related to immune functions, including HLA (encodes human leukocyte antigen), GPNMB (encodes glycoprotein NMB), BST1 (encodes bone marrow stromal cell antigen 1 (AKA CD157)), SYT11 (encodes synaptotagmin 11,), TMEM175 (encodes transmembrane protein 175) and GRN (encodes progranulin) (E.-K. Tan et al., 2020; Tansey et al., 2022) .

Autoimmune Diseases and PD

With increasing evidence that inflammation may play a role in PD pathology, studies have been conducted to better understand the possible overlap between autoimmune disease and PD. Autoimmune disease is a group of diseases in which the peripheral immune system is chronically activated producing inflammatory mediators that may be able to promote neuroinflammation and PD pathogenesis (Tansey et al., 2022). A 2017 GWAS study found 17 overlapping genes between PD and autoimmune diseases including type 1 diabetes, Crohn disease, ulcerative colitis, rheumatoid arthritis, celiac disease, psoriasis, and multiple sclerosis (Witoelar et al., 2017). Further an epidemiological study in Sweden showed a 33% increased risk of PD among patients with autoimmune disorders, specifically Multiple sclerosis (MS), Hashimoto disease, Graves' disease, and polymyalgia rheumatica (Li et al., 2012). Further studies have shown that that treatment with chloroquine/hydroxychloroquine, which is used to decrease inflammation in rheumatoid arthritis, is associated with a decreased risk of PD (Paakinaho et al., 2022).

Altered Microbiota and Inflammatory Markers in Feces of Patients with PD

The prodromal non motor symptoms experienced by patients often include gastrointestinal issues which has led researchers to analyze the involvement of the gut, specifically the gut-brain axis, and its possible role in PD onset and progression. Analysis of the gut microbiome has shown differences in the GI environment and microbial inhabitants in PD patients as compared to controls (Aho et al., 2019; Bhattarai & Kashyap, 2020; Boertien et al., 2019; Heinzel et al., 2020; Scheperjans et al., 2015). While many questions still remain, large

epidemiological studies have demonstrated that gut dysbiosis and inflammatory bowel disease (IBD) is associated with a 20%-90% increased risk of developing PD when compared to individuals without IBD(Park et al., 2019; Peter et al., 2018; Villumsen et al., 2019; Weimers et al., 2019; Zhu et al., 2022). Analysis of PD subject fecal matter has identified intestinal inflammatory markers, including IL-1a, IL-1beta, CXCL18, and calprotectin (Aho et al., 2021; Houser et al., 2018; Mulak et al., 2019; Schwiertz et al., 2018)

Anti-Inflammatory Drugs and Immunosuppressants are Associated with Lower PD Risk

As Alzheimer's disease (AD) and PD share some pathological features, the finding that non-steroidal anti-inflammatory drug (NSAID) exposure is linked to decreased AD risk (Zhang et al., 2018) prompted studies that revealed a similar link with PD as well. A large epidemiological study reported that patients with regular non-aspirin NSAIDs had a lower risk of developing PD as compared to controls (H. Chen et al., 2003; Wahner et al., 2007) , and acetaminophen or aspirin users had a 35% lower risk of developing PD (H. Chen et al., 2005). Interestingly, a 2006 study found that non-aspirin NSAIDS decreased the risk of developing PD by 20% in men while it increased the risk by 20% in women (Hernán et al., 2006) being the first study to show a difference in sex, PD risk and NSAID usage. The mechanism by which NSAIDs are neuroprotective is thought to be through the inhibition of cyclooxygenase 1 (COX1) and COX2 leading to a decrease in nitric oxide radicals and oxidative stress (Asanuma et al., 2001). Some studies have not shown a link between NSAIDs and decreased risk of PD (Poly et al., 2019; Ren et al., 2018) suggesting that further study is needed.

Disease Modifying Therapies for PD

While there are currently no FDA-approved medications that are disease modifying, according to clinicaltrials.gov there are more and 3,000 ongoing clinical trials for Parkinson's disease. These clinical trials are investigating early diagnosis biomarkers, or testing therapies aimed at decreasing L-DOPA dyskinesias, decreasing α-syn aggregation, slowing neuronal degeneration, and reducing chronic inflammation. One current anti-inflammatory trial involves the small molecule AKST4290 (McFarthing et al., 2021). This molecule is a G protein-coupled C-C chemokine receptor type 3 (CCR3) inhibitor leading to the blocking of the action of eotaxin resulting in reduced inflammatory signaling.

Preclinical Models of PD

Before potentially disease modifying treatments can be tested in the clinical setting, preclinical studies must be conducted. Over the years a handful of animal models have been created to study PD, with each model having its own inherent strengths and weaknesses.

Toxicant models

The earliest animal model of PD was a toxicant model developed in 1968 by Dr. Urban Ungerstedt when he found that an intracerebral injection of 6-hydroxydopamine (6-OHDA) leads to the selective degeneration of the dopaminergic neurons in the nigrostriatal system in rats through the production of reactive oxygen species leading to mitochondrial dysfunction (Ungerstedt, 1968). The next, and most used, PD toxicant model to be developed was the MPTP model. In the early 1980s, Dr. William Langston (Santa Clara Valley Medical Center, San Jose,

CA) was treating a group of patients with spontaneous parkinsonian symptoms following synthetic “designer” heroin ingestion (Langston, 2017). When treated with levodopa all patients showed relief in symptoms. It was then found that the drug the patients had used was contaminated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) which is metabolized by astrocytes into a toxic metabolite MPP+ (1-methyl-4-phenylpyridinium) (Markey et al., 1984). MPTP administration was subsequently used in mice and non-human primates to induce the death of the dopaminergic neurons following a systemic injection. MPP+ has a similar structure to DA and is therefore taken up by the DA transporter (DAT) into DA neurons, leading to mitochondrial dysfunction and cell death (Forno et al., 1993; Kim-Han et al., 2011).

The similarity of the structure of MPP+ to the known herbicide paraquat led researchers to investigate the possible association of pesticide exposure with PD (as discussed previously) (Betarbet et al., 2000; Brooks et al., 1999; Day et al., 1999). This information was also applied to development of a paraquat PD animal model in which systemic paraquat injection leads to the death of dopaminergic neurons through inhibition of the neurons’ ability to protect against reactive oxygen species (ROS). A second pesticide and piscicide, rotenone, has also been utilized in preclinical research, as it inhibits mitochondrial function (similar to MPTP) (Gao et al., 2003; Wu et al., 2015). However, these two models are currently not as commonly used due to their lack of selectivity for degeneration of the nigrostriatal pathway.

The aforementioned models represent the most commonly used toxicant models in preclinical PD research. One main feature that all these neurotoxicant models possess is that all result in degeneration of the nigrostriatal pathway, similar to what occurs in PD. Furthermore, all show an inflammatory response to the death of the neurons, including microgliosis and

astrogliosis. However, none display marked accumulation of Lewy body-like alpha syn inclusions preceding degeneration.

Transgenic and Viral Vector Mediated PD Models

Due to the association between multiplications and mutations in specific genes and PD risk, transgenic and viral vector-mediated PD models have been developed (Fernagut & Chesselet, 2004; Fischer et al., 2016; Ulusoy et al., 2010). The first genetic model, a SNCA mouse model, was created in 2000 and showed that human α-syn can be transcribed and produced in a mouse (Masliah et al., 2000) . Currently, transgenic preclinical rat and mice PD models include, but are not limited to: SNCA, LRRK2, PRKN, PINK1, and DJ-1 in which disease-associated point mutations, knock out or overexpression of the specific genes is studied (Konnova & Swanberg, 2018) . However, while many of these models result in robust α-syn pathology and neuroinflammation, the majority do not result in significant nigrostriatal degeneration. Viral vector-mediated models have been developed to allow for the introduction or overexpression of genes at specific neuroanatomical locations (e.g., α-syn overexpression in the nigrostriatal system) to better understand their role in pathogenesis (Fischer et al., 2016). These models can result in marked pathological α-syn accumulation, nigrostriatal degeneration and neuroinflammation, however the time course of these events is condensed, often occurring over only a few weeks. Further, viral vector-mediated α-syn overexpression results in α-syn levels that far exceed that which is observed in idiopathic and genetic forms of PD (Su et al., 2017; Zhou et al., 2011).

Alpha-Synuclein Pre-formed Fibril Model

The preclinical model used in the experiments in this dissertation is the most recent PD model that has been developed, the α-syn preformed fibril (PFF) model, first created in 2011 in the laboratory of Dr. Virginia Lee (Luk et al., 2012; Volpicelli-Daley et al., 2011, 2014) . In the α-syn PFF model pathology is triggered by an injection of fibrillized α-syn leading to the templating of endogenous α-syn, resulting in accumulation of α-syn aggregates, followed by prolonged neurodegeneration. The benefit of this model is that it allows for the analysis of pathogenic events, including neuroinflammation, at specific stages of Lewy-body like pathology and neurodegeneration (Duffy, Collier, Patterson, Kemp, Fischer, et al., 2018; Duffy, Collier, Patterson, Kemp, Luk, et al., 2018). A detailed overview and current understanding of the aggregation, neurodegeneration and neuroinflammation in the α-syn PFF model is reviewed in depth in Chapter 2.

Overall

The overarching question of this dissertation is: Does the inflammatory environment contribute to PD progression? Studies were undertaken to characterize the specific phenotype of microglia in association with Lewy body like pathology and to determine whether depletion of microglia can attenuate degeneration of the nigrostriatal system.

Aim 1

Does microglial depletion affect α-syn aggregation associated neuroinflammation and neurodegeneration? Utilizing the known time course of neuroinflammation in the α-syn PFF

model, aim 1 works to understand whether the inflammatory response to α-syn aggregation and neurodegeneration can be attenuated through partial microglia depletion. Aim 1 also analyzes whether partial depletion impacts the magnitude of α-syn aggregation or the ultimate neurodegeneration of the nigrostriatal system.

Aim 2

Characterization of the transcriptomic profile of the α-syn inclusion associated microglial (a-SAM) phenotype. Analysis of the microglial inflammatory response to the α-syn aggregation within the α-syn PFF model has been limited previously to evaluation of size and MHCII expression. Aim 2 seeks to further characterize the reactive microglial phenotype associated with α-syn aggregates.

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Chapter 2: Leveraging the preformed fibril model to distinguish between alpha-synuclein inclusion- and nigrostriatal degeneration-associated immunogenicity

Abstract

Neuroinflammation has become a well-accepted pathologic hallmark of Parkinson's disease (PD). However, it remains unclear whether inflammation, triggered by α-syn aggregation and/or degeneration, contributes to the progression of the disease. Studies examining neuroinflammation in PD are unable to distinguish between Lewy body-associated inflammation and degeneration-associated inflammation, as both pathologies are present simultaneously. Intrastriatal and intranigral injections of alpha-synuclein (α-syn) preformed fibrils (PFFs) results in two distinct pathologic phases: Phase 1: The accumulation and peak formation of α-syn inclusions in nigrostriatal system and, Phase 2: Protracted dopaminergic neuron degeneration. In this review we summarize the current understanding of neuroinflammation in the α-syn PFF model, leveraging the distinct Phase 1 aggregation phase and Phase 2 degeneration phase to guide our interpretations. Studies consistently demonstrate an association between pathologic α-syn aggregation in the substantia nigra (SN) and activation of the innate immune system. Further, major histocompatibility complex-II (MHC-II) antigen presentation is proportionate to inclusion load. The α-syn aggregation phase is also associated with peripheral and adaptive immune cell infiltration to the SN. These findings suggest that α-syn like aggregates are immunogenic and thus have the potential to contribute to the degenerative process. Studies examining neuroinflammation during the neurodegenerative phase reveal elevated innate, adaptive, and peripheral immune cell markers, however limitations of single time point experimental design hinder interpretations as to whether this neuroinflammation preceded, or was triggered by, nigral degeneration. Longitudinal studies across both the aggregation and degeneration phases of the model suggest that microglial

activation (MHC-II) is greater in magnitude during the aggregation phase that precedes degeneration. Overall, the consistency between neuroinflammatory markers in the parkinsonian brain and in the α-syn PFF model, combined with the distinct aggregation and degenerative phases, establishes the utility of this model platform to yield insights into pathologic events that contribute to neuroinflammation and disease progression in PD.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the United States (US), with motor symptoms that include tremor at rest, bradykinesia, rigidity, and postural instability. Nearly one million people in the US are living with PD and 60,000 people are diagnosed each year, with these numbers expected to rise to 1.2 million by 2030 (Marras et al., 2018). The yearly economic burden of PD is an estimated $51.9 billion (Yang et al., 2020). The hallmark pathology of PD is characterized by the presence of proteinaceous intraneuronal inclusions (Lewy bodies) and degeneration of dopaminergic neurons of the substantia nigra (SN) with axonopathy of striatal dopaminergic terminals preceding the degeneration of the soma (Kordower et al., 2013). In addition, loss of neurons in the pedunculopontine nucleus, locus coeruleus, dorsal motor nucleus of the vagus, and the nucleus basalis of Meynert is observed (Giguère et al., 2018) . Further, positron emission tomography (PET) imaging in early PD subjects or asymptomatic carriers of leucine-rich repeat kinase 2 (LRRK2) exhibit normal DA synthesis and storage, however DA turnover is increased and dopamine transporter (DAT) and vesicular monoamine transporter type 2 (VMAT2) are reduced (Adams et al., 2005; Sossi et al., 2002, 2010).

Lewy bodies are primarily composed of the misfolded, phosphorylated protein alpha-synuclein (α-syn). α-syn is abundantly expressed in neurons where it exists normally in its monomeric, soluble form, however this inherently disordered protein has a propensity to misfold into secondary and tertiary structures (Bisi et al., 2021). Misfolded a-syn exposes serine 129, allowing for its phosphorylation, however it is unclear whether this facilitates or inhibits aggregation (Fujiwara et al., 2002; Paleologou et al., 2008; Tenreiro et al., 2014). Within the

parkinsonian brain, Lewy bodies are widespread in numerous regions including areas in which neurodegeneration is observed, such as the SN pars compacta (SNpc).

Neuroinflammation in PD

Neuroinflammation is increasingly appreciated as being associated with PD. Research examining microglial reactivity in PD has focused on markers of microglial activation and measurements of microglial-associated inflammatory cytokines in PD brain, cerebrospinal fluid (CSF) and blood. Increases in cells immunoreactive for ionized calcium binding adaptor molecule 1 (Iba1), a macrophage marker which is highly expressed in microglia, are observed in the PD SN, as are increases in CD68 positive ameboid microglia (Croisier et al., 2005; Doorn et al., 2014). Further, increased expression of human leukocyte antigen related-D (HLA-DR, human analog for major histocompatibility complex-II, MHC-II) is observed in the PD SN and striatum (Imamura et al., 2003; McGeer et al., 1993; Mcgeer et al., 1988a, 1988b). HLA-DR is a cell surface protein found on antigen presenting cells (e.g. microglia) that interact with T-cells and the adaptive immune system (Schetters et al., 2018). Increased risk of PD is associated with HLA-DR variants that confer overactivity (Kannarkat et al., 2015). The SN and striatum of PD brains possess elevated cytokines produced by microglia and immune cells, including interleukin-6 (IL-6), interleukin-1-beta I (IL-1ß), interleukin-2 (IL-2), interleukin-4 (IL-4) and tumor necrosis factor-alpha (TNF-α) (Mogi et al., 1994a, 1994b; Nagatsu et al., 2000). Furthermore, PD CSF contains elevated levels of IL-1ß, IL-6 and transforming growth factor-beta 1 (TGF-ß1) (Chen et al., 2018). Analysis of blood levels in PD subjects reveals increased IL-6, TNF-α, IL-1ß, IL-2, interleukin-10 (IL-10), C reactive protein and regulated upon activation,

normal T cell expressed and presumably secreted (RANTES) (Qin et al., 2016). Positron emission tomography (PET) imaging of microglial activation reveals widespread neuroinflammation not only in the SN, but in brain regions associated with further PD progression (Gerhard et al., 2006; Ouchi et al., 2005). While most studies examining innate immune activation in the PD brain have focused on the response of microglia, astrocytes are, in fact, the most abundant glial cell type of the brain (Matejuk & Ransohoff, 2020). Microglia can convert astrocytes to a toxic proinflammatory A1 astrocyte and A1 astrocytes have been observed in the PD SN (Liddelow et al., 2017). Additionally, genetic forms of PD have been shown to involve genes expressed by astrocytes with implications in astrocyte biology (e.g. *GBA*, *LRRK2, PINK1*) (Booth et al., 2017). Collectively, the evidence of heightened innate immune activity in the parkinsonian brain is abundant.

Whereas the aforementioned studies have focused on understanding the response of the innate immune system in PD, the observed increase in MHC-II, neuronal MHC-I expression (Cebrián et al., 2014) as well as the presence of immunoglobulin G on dopaminergic neurons in PD brains (Orr et al., 2005) suggests an additional role for the adaptive immune system in PD. In 2009, Brochard et al. reported T-cell infiltration (Both CD4+ and CD8+) in postmortem PD brains. Additionally, T cells from PD subjects, and not control subjects, recognize different forms of α-syn peptides (Lindestam Arlehamn et al., 2020; Sulzer et al., 2017). Ongoing investigations continue to characterize the response of the innate immune system in PD.

Lewy Bodies as a Potential Immunogenic Signal

The overwhelming majority of studies characterizing neuroinflammation in PD are unable to distinguish between Lewy body-associated inflammation and degeneration-associated inflammation, as both pathologies are present simultaneously. It is widely accepted that the formation of Lewy bodies precedes neurodegeneration, and that the formation of Lewy bodies is ultimately associated with pathogenic consequences (Braak et al., 2003; Miller et al., 2021a). Some studies provide evidence to suggest immunogenicity of Lewy bodies specifically, prior to degeneration; specifically, MHC-II class receptor expression (*HLA-DQA1, HLA-DRA, HLA-DRB1*) is upregulated in premotor PD SN (Braak stage 1–2) compared with controls, but not in other stages of the pathological progression (Dijkstra et al., 2015). Further, studies that examine the relationship between abundance of Lewy body pathology and neuroinflammation report a positive association. MHC-II immunoreactivity is observed coincident with Lewy body pathology in PD (Imamura et al., 2003; McGeer et al., 1993; McGeer et al., 1988a, 1988b; Rostami et al., 2020) with a significant correlation observed between the level of MHC-II expression and Lewy body deposition (Croisier et al., 2005; Rostami et al., 2020). Understanding whether Lewy bodies initiate specific immunogenic signals that contribute to neurodegeneration will be required to develop effective neuroprotective strategies in PD.

Leveraging the α-syn Preformed Fibril Model to Understand Neuroinflammation in PD

Limitations of Preclinical PD Models

Animal models can serve as platforms to investigate the role of neuroinflammation in PD. Many studies have investigated the neuroinflammatory consequences of nigral degeneration and/or α-syn aggregation using neurotoxicant models (Cicchetti et al., 2002; Forno et al., 1993; Koprich et al., 2008; Kurkowska-Jastrze et al., 1999); transgenic overexpression models of human wild type or mutated asyn (A53T, A30P) (Gao et al., 2011; Gomez-Isla et al., 2003); and/or viral vector mediated α-syn overexpression models (Koprich et al., 2008; Sanchez-Guajardo et al., 2010). However, while these models are useful, they are limited in their ability to capture a prolonged interval of α-syn aggregation that culminates in dopaminergic neuron loss. Neurotoxicant models result in nigrostriatal neurodegeneration with little to no α-syn pathology. Transgenic α-syn overexpression models, despite widespread α-syn pathology, rarely show significant degeneration. A viral vector based α-syn overexpression approach produces robust neuroinflammation prior to degeneration (Fischer et al., 2016; Sanchez-Guajardo et al., 2010), but the accelerated aggregation and toxicity observed in this model confounds examination of distinct disease stages. Further, the contribution to the inflammatory response of supraphysiological α-syn levels, or the species difference of the α-syn overexpressed, is unclear. Importantly, in idiopathic PD total α-syn levels are not increased (Su et al., 2017; Zhou et al., 2011), rather phosphorylation of α-syn, and the ratio of soluble to insoluble α-syn, increases (Zhou et al., 2011). As such, models based on the overexpression of α-syn are not likely to accurately recapitulate the pathological state of idiopathic PD. Indeed, this raises the possibility that supraphysiological α-syn

expression may drive pathophysiological mechanisms that are not relevant to idiopathic PD. A model in which α-syn inclusions are triggered to form within the context of normal α-syn expression levels likely represents a more faithful animal model of idiopathic PD.

The α-syn Preformed Fibril Model

In the α-syn preformed fibril (PFF) model, widespread α-syn aggregation can be triggered to form within the context of normal levels of endogenous α-syn. This phenomenon was first developed in vitro using primary neuronal cultures (Volpicelli-Daley et al., 2011), in which recombinant α-syn fibrils are introduced to cell culture and taken up by neurons, which leads to the templating of the endogenous α-syn into accumulation of insoluble α-syn aggregates comprised of phosphorylated α-syn (Luk et al., 2009; Volpicelli-Daley et al., 2011, 2014) followed by neuronal dysfunction and degeneration. This toxicity is not due to the initial PFFs per se, but instead can be directly tied to the recruitment of endogenous α-syn into inclusions, as evidenced by the fact that PFFs do not induce toxicity when applied to α-syn$^{-/-}$ neurons (Volpicelli-Daley et al., 2011). Similarly, intraparenchymal injections of α-syn PFFs into mice, rats, marmosets and monkeys results in the templating, accumulation, and phosphorylation of endogenous α-syn (Abdelmotilib et al., 2017; Chu et al., 2019; Creed & Goldberg, 2020; Duffy et al., 2018; Henderson et al., 2019; Luk et al., 2012a, 2012b; Negrini et al., 2022; Patterson et al., 2019; Paumier et al., 2015; Shimozawa et al., 2017; Stoyka et al., 2020; Thakur et al., 2017). The observed α-syn inclusions share multiple features with Lewy bodies, including immunoreactivity for α-syn phosphorylated at serine 129 (pSyn), p62, and

ubiquitin, and are also thioflavin-S positive and proteinase-K resistant (Duffy et al., 2018; Patterson et al., 2019; Paumier et al., 2015).

pSyn immunoreactivity labels multiple stages of a-syn aggregation, ranging from oligomers to fibrils (Duffy et al., 2018; Patterson et al., 2019). It is unclear what role, if any, early forms of pathological a-syn (dimers, trimers, oligomers) (Bengoa-Vergniory et al., 2017; Garcia et al., 2022; Wan & Chung, 2012; Winner et al., 2011) or end stage Lewy body-like inclusions directly play in toxicity and immunogenicity. In addition, the process of Lewy body-like inclusion formation may interfere with multiple cellular functions (Mahul-Mellier et al., 2020) and/or the depletion of monomeric α-syn (Kanaan & Manfredsson, 2012) may also have detrimental consequences. The presence of pSyn inclusions is associated with all of these potential contributors to pathogenesis.

The pattern of pSyn accumulation in the PFF model following intracerebral injection appears dictated by the connectivity of the injection site. For example, following intrastriatal PFF injection aggregates are observed in neurons located in brain regions with abundant innervation to the striatum (Wall et al., 2013) including multiple cortical regions, SN, ventral pallidum and amygdala with pSyn eventually accumulating within striatal neurons surrounding the injection site (Duffy et al., 2018; Guo et al., 2020; Luk et al., 2012a, 2012b; Patterson et al., 2019; Paumier et al., 2015; Thomsen et al., 2021)

The experimental parameters that determine the magnitude of α-syn inclusion formation in the α-syn PFF model can be grouped into four main categories: species of fibrils used; quantity of fibrils injected; amount of endogenous α-syn; and post injection interval. Regarding fibril species, comparisons of seeding efficiencies of different fibril types have been

investigated in both rats and mice (Luk et al., 2016; Howe et al., 2021). Injections into WT mice of either mouse PFFs (mPFFs) or human PFFs (huPFFs) demonstrate that mPFFs possess a higher seeding efficiency, leading to more rapid peak aggregation accumulation than that observed following huPFF injection (Luk et al., 2016; Masuda-Suzukake et al., 2013; Sorrentino et al., 2017). Studies in rats comparing injections of mPFFs to rat PFFs (rPFFs) reveal superior seeding efficiency using mPFFs (Howe et al., 2021). Furthermore, seeding efficiency can also be influenced by the type of synuclein strain used (Peelaerts et al., 2015; Peng et al., 2018; Rey et al., 2016; Van der Perren et al., 2020). For the purposes of this paper we will focus on studies that utilize recombinant mouse, rat, and/or human fibrils.

Regarding levels of endogenous α-syn, it is well-established that the presence of endogenous α-syn is required for templating PFFs after internalization by neurons (Luk et al., 2012a, 2012b; Volpicelli-Daley et al., 2011). Further, the relative levels of intraneuronal α-syn also can influence the magnitude/rate of α-syn inclusion formation. Courte et al. (2020) demonstrated in vitro that neuronal cell types with lower α-syn expression levels (striatum) showed less efficient inclusion formation compared to neuronal cell types with higher levels of α-syn expression (cortex, hippocampus), despite an equivalent uptake of PFFs. In transgenic mice that exhibit α-syn overexpression (M20, M83, and A30P), α-syn inclusion formation is also more efficient compared with wild type (WT) mice (Gentzel et al., 2021; Sorrentino et al., 2017). Regarding the quantity of PFFs, it has been shown in rats that an increase in the quantity of mPFFs injected (8 μg versus 16 μg) results in increased α-syn inclusion formation as well as an increase in the subsequent magnitude of nigral degeneration (Patterson et al., 2019).

All the aforementioned factors contribute to the time course and magnitude of α-syn inclusion formation following injection of PFFs, resulting in a pathological cascade of events that can be understood in two main phases: Phase 1: The accumulation and peak formation of α-syn inclusions in nigrostriatal system and, Phase 2: Protracted dopaminergic neuron degeneration. In Phase 1, in WT mice or rats, intrastriatal or intranigral injection of mPFFs has an observed peak of α-syn aggregation in the SN at 1-3 months post injection (Duffy et al., 2018; Harms et al., 2017; Izco et al., 2021; Patterson et al., 2019) whereas injection of huPFFs into the striatum of WT mice delays this peak to approximately 4-6 months post injection (Earls et al., 2019; Sorrentino et al., 2017). The use of transgenic mice with higher levels of α-syn expression offsets the liability of using less efficient huPFFs, allowing for peak nigral α-syn inclusion formation at approximately 1-2 months post injection (Earls et al., 2020; Gentzel et al., 2021; Luk et al., 2012a). Importantly, α-syn inclusion formation in striatal neurons, despite direct injections to this location, is significantly delayed following injections of PFFs into WT rats and mice, likely due to lower levels of endogenous α-syn in this cell population (Courte et al., 2020). In WT mice and rats robust intraneuronal pSyn inclusion formation is not observed in the striatum until 5-6 months following intrastriatal mPFF injection, and pSyn observed in the striatum at earlier time points is generally localized to terminals of inclusion-bearing neurons of the SN and cortex (Duffy et al., 2018; Luk et al., 2012b; Patterson et al., 2019).

pSyn inclusions ultimately lead to neuronal dysfunction and degeneration (Osterberg et al., 2015; Volpicelli-Daley et al., 2011). In long term in vivo studies, data strongly suggest that the SN neurons that form pathological α-syn inclusions during Phase 1 are the same SN neurons that ultimately degenerate in Phase 2; for example, in WT mice and rats injected intrastriatally

with mPFFs, the peak pSyn inclusion formation observed in the SN at two months is followed by a progressive reduction of nigral α-syn inclusions, in parallel to the loss of tyrosine hydroxylase (TH) and dopamine transporter (DAT) expression over the course of a six-month post injection interval (Duffy et al., 2018; Luk et al., 2012a; Patterson et al., 2019; Paumier et al., 2015). However, overt neuronal death (in contrast to loss of dopaminergic phenotype) is not observed until 5-6 months (Duffy et al., 2018; Luk et al., 2012a; Patterson et al., 2019; Thomsen et al., 2021) corresponding to the time point when the lowest numbers of pSyn containing nigral neurons are observed. Collectively, this pattern of observations suggests that in WT rats and mice nigral neurons survive with α-syn inclusions for a 3-month interval (Phase 1) after which undefined pathological sequelae lead to neuronal dysfunction and ultimately degeneration (Phase 2). Furthermore, the pattern of striatal terminal degeneration in the PFF model shares similarities with that observed in early-stage PD subjects (Adams et al., 2005; Sossi et al., 2002, 2010). Specifically, PET imaging in mPFF injected rats demonstrates that DA synthesis and storage is relatively preserved in the ipsilateral striatum at early time points, whereas loss of dopamine transporter (DAT) occurs earlier and progresses over time (Sossi et al., 2022). Reduction in vesicular monoamine transporter type 2 (VMAT2) is also observed in the ipsilateral striatum prior to overt nigral degeneration (Thomsen et al., 2021). Overall, the pattern of nigrostriatal degeneration observed following intrastriatal PFF injection to rats and mice is one of early nigrostriatal terminal dysfunction and loss of dopaminergic phenotype (Phase 1) followed by overt nigral degeneration (Phase 2) with the magnitude of these events dependent upon the efficiency of initial α-syn inclusion formation.

It should be noted that in some studies in which mPFFs are unilaterally injected into the striatum of WT rats, degeneration of both the ipsilateral and contralateral SN can be observed (Duffy et al., 2018; Patterson et al., 2019; Paumier et al., 2015), although not consistently (Sossi et al., 2022; Thomsen et al., 2021). When contralateral SN degeneration does occur it is of a lesser magnitude than the ipsilateral SN degeneration, and is independent of pSyn inclusion formation which remains exclusively ipsilateral to the injected striatal hemisphere (Duffy et al., 2018; Patterson et al., 2019; Paumier et al., 2015). Loss of contralateral nigral TH immunoreactivity and ultimately neuronal loss proceeds on a delayed time course relative to the ipsilateral degeneration, suggesting that an imbalance in striatal DA may participate in this phenomenon (Patterson et al., 2019). We speculate that the contralateral intact striatum may increase DA synthesis/release in response to loss of striatal DA in the PFF injected hemisphere (Paumier et al., 2015), perhaps resulting in toxic levels of reactive oxygen species that impact the survival of contralateral nigral neurons. Ultimately, the specific PFF model parameters eliciting bilateral degeneration and the mechanism of this phenomenon remain to be determined.

An appreciation of the timing of the pathological events, and the factors that contribute to them, provides a framework to interpret results of studies examining neuroinflammation in the α-syn PFF model. Specifically, to characterize α-syn inclusion-associated immunogenicity it is necessary to know whether significant inclusion formation is present in the region examined at the time of analyses (Phase 1). Conversely, immunogenic signals that are associated with nigral degeneration can be appreciated by examining nigrostriatal tissue at later intervals (Phase 2), ideally with parallel analyses that confirm neurodegeneration. In the following

section we summarize the current understanding of neuroinflammatory observations in the α-syn PFF model, leveraging the distinct Phase 1 aggregation phase and Phase 2 degeneration phase to guide our interpretations.

α-syn Inclusion Associated Neuroinflammation

Considerations of Experimental Parameters

Intraparenchymal injections of α-syn PFFs result in a relatively synchronous wave of inclusion formation and accumulation in the SNpc (Phase 1) followed by dopaminergic phenotype loss and nigrostriatal degeneration (Phase 2). For the purposes of this review, we will define the Phase 1 aggregation as the interval during which abundant intraneuronal pSyn immunoreactive inclusions form and accumulate in the SNpc. Of note, studies have examined the neuroinflammatory response to PFF injections, in mice and rats, within 1-3 days following injection, prior to the formation of pSyn inclusions, and have observed increases in Iba1 and MHC-II immunoreactivity (Harms et al., 2017; Karampetsou et al., 2017). This preaggregation phase represents an acute inflammatory response to the PFFs themselves, and not an inflammatory response to inclusion-bearing neurons. In support of this, in vitro studies using microglial cell lines (Sarkar et al., 2020) or isolated primary microglia (Harm et al., 2013; Hoenen et al., 2016) demonstrate an inflammatory response to the addition of α-syn PFFs, in the absence of inclusion formation. Further, when examining neuroinflammatory responses in the PFF model careful comparisons, ideally to monomer-injected, or vehicle-injected controls, are necessary to differentiate between the effect of inclusions and the effect of surgical injection. Although inflammation associated with surgical injection is expected to be particularly

pronounced within days of PFF injection at the injection site (e.g. analysis of striatum following intrastriatal PFF injection), we have observed a significant neuroinflammatory response in the distal SN for up to 1 month following control intrastriatal injections in rats (Duffy et al., 2018). Further, analysis of neuroinflammation in the striatum in Phase 1, in both rats and mice, is less than ideal for identification of α-syn inclusion-associated neuroinflammation since pSyn accumulation at this phase is limited to the terminals of inclusion-bearing neurons in the SN and cortex (Duffy et al., 2018; Luk et al., 2012a; Patterson et al., 2019). Inflammasome activation has been identified in the striatum one month following intrastriatal injections of mPFFs in WT mice (Gordon et al., 2018), however the inclusion burden in the striatum was likely not significant at this time point. To identify the strongest evidence of neuroinflammation associated with the accumulation of pathological α-syn inclusions specifically, we highlight and summarize the results from studies examining neuroinflammation in the SN in which the experimental design facilitates interpretations in light of these potentially confounding factors.

Nigral α-syn Inclusions are Associated with Increased Innate Immune System Markers

Multiple studies have revealed that the presence of pathological α-syn inclusions in the SNpc is associated with changes in the innate immune system, as assessed by immunohistological markers of microglial and/or astrocytic activation and increased expression of inflammatory cytokines. Changes in microglial shape, size and an increase in number have been reported in the SN of α-syn overexpressing mice, WT mice, and WT rats, in studies in which mouse or human α-syn PFF experimental conditions generated pSyn pathology in the SNpc (Duffy et al., 2018; Earls et al., 2019, 2020; Garcia et al., 2022; Gentzel et al., 2021; Guo et

al.,2020; Izco et al., 2021). Similarly, studies in WT rats (Duffy et al., 2018; Harms et al., 2017; Miller et al., 2021b; Thomsen et al., 2021) and one study in WT mice (Earls et al., 2019a) reveal increased MHC-II expression in the SN in association with robust nigral pSyn pathology. Of note, when longer experimental intervals allow for analysis of the relationship between nigral pSyn burden and number of MHC-II presenting cells in the SNpc of rats, a strong positive correlation is observed (Duffy et al., 2018).

Although the main focus to date has been to examine microglial alterations in association with the formation of intraneuronal nigral inclusions, it is possible that microglia directly contribute to α-syn aggregation or spread. Microglia experimentally induced to form pSyn inclusions are capable of releasing α-syn-containing exosomes that, in turn, can induce the formation of pSyn inclusions in neurons both in vitro and in vivo (Guo et al., 2020). Further, microglial/macrophage-derived exosomes derived from PD patient CSF contain α-syn oligomers and can similarly induce aggregation of α-syn in neuronal cultures (Guo et al., 2020). Further study will be required to determine whether microglia significantly impact the accumulation of pathological α-syn in the brain.

Analysis of the astrocytic inflammatory response to nigral pSyn pathology resulting from mouse and human PFF injections to WT mice and WT rats provides evidence of increased astrocytic branching and glial fibrillary acidic protein (GFAP) immunoreactivity (Earls et al., 2019; Garcia et al., 2022; Izco et al., 2021; Miller et al., 2021a), with a positive association observed between pSyn and GFAP immunoreactivity (Miller et al., 2021b). Beyond immunohistochemical approaches, nigral pSyn pathology also is associated with increased mRNA expression of the proinflammatory cytokines TNF-α and IL-1ß (Izco et al., 2021) in studies

using mPFF injections into WT mice. Additionally, transcriptomic analysis in WT mice following a mPFF striatal injection also has shown an enrichment of innate inflammatory pathways in response to α-syn aggregation (e.g. cytokine regulation, production and secretion, regulation of ROS production, and regulation of phagocytosis and endocytosis) (Garcia et al., 2022).

Nigral α-syn Inclusions are Associated with Peripheral and Adaptive Cell Infiltration

Although fewer studies have examined peripheral and adaptive immune involvement in the α-syn PFF model, three reports provide evidence of infiltration of lymphocytes and macrophages in WT mice and rats, and natural killer (NK) cells in α-syn overexpressing mice during the pathological α-syn aggregation phase. Peripheral cell infiltration, comprised of increased percentages of B lymphocytes (CD19+), T-helper lymphocytes (CD4+), T-cytotoxic lymphocytes (CD8+), activated myeloid cells (CD11b+/CD45 high) and natural killer cells (TCRß -/MK1.1+), is observed in the CNS parenchyma during the pSyn aggregation phase following intrastriatal hPFF injections to WT mice, as well as a decrease in the percentage of resting myeloid cells (CD11b+/CD45 low) (Earls et al., 2019). The infiltration of monocytes/macrophages (CD11b+/CD45 high and CD163+) and T-helper lymphocytes (CD4+), within pSyn inclusion-bearing nigral tissue specifically, has also been observed following intranigral injection of mPFFs into rats (Harms et al., 2017). In α-syn overexpressing mice given an intrastriatal injection of hPFFs , pSyn accumulation in the SNpc was accompanied by a 5-fold increase in infiltration of natural killer (NK) cells (Earls et al., 2020), a type of cytotoxic lymphocyte that can inhibit proinflammatory microglia (Earls & Lee, 2020). Additionally, transcriptomic analysis in the mouse striatum following a mPFF striatal injection also has shown

an enrichment of peripheral inflammatory response in response to α-syn aggregation (e.g. *Ptprc* (CD45) and CD4 antigen) (Garcia et al., 2022). Collectively, these studies implicate multiple peripheral and adaptive immune cell types associated with the accumulation of pathological α-syn aggregates following PFF injection.

Conclusions: α-syn Inclusion Associated Neuroinflammation

Numerous studies consistently demonstrate that pathological α-syn aggregation in the SN is associated with activation of the innate immune system, supported by findings of multiple markers of microgliosis and astrogliosis. Although the present summary focuses on PFF-induced pSyn accumulation in the SN, support for the relationship between α-syn aggregation, microgliosis and astrogliosis also comes from studies in which mouse and human PFFs are used to induce pSyn aggregates in other brain regions, including the olfactory bulb and hippocampus (Luk, et al., 2012; Rey et al., 2016; Sacino et al., 2014; Uemura et al., 2021). Strong evidence also demonstrates that pSyn accumulation is associated with MHC-II immunoreactivity, which is proportionate to inclusion load. Our evolving understanding of the neuroinflammatory response to α-syn aggregation in the SN also suggests a role for the involvement of peripheral and adaptive immune cell infiltration. Importantly, the defined stages of the α-syn PFF model, with prolonged aggregation preceding degeneration, provides evidence that intraneuronal, Lewy-body like aggregates are immunogenic, and that α-syn inclusion associated neuroinflammation may contribute to the degenerative process.

Nigral Degeneration-Associated Neuroinflammation

Considerations of Experimental Parameters

Following the α-syn inclusion formation and accumulation observed in the SNpc in Phase 1, protracted dopaminergic phenotype loss and nigrostriatal degeneration occurs during Phase 2 over the course of several months. For the purposes of this review, we will define the Phase 2 as the interval during which pSyn immunoreactive inclusions are decreasing as nigral dopamine neurons degenerate, confirmed histologically using stereologic assessments of nigral TH immunoreactive neurons, ideally including additional stereological assessments to confirm overt neurodegeneration (Duffy et al., 2018; Ma et al., 2021; Patterson et al., 2019; Paumier et al., 2015; Thomsen et al., 2021). In some cases, neurodegeneration has been presumed in the studies detailed below based on predictions informed by the specific experimental parameters used and the timing of the assessments.

Nigral Degeneration Increases Innate Immune System Markers

Intrastriatal mPFF injections into WT mice results in increased Iba1 immunoreactivity and Iba1 immunoreactive microglia at a time when nigral dopamine neurons have undergone significant degeneration (Yun et al., 2018). Under identical nigral degeneration conditions, mRNA expression levels of TNF-α, interleukin-1 alpha (IL-1 α) and complement component 1 Q subcomponent alpha (C1qa) are elevated in the SN compared with controls (Yun et al., 2018). Three studies to date, conducted using either intrastriatal or intranigral injections of mPFFs into WT rats, have examined the longitudinal microglial response in the SN during both the aggregation and dopamine neuron degeneration phases of the model (Duffy et al., 2018; Harms

et al., 2017; Thomsen et al., 2021). Results of all three are in agreement: that inclusion-triggered nigral degeneration observed 5-6 months following PFF injection is associated with increased MHC-II immunoreactivity compared with control rats (Duffy et al., 2018; Harms et al., 2017; Thomsen et al., 2021), with some studies indicating that the magnitude of MHC-II immunoreactivity observed during the degenerative phase is considerably less than that observed previously during the aggregation phase (Duffy et al., 2018; Thomsen et al., 2021). Markers indicative of nucleotide-binding oligomerization domain, leucine-rich repeat-containing protein 3 (NLRP3) inflammasome activation, associated with a proinflammatory microglial response, are significantly increased following nigral degeneration (Gordon et al., 2018). Specifically, protein levels of NLRP3 and apoptosis-associated speck-like protein containing a CARD (ASC) are significantly increased in the SN 8 months after intrastriatal mPFF injections into mice (Gordon et al., 2018).

The response of astrocytes also has been investigated during the nigral degeneration phase of the PFF model, specifically examining components of the complement system, associated with both A1 proinflammatory astrocytes and activated microglia. Components of the complement system label neurons, tagging them for phagocytosis (Liddelow et al., 2017; Zamanian et al., 2012). In WT mice injected with intrastriatal mPFF, GFAP protein and complement component 3d (C3d) are increased in the SN in association with significant nigral degeneration, as is the percentage of GFAP+ astrocytes expressing C3d and many elevated transcripts associated with the A1 astrocytic phenotype (Yun et al., 2018). Further evidence of a role for the complement system during the degenerative phase of the SN following intrastriatal

mPFF injections in mice comes from a comprehensive analysis of the proteome (Ma et al., 2021).

Nigral Degeneration is Associated with Peripheral Cell Infiltration

To our knowledge, only one study to date reports on peripheral immune cell markers in the SN during the degenerative phase induced by PFF injections. Six months following mPFF intranigral injections in rats, the infiltration of monocytes/macrophages (CD163+) is observed, indicating the earlier increase first observed during the aggregation phase was sustained long term (Harms et al., 2017).

Conclusions: Nigral Degeneration Associated Neuroinflammation

Studies demonstrate that the nigral degenerative phase induced in the α-syn PFF model is associated with activation of the innate immune system supported by findings of multiple markers of microgliosis and astrogliosis. However, studies in which a single time point during the degenerative phase is examined cannot rule out if neuroinflammation preceded, or was triggered by, nigral degeneration. Interestingly, the few reports that compare the neuroinflammatory response in the SN across both the aggregation and degeneration phases suggest that microglial activation, as assessed by MHC-II immunoreactivity, is greater in magnitude in the aggregation phase preceding degeneration. Relatively little is known about involvement of the peripheral and adaptive immune system during the degenerative phase of the α-syn PFF model.

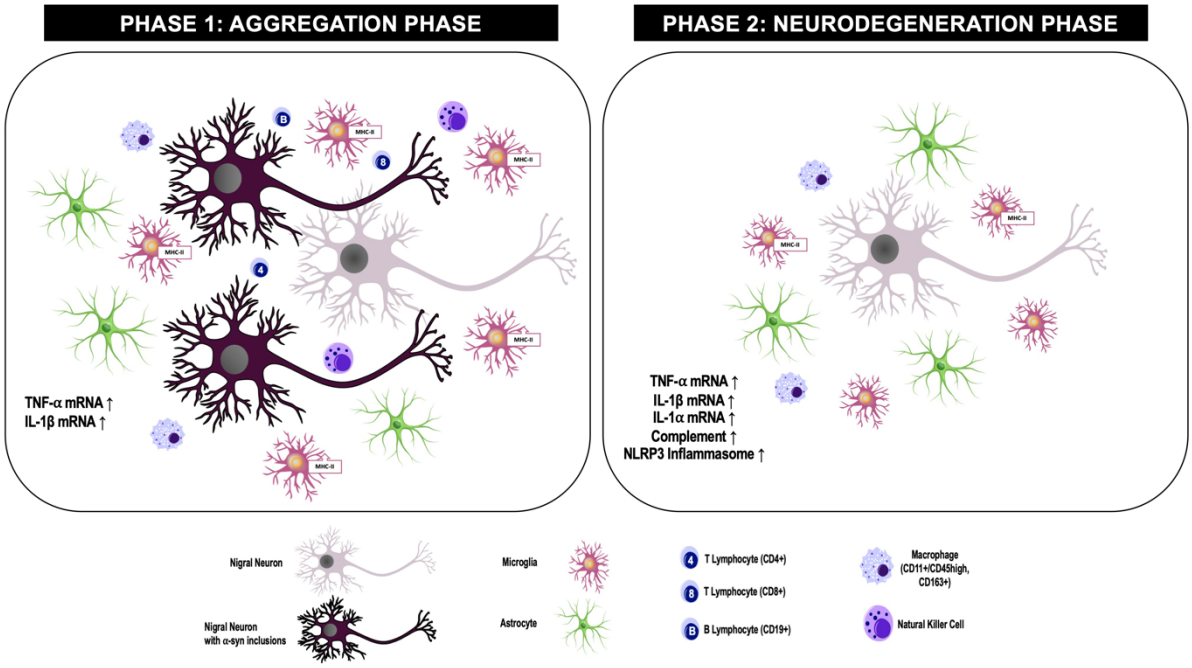

Figure 2.1. Inflammation markers identified in the substantia nigra during the aggregation or degeneration phases of the alpha-synuclein (α-syn) preformed fibril (PFF) model.

Aggregation Phase: *α-syn Inclusions*: pSyn accumulates and the number of pSyn immunoreactive neurons peaks. *DA Neurons:* No significant loss of DA neurons is observed. *Microglia:* Microglial immunoreactivity (Iba1), microglial soma size and MHC-II immunoreactivity is significantly elevated relative to control animals without pSyn inclusions. A positive association between MHC-II+ microglia and number of pSyn+ neurons is observed. *Astrocytes:* Astrocytic immunoreactivity (GFAP), length and branching of processes increases relative to control animals without pSyn inclusions. A positive association between GFAP immunoreactivity and number of pSyn+ neurons is observed. *Natural Killer (NK) Cells:* Infiltrating NK cells are increased relative to control animals without pSyn inclusions. *Macrophages:* Infiltrating macrophages (CD11b+/CD45+, CD163+) are increased relative to

Figure 2.1. (Cont'd)

control animals without pSyn inclusions. *T Lymphocytes:* CD4+ and CD8+ T Lymphocytes are increased relative to control animals without pSyn inclusions. *B lymphocytes:* B Lymphocytes are increased relative to control animals without pSyn inclusions. *Cytokines:* TNF-α and IL-1ß mRNA is increased relative to control animals without pSyn inclusions. *NLRP3 Inflammasome and Complement:* Unknown.

Degeneration Phase: *α-syn Inclusions:* Neurons in which pSyn inclusion previously formed have degenerated, pSyn inclusions are nearly absent. *Nigral Dopamine Neurons:* Significant loss of DA neurons has occurred. *Microglia:* MHC-II+ positive microglia are increased relative to controls without degeneration but decreased relative to the number of MHC-II+ microglia observed during the aggregation phase. *NLRP3 Inflammasome:* NLRP3 inflammasome activation (NLRP3, ASC protein) is increased relative to control animals without degeneration. *Astrocytes:* Astrocytic immunoreactivity (GFAP) is increased relative to control animals without degeneration. *Complement:* Complement component 3d (C3d), GFAP+/C3d+ astrocytes and transcripts associated with the A1 astrocytic phenotype are increased relative to control animals without degeneration. *Natural Killer Cells:* Not observed in the degeneration phase. *Macrophages:* Infiltrating macrophages (CD163+) are increased relative to control animals without degeneration. *Cytokines:* TNF-α, IL-1ß and IL-1 α mRNA is increased relative to control animals without degeneration. *T and B Lymphocytes:* Unknown.

pSyn = α-syn phosphorylated at serine 129; DA = dopamine; Iba1 = ionized calcium binding adaptor molecule 1; MHC-II = major histocompatibility complex-II; GFAP = glial fibrillary acidic protein; NLRP3 = nucleotide-binding oligomerization domain, leucine-rich repeat-containing

Figure 2.1. (Cont'd)

protein 3; ASC = apoptosis-associated speck-like protein containing a CARD; TNF-α = tumor necrosis factor-alpha; IL-1ß = interleukin 1-beta; IL-1 α = interleukin 1-alpha

Overall Conclusions and Future Directions

The α-syn PFF model provides an experimental platform that allows for investigation of the neuroinflammatory consequences of the progression of pathological α-syn to neurodegeneration (*Figure 1*). The concordance between the neuroinflammatory events documented in the parkinsonian brain and those observed in the α-syn PFF model (microgliosis, association of MHC-II immunoreactivity with α-syn inclusions, cytokine expression, astrogliosis, and T-cell infiltration) suggests that this model platform can prove useful for design of anti-inflammatory neurotherapeutics. However, there is much that we still need to learn about neuroinflammation in this model and whether it fully recapitulates neuroinflammation in PD. For example, proinflammatory cytokines and chemokines are elevated in the blood (IL-6, TNF-α, IL-1ß, IL-2, IL-10, C reactive protein, RANTES) and CSF (IL-1ß, IL-6, TGF-ß1) (Chen et al., 2018; Qin et al., 2016) of PD subjects. It is presently unknown whether a similar elevation is observed in the α-syn PFF model. The observation that any of these factors are increased during the α-syn aggregation phase may support their use as a biomarker in prodromal PD. Further, whereas CD68, MHC-II, and C3d expression has been observed in the parkinsonian SN (Croisier et al., 2005; Doorn et al., 2014; Garcia et al., 2022; Liddelow et al., 2017; Rostami et al., 2020), our overall understanding of the microglial and astroglial phenotype in response to α-syn inclusions and/or degeneration is in its infancy. The α-syn PFF model could be harnessed to fully

characterize the neuroinflammatory response of microglia and astrocytes and, if ultimately validated in PD brains, this approach may yield insight into the design of novel targeted therapeutics.

Regarding the role of the adaptive and peripheral immune system, in general, relatively little is known about the time course and pattern of its involvement across the progressive stages of pathology in PD. Although infiltration by T and B lymphocytes, macrophages and natural killer cells has been identified during the α-syn aggregation phase of the PFF model (Earls et al., 2019, 2020; Harms et al., 2017), it remains unclear whether all these cell types persist and if the magnitude of their involvement is altered during the degenerative phase. Additional investigation of the temporal pattern of adaptive and peripheral immune cell infiltration using longitudinal analysis in the PFF model is warranted. Another area worthy of investigation is whether cytotoxic T cells isolated from brains of PFF model animals are able to recognize different epitopes of pathological α-syn when presented by MHCII, as has been shown with cytotoxic T cells isolated from PD brains (Sulzer et al., 2017).

Neuroinflammation has become a well-accepted pathological hallmark of PD. However, whether inflammation triggered by either α-syn aggregation and/or degeneration contributes to the progression of the disease remains one of the most critical questions facing the field. By leveraging the relatively synchronous wave of α-syn aggregation followed by the relatively synchronous wave of degeneration of the SNpc of the α-syn PFF model, numerous studies have identified a significant and early neuroinflammatory response of the innate, adaptive, and peripheral immune systems that is associated with the formation of pathological α-syn aggregates in the SN. These findings put the neuroinflammation suspect at the scene of the

crime, prior to degeneration. Further investigation will be required ultimately to reveal how, and if, this early inflammation participates in PD progression.

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Chapter 3: Partial microglial depletion does not impact alpha-synuclein aggregation triggered degeneration and may induce a heightened proinflammatory state in remaining microglia

Abstract

Parkinson's disease (PD) is a neurodegenerative disorder that is characterized by the presence of proteinaceous alpha-synuclein (α-syn) inclusions (Lewy bodies), the progressive loss of the nigrostriatal dopamine (DA) neurons and markers of neuroinflammation. These pathological features can be recapitulated *in vivo* using the α-syn preformed fibril (PFF) model of synucleinopathy. We have previously described the time course of microglial major-histocompatibility complex-II (MHC-II) expression and alterations in microglia morphology in the PFF model in rats. Specifically, the peaks of α-syn inclusion formation, MHC-II expression, and reactive morphology in the substantia nigra pars compacta (SNpc) all occur two months post PFF injection, months before neurodegeneration occurs. These results suggest that activated microglia may contribute neurodegeneration and could represent a potential target for novel therapeutics. The goal of this study was to determine whether partial microglial depletion could impact the magnitude of α-syn aggregation, nigrostriatal degeneration, or related microglial activation during the α-syn PFF model. Previous studies demonstrated that Pexidartinib (PLX3397), a colony stimulating factor-1 receptor (CSF1R) inhibitor, significantly decreases microglia levels. In this study, male Fischer 344 rats were injected intrastriatally with either α-syn PFFs or saline as a control condition. Rats were administered PLX3397B (600mg/kg) or no drug in their chow for a period of either two months or six months. PLX3397B was associated with a partial (45-53%) depletion of ionized calcium-binding adapter molecule 1 immunoreactive (Iba-1ir) microglia within the SNpc. This partial microglial depletion did not alter the number of α-syn inclusion-containing SNpc neurons or the increased soma size and expression of MHC-II on microglia within the α-syn inclusion bearing SNpc. Despite significant

microglial depletion, the number of localized, inclusion associated MHC-II expressing microglia were completely maintained. Further, partial microglia depletion did not impact degeneration of tyrosine hydroxylase immunoreactive dopaminergic neurons in the substantia nigra. Paradoxically, long term partial microglial depletion resulted in increased soma size of remaining microglia in both control and PFF rats, as well as widespread MHC-IIir expression in extranigral regions. Collectively, our results suggest that partial microglial depletion is not a promising anti-inflammatory therapeutic strategy for PD and that this approach may induce a heightened proinflammatory state in remaining microglia.

Introduction

Parkinson's Disease, the second most common neurodegenerative disease, affects around 1 million people in the USA with 60,000 newly diagnosed people each year (Marras et al., 2018). Pathologically, PD is characterized by the presence of proteinaceous alpha-synuclein (α-syn) inclusions (Lewy bodies) and the progressive loss of the nigrostriatal dopamine (DA) neurons (Kordower et al., 2013). While the exact cause of PD is still unknown, mounting evidence has suggested that neuroinflammation, mediated by microglia, may play a significant role in PD progression and neuropathology.

Microglia, macrophages found in the central nervous system (CNS), have many roles in helping maintain healthy homeostasis in the brain, including synaptic pruning, neurogenesis, and neuronal surveillance (Menassa & Gomez-Nicola, 2018; Nimmerjahn et al., 2005; Tan et al., 2020). However, microglia are main players in the immune response to an insult and allow for the bridging of the innate and adaptive immune system (Cartier et al., 2014; Schetters et al., 2018). Postmortem PD brain analysis has shown levels of inflammatory markers including increases in cells immunoreactive for ionized calcium binding adaptor molecule 1 (Iba1), human leukocyte antigen (HLA-DR), and phagocytic marker CD68 in the vicinity of Lewy pathology, specifically the substantia nigra (Croisier et al., 2005; Doorn et al., 2014; Imamura et al., 2003; Mcgeer et al., 1988; McGeer et al., 1988). Patients with PD have elevated levels of proinflammatory cytokines (i.e., interleukin 1-beta, interleukin-6, interferon gamma, and tumor necrosis factor-alpha) in their cerebral spinal fluid (CSF) and plasma, all produced by microglia and immune cells (Mogi et al., 1996; Mogi, Harada, Kondo, et al., 1994; Mogi, Harada, Riederer, et al., 1994; Nagatsu' et al., 2000).

These pathological features, α-syn inclusions, loss of dopaminergic neurons and neuroinflammation, can be recapitulated *in vivo* using the α-syn preformed fibril (PFF) model of synucleinopathy (Luk, Kehm, Carroll, et al., 2012; Luk, Kehm, Zhang, et al., 2012; Patterson et al., 2019; Thakur et al., 2017). We have previously described the time course of the accumulation of α-syn inclusions, nigrostriatal degeneration, and microglial response in this model in rats (Duffy et al., 2018; Patterson et al., 2019). Specifically, the peak of α-syn inclusion formation, number of major-histocompatibility complex-II (MHC-II) immunoreactive (MHC-IIir) microglia and increased microglial soma size in the substantia nigra pars compacta (SNpc) occurs during the aggregation phase, two months post PFF injection, months before the neurodegeneration phase occurring at 5-6 months (Duffy et al., 2018). Of importance, a localized subpopulation of MHC-IIir microglia is observed immediately adjacent to nigral α-syn inclusions, with the size of this reactive microglial subpopulation dependent on nigral inclusion load. These results, along with results from other laboratories (Earls et al., 2019; Harms et al., 2013, 2017; Karampetsou et al., 2017) suggest that α-syn inclusions are immunogenic, provoking a microglial proinflammatory response that may contribute to neurodegeneration. Thus, therapeutic strategies that target microglia may have potential to slow disease progression.

Pexidartinib (PLX3397B; Plexxikon inc.), a selective tyrosine kinase inhibitor, targets the macrophage (i.e. microglia) colony stimulating factor 1 receptor (CSF1R). The CSF1R is required for the activation, proliferation, and survival of microglia and, when inhibited, leads to microglial death resulting in microglial depletion within the brain parenchyma (Elmore et al., 2014). Microglia depletion has been analyzed in mouse models of disease to understand the

role microglia may play in disease progression (Barnett et al., 2021; Bennett et al., 2018; Fu et al., 2020). However, microglia are required to maintain healthy brain homeostasis and as such, complete microglia depletion may not be a viable therapeutic strategy. In the present study we examined the effect of partial microglia depletion on α-syn aggregation and neurodegeneration within the rat PFF model. We demonstrate that partial microglia depletion (36-46%) did not affect α-syn inclusion load in the SNpc 2-months following α-syn PFF injection (aggregation phase). This partial depletion further did not impact the inclusion-associated microglial responses; increase in microglial soma and increase in MHCIIir microglia subpopulation, nor did it impact the THir loss. However, at 6-months post α-syn PFF injection (neurodegeneration phase) partial depletion did not affect α-syn aggregation, MHCIIir cells within the SNpc, or the THir cell loss seen, long term partial depletion does lead to a widespread MHCIIir microglia expression that is not seen in no Pexidartinib treated animals. While research has shown that partial microglia depletion does not impact animal behavior, these results suggest that long term partial depletion may lead to imbalance in immune gene expression and further analysis will be needed to better understand whether microglia depletion can be a possible therapeutic target.

Methods

Experimental Overview

This experiment was designed to investigate whether microglia depletion impacts accumulation of phosphorylated alpha-synuclein (pSyn) in the substantia nigra (SN), the microglial response associated with pSyn accumulation, or nigral degeneration. Rats were unilaterally injected with either alpha-synuclein preformed fibrils (α-syn PFFs) or and equal volume of PBS and were orally administered the colony-stimulating factor 1 receptor (CSF1R) inhibitor PLX3397B or control chow for either 60- or 180-days. At the conclusion of the experiment rats were euthanized and brain tissue analyzed. Figure 3.1a illustrates the experimental design.

Animals

Three-month old, male Fischer 344 rats (Charles River) were housed, 2-3 per cage, at the Grand Rapids Research Center which is fully approved through the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Rats were in a room with a 12-hour light/dark cycle and provided food and water *ad libitum.* All procedures were done in accordance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University.

α-syn PFF Preparation and Fibril Measurements

α-syn PFFs were generated from wild-type-full length, recombinant mouse α-syn monomers as previously described (Luk, Kehm, Zhang, et al., 2012; Polinski et al., 2018;

Volpicelli-Daley et al., 2011, 2014). Quality control was completed on full length fibrils to ensure fibril formation (transmission electron microscopy), amyloid structure (thioflavin T assay), pelletability as compared to monomers (sedimentation assay), and low endotoxin contamination (*Limulus* amebocyte lysate assay; <0.5 endotoxin units/mg of total protein). On surgery day, PFF were thawed to room temperature and diluted to 4 ug/ul in sterile Dulbecco's phosphate buffered saline and sonicated with an ultrasonic homogenizer (300 VT; Biologics, Inc.) for 60- 1s pulses with the pulser set at 20% and power output at 30%. PFFs were prepared on Formvar/carbon-coated copper grids (EMSDIASUM, FCF300-Cu). Fibrils were then imaged with a JEOL JEM-1400+ transmission electron microscope (Following procedure in Patterson et al 2019). The length of ~650 fibrils was determined using ImageJ 1.53K (Wayne Rasband and contributors, National Institutes of Health, USA). **(**Figure 3.1B,C). The mean length for the 2-month surgical cohort was 35.9 ± 0.06 nm and for the 6-month surgical cohort was 34 ± 0.57 nm. Fibril length < 50 nm is required for successful seeding of endogenous α-syn inclusions (Tarutani et al., 2016).

Stereotaxic Injections

Unilateral intrastriatal α-syn PFF injections were conducted as previously described (Patterson et al., 2019). Rats were anesthetized with isoflurane (5% induction and 1.5% maintenance) and received unilateral intrastriatal injections to the left hemisphere (2 x 2 µl, AP +1.6, ML +2.0, DV -4.0; AP +0.1, ML +4.2, DV -5.0, AP and ML coordinates relative to Bregma, DV coordinates relative to dura). PFFs (4 ug/µl; 16 ug total) or an equal volume of PBS were injected at a rate of 0.5 µl/min with a pulled glass capillary tube attached to a 10 µl Hamilton

syringe. After each injection the needle was left in place for 1 minute, retracted 0.5 mm and left for 2 minutes to avoid PFF displacement, and then fully retracted. All animals received analgesic (1.2 mg/kg of sustained release buprenorphine) after surgery and were monitored until euthanasia.

Pexidartinib Dosing

Animals were fed Pexidartinib chow (PLX3397B, 600mg/kg; Plexxikon Inc.; Research Diets Inc.) or control chow *ad libitum* for either 60 or 180 days starting on the day of PFF injections. Animal weights and collective cage food intake was tracked weekly (Figs. A3.8A-B, A3.9A-B).

Euthanasia

Animals were euthanized at 60 days (peak inclusion formation in the SN) or 180 days (peak nigral degeneration) post-surgery, pathological intervals that have been previously identified in this model (Duffy et al., 2018; Patterson et al., 2019; Paumier et al., 2015). Rats were given a 30 mg/kg pentobarbital injection (i.p.) (Euthanasia-III Solution, MED-PHARMEX, Inc.) and perfused intracardially with heparinized 0.9% saline. Livers were removed and weighed (Figs. A3.8C, A3.9C). Brains were removed and post-fixed in 4% paraformaldehyde (PFA) for one week and then transferred to 30% sucrose in 0.1M phosphate buffer until sunk. Brains were frozen with dry ice and cut at 40µm thick on a sliding microtome and stored in cryoprotectant (30% sucrose, 30% ethylene glycol, in 0.1M Phosphate Buffer (PB), pH 7.3) at -20°C.

Immunohistochemistry

Free floating sections were washed 4 x 5minutes in 0.1M tris buffered saline (TBS) containing 0.5% Triton-X100 (TBS-Tx), quenched in 3% H_2O_2 for 1 hour, blocked in 10% normal goat serum (NGS) in TBX-Tx, and incubated overnight in primary antibody in 1% NGS/TBS-Tx at 4°C on a shaker. Primary antibodies used included: mouse anti-α-syn phosphorylated at serine 129 (pSyn) (1:10,000; Abcam, AB184674), mouse anti-tyrosine hydroxylase (TH) (1:4000; Millipore, MAB318), rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1) (1:1,000; Wako, 019-09741), mouse anti-major histocompatibility complex-II (MHC Class II RT1B clone OX-6) (1:2,000; BioRad, MCA46G). Sections were washed in TBS-Tx and then incubated for 2-hours at room temperature with biotinylated secondary antibodies in 1% NGS/TBS-Tx. Secondary antibodies used included: goat anti-mouse IgG (1:500; Millipore, AP124B), goat anti-rabbit IgG (1:500, Millipore, AP132B), and horse anti-mouse IgG rat preabsorbed (for TH; 1:500; Vector Laboratories, BA-2001). Sections were washed 4 x 5 minutes in TBS-Tx and incubated in standard avidin-biotin complex detection kit (ABC, Vector Laboratories, PK-6100). Visualization for pSyn was done using 2.5 mg/ml nickel ammonium sulfate hexahydrate (Fisher, N48–500), 0.5 mg/ml diaminobenzidine (Sigma-Aldrich, D5637), and 0.03% H_2O_2 in TBS-Tx. TH was visualized with 0.5 mg/ml diaminobenzidine (Sigma-Aldrich, D5637), and 0.03% H_2O_2 in TBS-Tx. MHC-II was visualized using Vector ImmPACT DAB (brown) Peroxidase kit (Vector Laboratories; SK-4105). Iba1 was visualized with ImmPACT VIP (purple) Peroxidase Kit (Vector Laboratories; SK-4605). Sections were mounted, allowed to dry, rehydrated, then dehydrated in ascending ethanol washes and cleared with xylene before cover slipping using Epredia Cytoseal-60

(Thermo-Fischer, 22-050-262). pSyn sections were counterstained with cresyl violet before dehydration.

Immunofluorescence

Free floating sections were washed 5 x 5 minutes in TBS-Tx, blocked in 10% NGS in TBX-Tx, then incubated overnight in primary antibodies in 1% NGS/TBS-Tx at 4°C on a shaker. Primary antibodies used included: mouse anti-pSyn (1:10000; Abcam, AB184674) and rabbit anti-Iba1 (1:1,000; Wako, 019-09741). Sections were washed in TBS-Tx and then incubated for 2-hours, in the dark, at room temperature, with fluorescent conjugated secondary antibodies in 1%NGS/TBS-Tx. Secondary antibodies used included: Alexa Fluor 568 goat anti-mouse IgG (1:500, Invitrogen, A-11004), and Alexa Fluor 647 goat anti rabbit IgG (1:500, Invitrogen, A32733). Sections were then rinsed 5 x 5 minutes in TBS-Tx, incubated 1 x 5 minutes in 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) made in TBS-Tx (1:10,000, Invitrogen, D1306) and placed back in TBS-Tx for mounting. Sections were mounted and cover-slipped with VECTASHIELD Vibrance antifade mounting medium (Vector Laboratories, H-1700) and kept in the dark until imaging utilizing the Zeiss Axioscan.Z1 scanning microscope.

Total Enumeration for pSyn and MHC-II

Due to heterogeneity in the distribution of both pSyn and MHC-II immunoreactive profiles within the SN, total enumeration rather than stereological counting frames was used for quantification. The investigator was blinded to treatment groups. Total enumeration of pSyn immunoreactive (pSyn-ir) neurons and MHC-II-ir cells was conducted utilizing Microbrightfield

Stereoinvestigator (MBF Bioscience). Sections containing the SN pars compacta (SNpc, 1:6 series) were used. Contours were drawn around the SNpc at 4X, a 20x magnification was then used for identification and counting. Counts represent the raw total number multiplied by six. Data are reported as total estimates of pSyn-ir neurons or MHC-II-ir cells in each hemisphere.

Stereological Assessment of Nigral TH Immunoreactive Neurons

The number of TH immunoreactive neurons in the ipsilateral and contralateral SNpc was estimated using unbiased stereology with the optical fractionator principle. The investigator was blinded to treatment groups. Using a Nikon Eclipse 80i microscope, Retiga 4000R camera (QImaging) and Microbrightfield StereoInvestigator software (Microbrightfield Bioscience, Williston, VT), THir neuron quantification was completed by drawing a contour around the SNpc borders using the 4X objective on every sixth and counting neurons according to stereological principles at 60X magnification. Briefly, counting frames (50 µm x 50 µm) were systematically and randomly distributed over a grid (183 µm x 122 µm) overlaid on the SNpc. A coefficient of error < 0.10 was accepted. Data are reported as total estimate of THir neurons in each hemisphere.

Microglia Soma Size and Number

Nigral sections were fluorescently labeled for pSyn and Iba1. The investigator was blinded to treatment groups. Utilizing the Zeiss Axioscan.Z1 scanning microscope, Z-Stacks images at 20X were obtained and three consecutive nigral sections representing the sections with the highest number of pSyn-ir neurons were analyzed with Nikon Elements AR (Version

4.50.00, Melville, NY). Iba1-ir soma were outlined, excluding processes. Data for soma size is reported as the number of pixels per outlined microglia soma and all microglia per treatment group was grouped into 2-pixel bins. Number of microglia was determined based on the number of microglia somas that were outlined. For striatal pSyn-ir analysis HALO (Indica Labs), was used for analyzing the IHC signal present in regions of interest using the HALO module: "Area quantification v1.0 for brightfield."

Statistical Analysis

All statistical tests of the results were completed using GraphPad Prism software (version 9, GraphPad, La Jolla, CA). Outliers were assessed with the absolute deviation from the median method (Leys et al., 2013) utilizing the very conservative difference of 2.5X median absolute deviation as the exclusion criteria. Statistical significance was set to $\alpha \leq 0.05$. Comparisons were made across all groups using two-way ANOVA with a *post-hoc* Tukey test, except for: pSyn load was analyzed using a Student's T-test and THir neurons in the SNpc comparisons were made within each brain hemisphere: 2-way ANOVA with *post-hoc* Tukey test was done for each brain hemisphere separately. Comparisons of food intake was measured with repeated measures two-way ANOVA.

Results

Impact of microglial depletion during the nigral aggregation phase of the α-syn PFF model

Pexidartinib (PLX3397B) partially depletes microglia in both α-syn PFF and PBS injected rats after two months of treatment

Treatment with Pexidartinib (PLX3397B; 600mg/kg) led to the partial depletion of microglia within the SNpc in both PBS and α-syn PFF injected rats after 2-months of treatment. PBS injected animals displayed a 45% decrease ($p=0.001$) and PFF animals showed a 36.6% decrease ($p<0.001$) in number of Iba-1 immunoreactive microglia as compared to the control fed rats (Figure 3.2A-E). Significantly more microglia were observed PFF rats compared to PBS rats in both chow treatment groups ($p<0.05$). Specifically, PFF injected rats without PLX3397B treatment showed a mean of 437.3 ± 32.17 microglia within the SNpc as compared to the PBS injected average of 368.0 ± 10.25. PLX3397B treatment showed significantly less microglia within the SNpc after PBS injection (202 ± 16.26) as compared to α-syn PFF injected average of 277.1 ± 28.18 microglia ($p=0.030$). These data suggest that PLX3397B treatment for 2 months results in marked, but incomplete, microglial depletion; however, inclusion-associated increases in microglia persist.

Partial microglial depletion does not impact accumulation of pSyn aggregates in nigral neurons or early loss of TH phenotype

Our previous work has demonstrated that unilateral intrastriatal injection of mouse α-syn PFFs results in peak intraneuronal phosphorylated α-syn (pSyn) aggregation in the SNpc 2 months post intrastriatal injection. In the present study, PFF injection in rats without PLX3397B

treatment resulted in 4826 ± 229.3 pSyn containing neurons in the SNpc whereas PFF injection with PLX3397B treatment resulted in 4760 ± 148.8. No significant difference in the number of pSyn immunoreactive SNpc neurons was observed due to PLX3397B treatment ($p>0.05$, Figure 3.3A, B).

In our previous studies utilizing identical PFF surgical parameters in rats we observe a gradual loss of ipsilateral SNpc TH phenotype as early as 1-2 months after surgery, in the absence of overt degeneration (Miller et al., 2021; Patterson et al., 2019). In the present study, 2 months following surgery we observed a 33% reduction ($p= 0.0058$) in THir neurons in the ipsilateral SNpc of α-syn PFF injected rats (8972 ± 1062) as compared to the control injected animals (13585 ± 549.4), both with and without PLX3397B treatment (Fig. 3.1C, D). The PLX339B treated animals showed a 24% reduction ($p=0.0330$) in the ipsilateral SNpc of α-syn PFF injected rats (10587 ± 362.3) as compared to the ipsilateral SNpc of control injected animals (14089 ± 1102). However, no significant differences were observed in THir neurons between the α-syn PFF injected rats with or without PLX3397B. These results suggest that PLX3397B does not impact the modest loss of TH phenotype of SNpc neurons during the aggregation phase.

Partial microglial depletion does not impact reactive microglia morphology or MHC-II expression associated with α-syn inclusions

Our previous work has shown that pSyn inclusions in the SNpc are associated with an increase in microglial soma size and a localized expression of MHC-II that correlates with α-syn inclusion load (Duffy et al 2018). In the present study, we observed a similar significant increase

in MHC-IIir microglia within the SNpc after α-syn PFF compared to PBS control rats in both PLX3397B treated ($p<0.0001$) and untreated ($p<0.0001$) rats (Figure 3.4A-B). Specifically, PFF rats not receiving PLX3397B possessed 957.0 ± 49.20 MHC-IIir microglia compared to 76.29 ± 15.75 in PBS injected rats not receiving PLX3397B. PFF PLX3397B rats possessed 1094 ± 110.9 MHC-IIir microglia compared to 169.7 ± 29.50 in PBS PLX3397B rats. No significant differences were observed in the number of MHC-IIir microglia due to PLX3397B treatment ($p>0.05$, Figure 3.4A-B).

Rats with nigral pSyn inclusions exhibited significantly larger microglial soma size compared to PBS control rats regardless of PLX3397B treatment ($p<0.0001$, Figure 3.4C-G). In general, Iba-1 immunoreactive microglia were approximately 15-20% larger in PFF injected rats. No significant differences in microglial soma size were observed within PBS or PFF treatment groups due to PLX3397B ($p>0.05$). Collectively, these results suggest that despite significant depletion of microglia, the localized inflammatory response to pSyn inclusions in the SN is preserved.

Impact of microglial depletion during the nigrostriatal degeneration phase of the α-syn PFF model

Pexidartinib partially depletes microglia in both α-syn PFF and PBS injected animals after 6-months of treatment

Similar to what we observed with 2 months of Pexidartinib treatment, six months of treatment (PLX3397B; 600mg/kg) resulted in partial microglial depletion within the SNpc of both PBS and α-syn PFF injected animals. Both PBS PLX3397B ($p<0.0001$) and PFF PLX3397B rats

(p<0.001) displayed significantly fewer Iba-1 immunoreactive microglia compared to control fed rats (Figure 3.5A-E). α-syn PFF injected rats without PLX3397B treatment possessed a mean of 512.2 ± 20.95 microglia within the SNpc as compared to the PBS injected mean of 503.2 ± 11.76. PLX3397B treatment possessed a mean of 219.1 ± 13.17 microglia within the SNpc after PBS injection as compared to the PFF injected average of 332.8 ± 20.75 microglia. However, in contrast to what we observed with 2 months of PLX3397B treatment in which microglial depletion was similar between surgical treatment groups, depletion of microglia following 6 months of PLX3397B treatment in PFF rats (35%) was significantly less effective compared to the magnitude of depletion observed in PBS injected rats (56%) (p=0.0002).

Partial microglial depletion does not impact pSyn inclusion triggered degeneration of nigral dopamine neurons

Our previous work has demonstrated that few pSyn inclusions remain in the SNpc 6 months following PFF injection due to the loss of the SNpc neurons that were initially seeded (Duffy et al., 2018; Patterson et al., 2019). In general, the number of pSyn immunoreactive SNpc neurons observed at 6 months represents 10-20% what is observed during the peak 2-month aggregation phase (Patterson et al., 2019). In the present study we similarly observed an approximate 80% reduction in pSyn immunoreactive neurons in the SNpc at 6 months when compared to 2 months post α-syn PFF injection (p<0.0001). PFF injection in rats without PLX3397B treatment resulted in 994.8 ± 33.89 pSyn containing neurons whereas PFF injection with PLX3397B treatment resulted in 1137 ± 58.93 (Figure 3.6A, B). Interestingly, a modest yet significant increase in pSyn positive SNpc neurons was observed in PFF PLX3397B rats

compared to PFF rats fed control chow (p=0.0470; Figure 3.6A,B). Due to the fact that pSyn+ accumulation is relatively minor in the SNpc at the 6-month time point we also evaluated the impact of PLX3397B on pSyn in the striatum, a structure in which pSyn accumulation is abundant at the 6-month time point (Patterson et al., 2019). PSyn accumulation in the striatum of PFF PLX3397B rats was identical to PFF rats that were fed control chow (p>0.05, Figure A3.10).

Previous PFF studies using identical surgical parameters reveal significant loss of ipsilateral SNpc THir neurons 5-6 months post intrastriatal α-syn PFF injection that parallels frank neuronal loss (Patterson et al., 2019). In the present study, 6 months following surgery, we observed a 52-55% reduction in THir neurons in the ipsilateral SNpc of PFF rats as compared to the ipsilateral hemisphere of PBS injected rats (p<0.0001), both with and without PLX3397B treatment (p<0.0001, Figure 3.6 C,D). Specifically, the ipsilateral SNpc of PFF rats fed control chow possessed 6333 ± 349.5 THir neurons whereas the ipsilateral SNpc of PBS rats fed control chow possessed 13221 ± 838.1 THir neurons. The ipsilateral SNpc of PFF PLX3397B rats possessed 5658 ± 967.2 THir neurons compared to 12536 ± 896.8 THir neurons in the ipsilateral SNpc of PBS PLX3397B rats. No significant differences were observed in ipsilateral SNpc THir neurons in PFF rats due to PLX3397B (p>0.05). These results suggest that microglial depletion does not impact the loss of THir SNpc neurons during the degeneration phase of the PFF model.

Partial microglial depletion results in increased microglia soma size and emergence of MHC-II expression in areas outside the SNpc

MHC-IIir microglia peak in abundance in the SN 2 months after PFF injection, in proximity to pSyn inclusions (Duffy et al., 2018). Although less abundant relative to 2 months, MHC-IIir microglia remain elevated compared to controls during the degenerative phase at 6 months (Duffy et al., 2018). In alignment with our earlier observations, we observed a significant decrease in the number of MHC-IIir microglia in the SNpc of PFF injected rats at six months compared to two months ($p<0.0001$) representing a reduction of approximately 70%. Despite the reduced population of MHC-II microglia compared to 2 months, we observed a significant increase in SNpc MHC-IIir microglia in PFF rats compared to controls, in both PLX3397B treated ($p=0.0001$) and untreated ($p<0.0001$) groups (Figure 3.7A, B). Specifically, PFF rats not receiving PLX3397B possessed 310 ± 18.9 MHC-IIir microglia compared to 77.33. ± 7.1 MHC-IIir microglia in PBS injected rats not receiving PLX3397B. PFF PLX3397B rats possessed 352.7 ± 48.9 MHC-IIir microglia compared to 138 ± 25.66 in PBS PLX3397B rats. Within surgical treatment groups, no significant differences were observed in the number of MHC-IIir microglia in the SNpc due to PLX3397B treatment ($p>0.05$, Figure 3.7A, B). However, qualitative analysis outside the nigral region revealed widespread MHC-II expression in both PFF and PBS injected rats that received PLX3397B treatment that was not seen in control fed rats (Figure 3.7H).

Analysis of the microglial soma size at six months revealed that PFF injected rats possessed larger microglia in the SNpc compared to PBS control rats regardless of PLX3397B treatment ($p=0.0194$, Figure 3.7C-G). Further, six months of partial microglial depletion led to a significant increase in microglial soma size in both PBS and PFF injected animals ($p<0.001$).

These results suggest that despite significant microglial depletion, the localized inflammatory response to nigral degeneration is preserved.

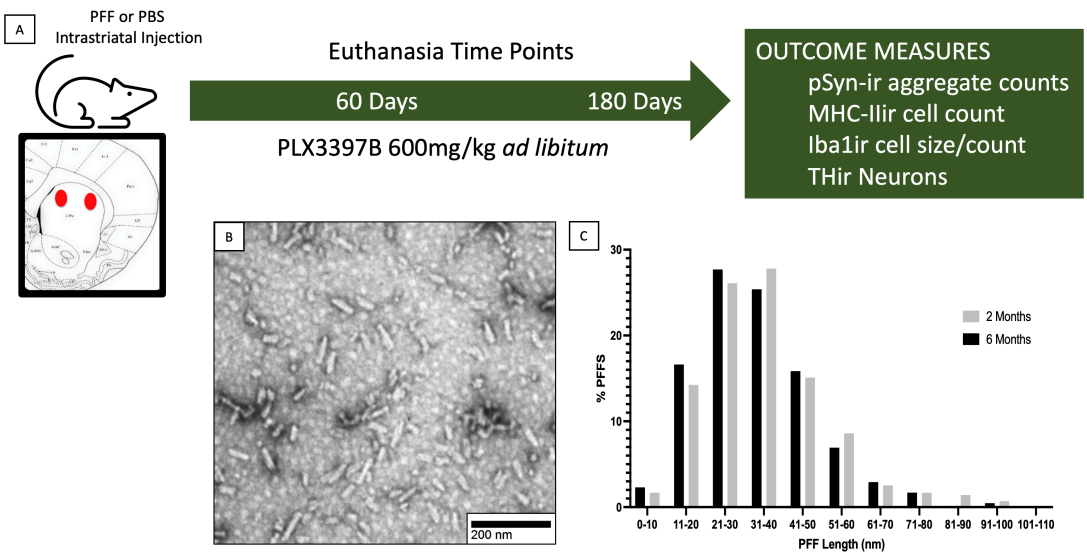

Figure 3.1. Experimental Design and PFF Size Distribution.

A: Young 3-month-old male Fischer 344 rats received two intrastriatal injections of sonicated α-syn PFFs or an equal volume of PBS as a control for surgical injection. Rats were fed PLX3397B or control chow *ad libitum* starting on the day of surgery until euthanasia at either 2 months or 6 months post-surgery. Brains were collected for postmortem endpoints including quantification of pSyn immunoreactive neurons, MHC-IIir cells, THir neurons and Iba1ir microglia and soma size in the SNpc. **B:** Representative electron micrograph of sonicated α-syn fibrils. **C:** Measurement distribution of ~650 sonicated fibrils prior to injection (mean fibril size- 2 months: 35.9 +/- 0.06 nm, 6-months: 34 +/- 0.57nm).

Abbreviations: α-syn PFF= alpha-synuclein pre-formed fibrils; PBS= phosphate buffered saline; PLX3397B= Pexidartinib containing rodent chow (600ppm); pSyn= phosphorylated alpha-synuclein phosphorylated at serine 129; MHC-IIir= major histocompatibility complex II immunoreactive; THir= tyrosine hydroxylase immunoreactive; SNpc= substantia nigra pars compacta; Iba1= Ionized calcium-binding adaptor molecule 1.

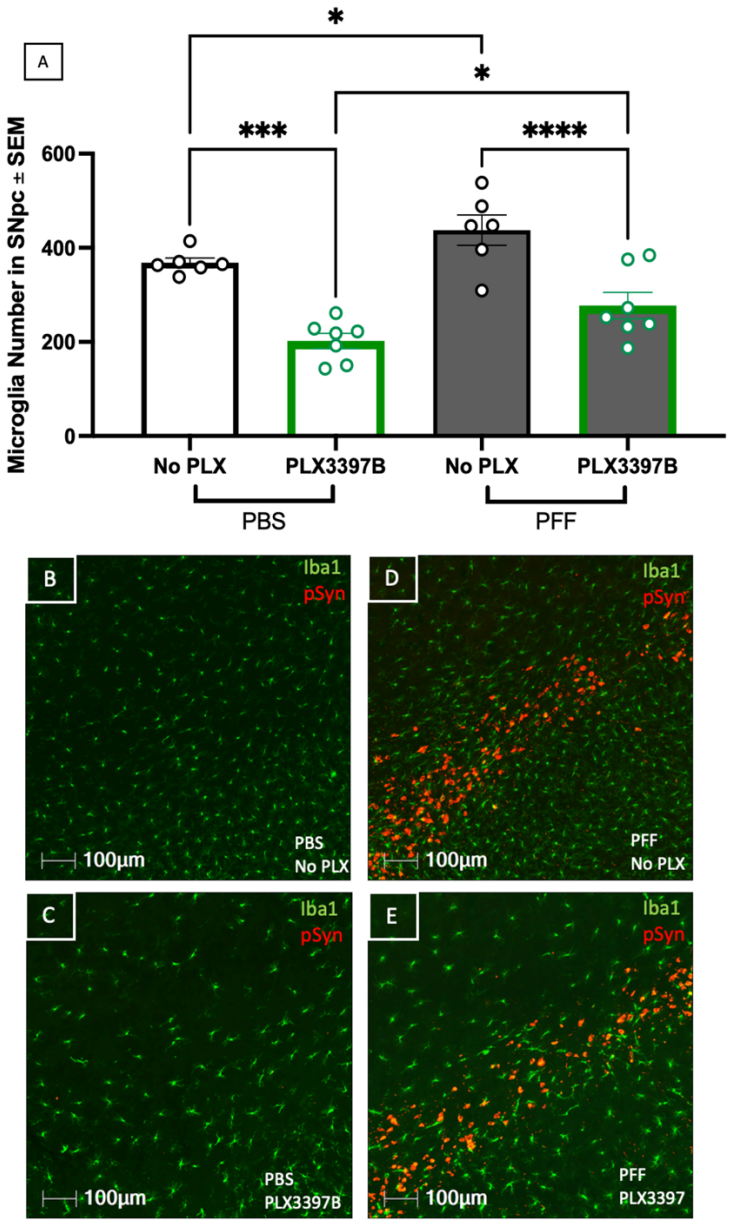

Figure 3.2. PLX3397B treatment for 2 months (aggregation phase) results in partial microglial depletion in the SNpc in both α-syn PFF and PBS injected rats.

A: PLX3397B (600mg/kg formulated in chow) treatment for 2-months results in partial microglia depletion (Iba-1) in the ipsilateral SNpc in both PBS and α-syn PFF injected animals. PFF injected rats possess significantly more microglia than PBS controls, in both control fed and PLX3397B

Figure 3.2. (Cont'd)

fed conditions. **B-E:** Representative images of ipsilateral SNpc dual labeled for Iba1 (green) and pSyn (red) 2 months post α-syn PFF or PBS injection, with or without PLX3397B treatment. PBS injection = white bars, PFF injection = grey bars, no PLX3397B = black outline, PLX3397B = green outline. ****p<0.0001 ***p=0.0001 *p<0.04. Values represent the mean ± SEM.

Abbreviations: α-syn PFF= alpha-synuclein pre-formed fibrils; SNpc= substantia nigra pars compacta; PBS= phosphate buffered saline; pSyn= phosphorylated alpha-synuclein phosphorylated at serine 129; Iba1= Ionized calcium-binding adaptor molecule 1.

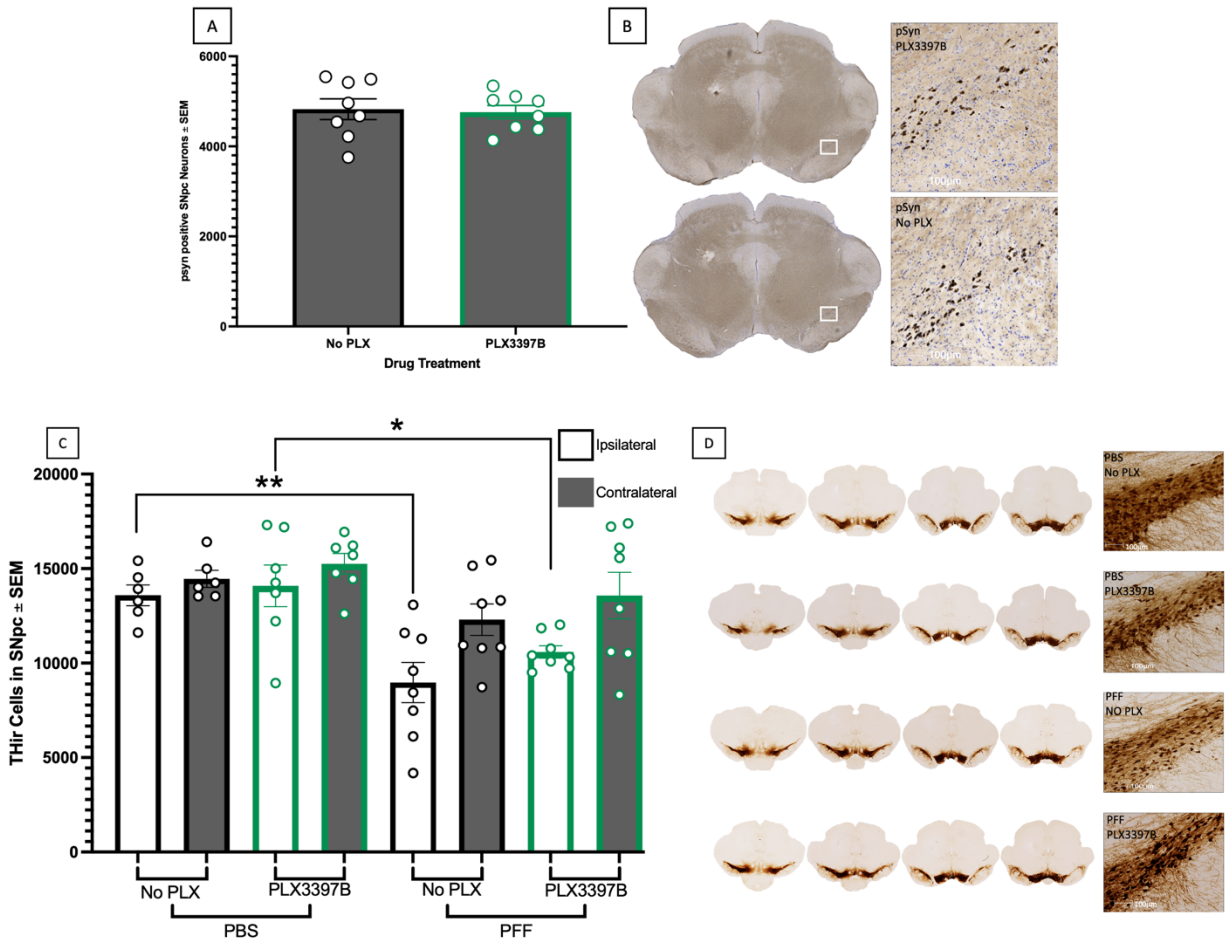

Figure 3.3. **Partial microglial depletion does not impact accumulation of pSyn aggregates or early loss of TH phenotype in the SNpc 2 months after α-syn PFF injection.**

A: Quantification of pSyn containing neurons in the ipsilateral SNpc in rats 2 months after α-syn PFF injection. No significant difference was observed between rats fed PLX3397B. PFF injection No PLX3397B = black outline, PLX3397B = green outline. **B:** Representative images of pSyn inclusions in the ipsilateral SNpc at 2 months post α-syn PFF injection in both PLX3397B and control fed animals. **C:** Quantification of THir neurons in the SNpc (Ipsilateral SNpc = white bars, Contralateral SNpc = gray bars, PLX3397B = green outline, no PLX3397B = black outline). **D:** Representative whole nigra stitched images and high magnification ipsilateral boxed inset of

Figure 3.3. (Cont'd)

THir neurons in the SNpc of the PFF and PBS injected rats with and without PLX3397B treatment. *p= 0.0330; **p=0.0058. Values represent the mean ± SEM.

Abbreviations: α-syn PFF= alpha-synuclein pre-formed fibrils; SNpc= substantia nigra pars compacta; THir= tyrosine hydroxylase immunoreactive; PBS= phosphate buffered saline; pSyn= alpha-synuclein phosphorylated at serine 129.

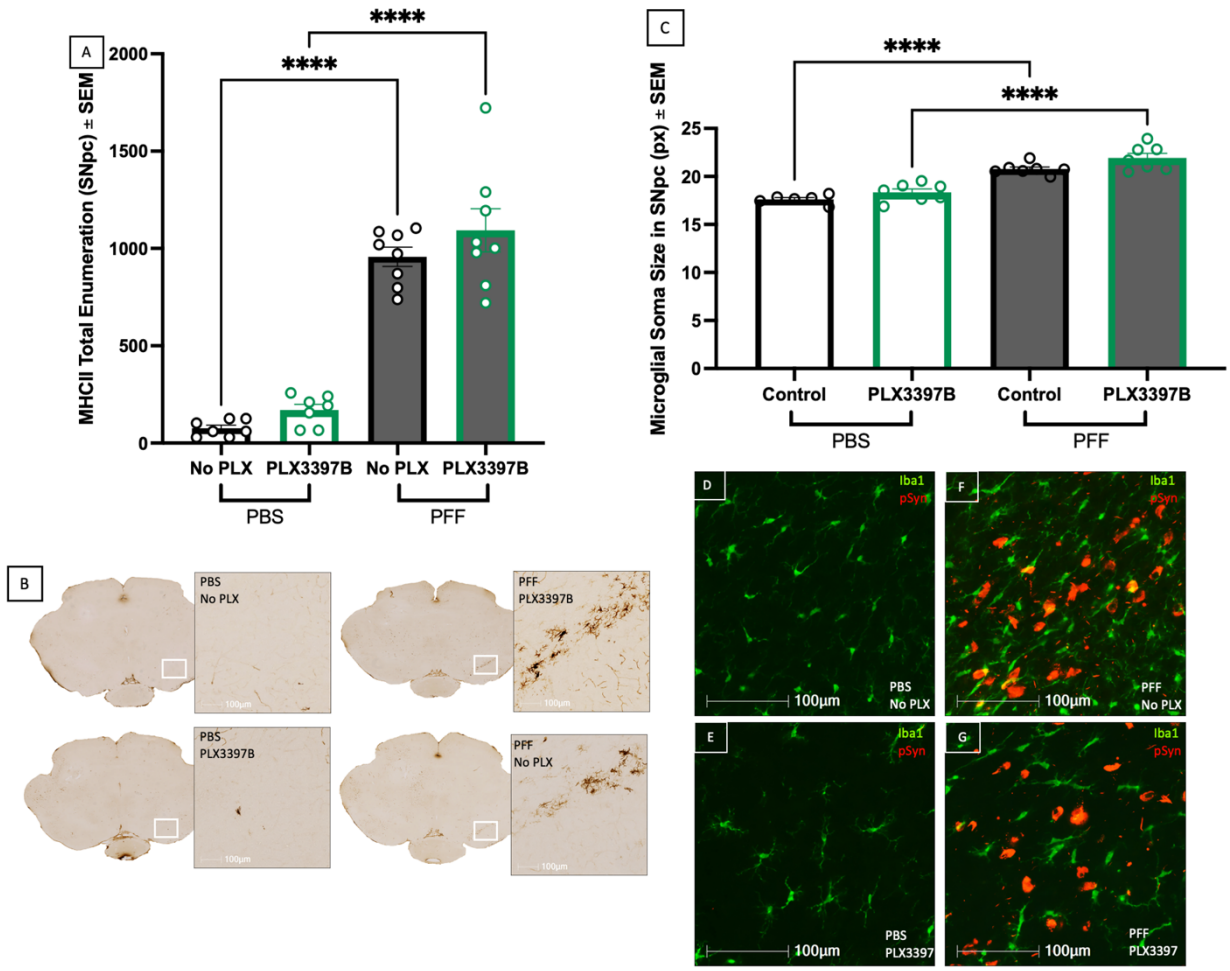

Figure 3.4. Partial microglial depletion does not impact reactive microglial morphology or MHC-II expression associated with α-syn inclusions 2 months after α-syn PFF injection.

A: Quantification of MHC-IIir microglia in the SNpc demonstrates a significant inclusion-associated increase as compared to PBS injected rats at 2-months, that is not affected by PLX3397B treatment. **B:** Representative images of MHC-IIir microglia in the ipsilateral SNpc. **C:** Analysis of Iba1ir microglia soma sizes in both PBS and α-syn PFF injected rats with and without PLX3397B treatment. Quantification demonstrates a significant increase in microglia soma size following α-syn PFF injection as compared to PBS. PLX3397B treatment for 2 months does not lead to a significant increase in microglia soma size. **D-G:** Representative images of ipsilateral

Figure 3.4. (Cont'd)

SNpc sections dual labeled for Iba1ir microglia (green) and pSyn (red) 2-months post α-syn PFF or PBS injection, with or without PLX3397B treatment. PBS injection = white bars, PFF injection = grey bars, no PLX3397B = black outline, PLX3397B = green outline. ****p<0.0001. Values represent the mean ± SEM.

Abbreviations: MHC-IIir= major histocompatibility complex II immunoreactive; α-syn PFF= alpha-synuclein pre-formed fibrils; SNpc= substantia nigra pars compacta; PBS= phosphate buffered saline; Iba1= Ionized calcium-binding adaptor molecule 1.

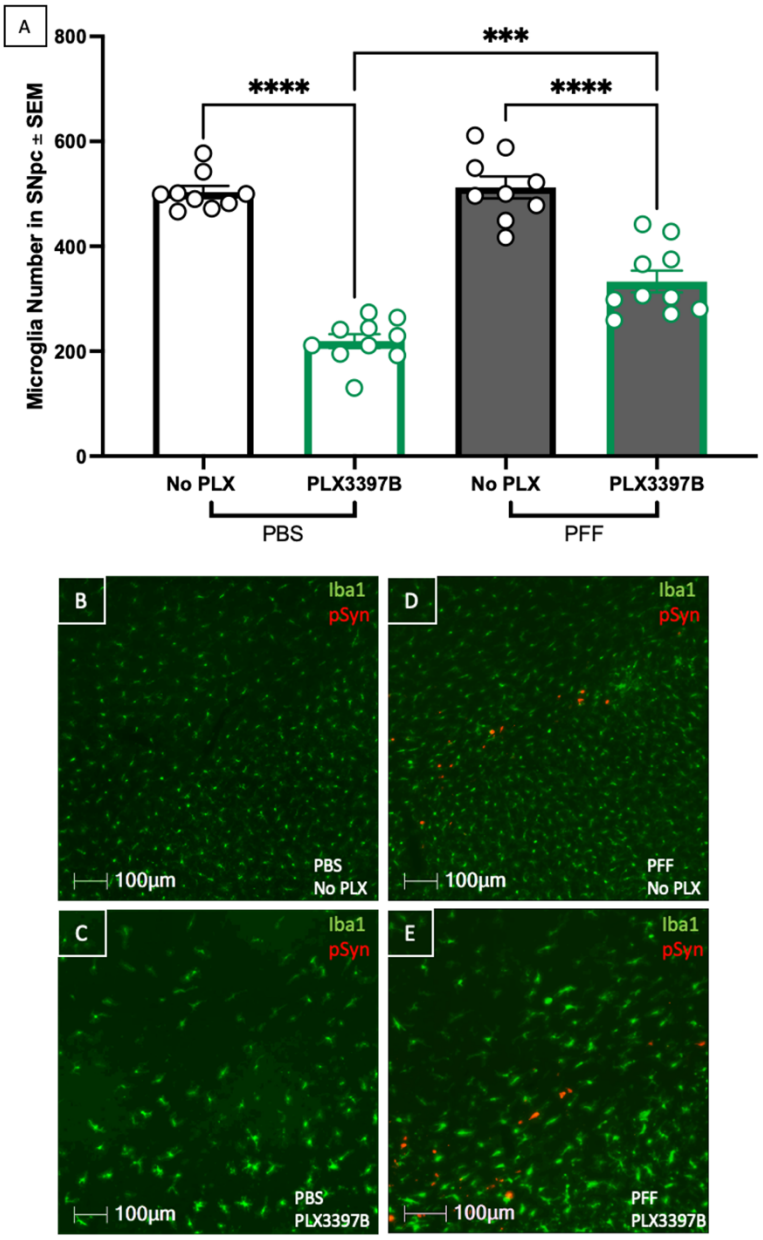

Figure 3.5. PLX3397B treatment for 6 months (degeneration phase) results in partial microglial depletion in the SNpc in both α-syn PFF and PBS injected rats.

A: PLX3397B (600mg/kg formulated in chow) treatment for 6-months results in partial microglia depletion (Iba-1) in the ipsilateral SNpc in both PBS and α-syn PFF injected animals. PLX3397B PFF injected rats possess significantly more microglia than PLX3397B PBS controls.

Figure 3.5. (Cont'd)

B-E: Representative images of ipsilateral SNpc dual labeled for Iba1 (green) and pSyn (red) 2 months post α-syn PFF or PBS injection, with or without PLX3397B treatment. Fewer pSyn immunoreactive SNpc neurons are evident at the 6-month time point. PBS injection = white bars, PFF injection = grey bars, no PLX3397B = black outline, PLX3397B = green outline. ****p<0.0001 ***p=0.0001. Values represent the mean ± SEM.

Abbreviations: α-syn PFF= alpha-synuclein pre-formed fibrils; SNpc= substantia nigra pars compacta; PBS= phosphate buffered saline; pSyn= phosphorylated alpha-synuclein phosphorylated at serine 129; Iba1= Ionized calcium-binding adaptor molecule 1.

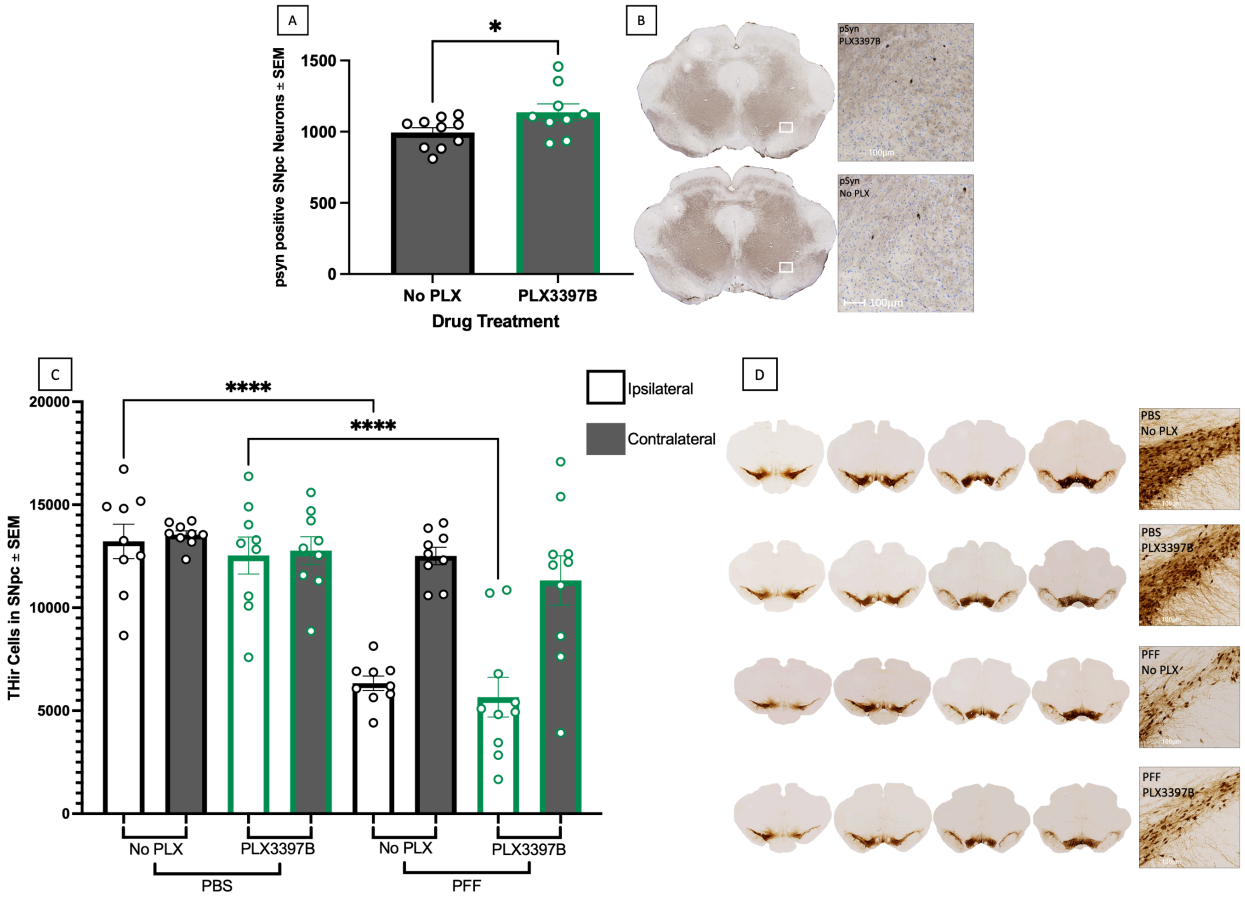

Figure 3.6. Partial microglial depletion does not impact degeneration of SNpc neurons.

A: Quantification of pSyn containing neurons in the ipsilateral SNpc in rats 6 months after α-syn PFF injection. Significantly fewer pSyn SNpc neurons are observed in PFF rats fed PLX3397B. No PLX3397B = black outline, PLX3397B = green outline. **B:** Representative images of pSyn inclusions in the ipsilateral SNpc at 6 months post α-syn PFF injection in both PLX3397B and control fed animals. **C:** Quantification of THir neurons in the SNpc 6 months following surgery. PFF injection resulted in significant THir SNpc neuron loss at 6 months in both PLX3379B and control fed rats (Ipsilateral SNpc = white bars, Contralateral SNpc = gray bars, PLX3397B = green outline, no PLX3397B = black outline). **D:** Representative whole nigra stitched images and high magnification ipsilateral boxed inset of THir neurons in the SNpc of the PFF and PBS injected

Figure 3.6. (Cont'd)

rats with and without PLX3397B treatment. ****$p<0.0001$ *$p<0.05$. Values represent the mean ± SEM.

Abbreviations: α-syn PFF= alpha-synuclein pre-formed fibrils; SNpc= substantia nigra pars compacta; THir= tyrosine hydroxylase immunoreactive; PBS= phosphate buffered saline; pSyn= alpha-synuclein phosphorylated at serine 129.

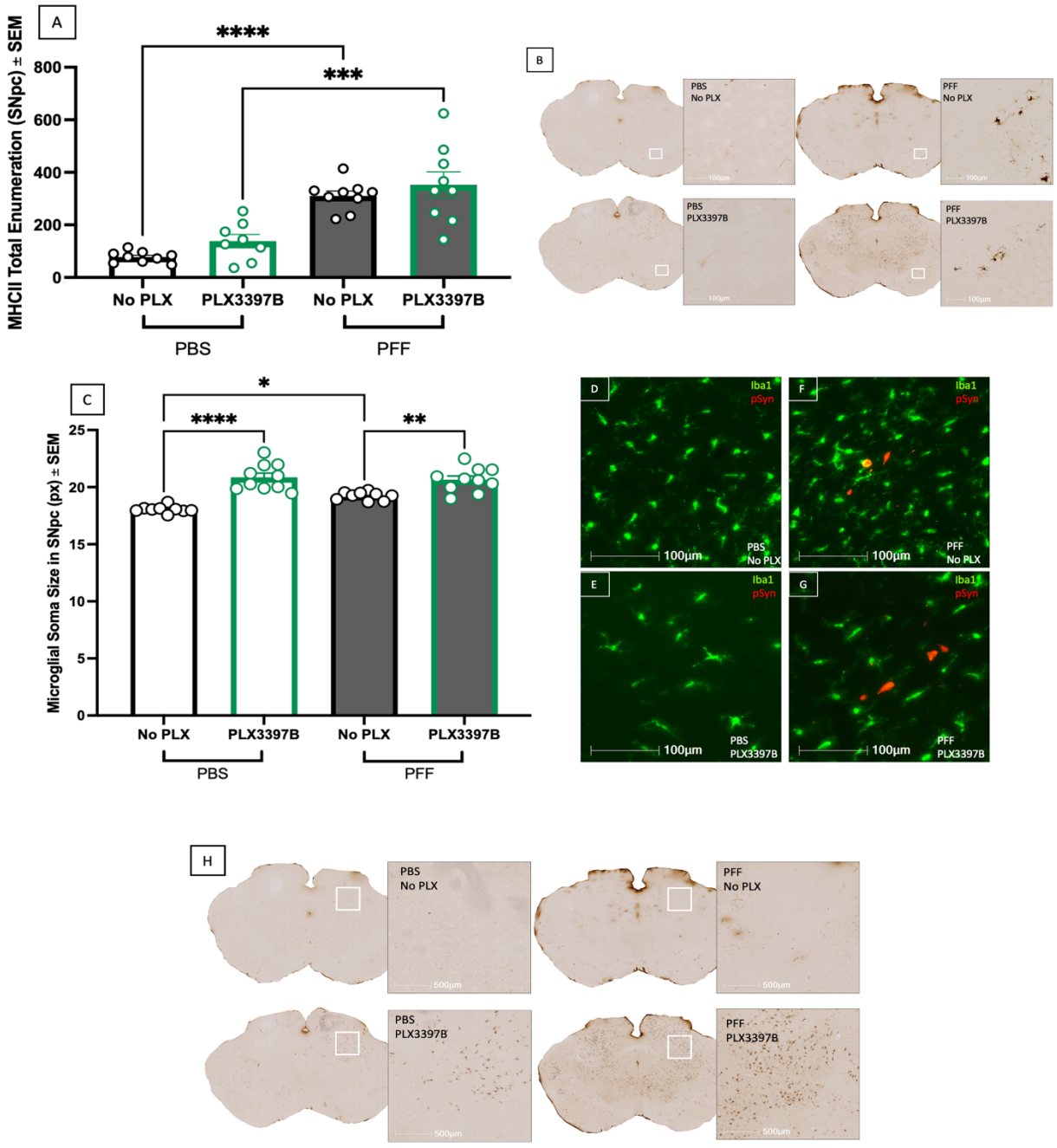

Figure 3.7. **Partial microglial depletion for 6 months results in increased microglia soma size and emergence of MHC-II expression in areas outside the SNpc.**

A: Quantification of MHC-IIir microglia in the SNpc demonstrates a significant degeneration-associated increase as compared to PBS injected rats at 6-months that is not affected by

Figure 3.7. (Cont'd)

PLX3397B treatment. **B:** Representative images of MHC-IIir microglia in the ipsilateral SNpc. **C:** Analysis of Iba1ir microglia soma sizes in both PBS and α-syn PFF injected rats with and without 6 months of PLX3397B treatment. Quantification demonstrates a significant increase in microglia soma size following α-syn PFF injection as compared to PBS in rats that were not fed PLX3397B, however microglia soma size was not increased in PLX3397B PFF rats compared to PLX3397B PBS rats. Further, within both surgical treatment groups, 6 months of PLX3397B treatment resulted in increased microglia soma size. **D-G:** Representative images of ipsilateral SNpc sections dual labeled for Iba1ir microglia (green) and pSyn (red) 2-months post α-syn PFF or PBS injection, with or without PLX3397B treatment. **H:** Representative images demonstrating increased MHC-II expression in cells outside of the SNpc in both α-syn PFF and PBS injected animals after 6-months PLX3397B treatment. PBS injection = white bars, PFF injection = grey bars, no PLX3397B = black outline, PLX3397B = green outline. ****$p<0.0001$ ***$p=0.0001$ ** $p<0.005$ *$p<0.04$. Values represent the mean ± SEM.

Abbreviations: MHC-IIir= major histocompatibility complex II immunoreactive; α-syn PFF= alpha-synuclein pre-formed fibrils; SNpc= substantia nigra pars compacta; PBS= phosphate buffered saline; Iba1= Ionized calcium-binding adaptor molecule 1.

Discussion

Imaging and histological studies provide support for the presence of ongoing neuroinflammatory processes in PD (McGeer et al., 1988; Mogi, Harada, Kondo, et al., 1994; Nagatsu' et al., 2000; Stojkovska et al., 2015; Tansey et al., 2022; Walker et al., 2016). The ability to attenuate these inflammatory processes through microglial depletion has yielded mixed results in both AD (Tau; Bennett et al., 2018)and PD (MPTP; Neal et al., 2020) animal models. In some studies, microglial depletion has led to the exacerbation of neurodegeneration (Yang et al 2018; Li et al 2021; Shabestari et al 2022) whereas in others neuroprotection is observed (Neal et al 2020; Oh et al 2020). All previous PD models used CSF1R inhibitors in mice with dosing strategies that resulted in near complete microglial depletion (~90%) (Elmore et al., 2014; Han et al., 2019a; Oh et al., 2020). However, microglia play many roles in maintaining healthy homeostasis in the brain (Lazdon et al., 2020; Menassa & Gomez-Nicola, 2018; Tan et al., 2020)and complete microglia depletion may not be a viable therapeutic strategy. Therefore, in the present study we employed a PLX3397B dosing strategy that elicited partial (~40%) microglial depletion in the SNpc. Our results demonstrate that partial microglial depletion does not prevent α-syn aggregation in the SNpc or the striatum, attenuate the inflammatory response to aggregation or degeneration, or prevent nigral degeneration following intrastriatal PFF injection.

Our previous studies have revealed that microglia respond to the aggregation and degeneration phases of the rat α-syn PFF model in a consistent, measurable manner (Duffy et al., 2018; Miller et al., 2021). During the peak aggregation phase in the SNpc at 2 months, microglia increase in number, soma size and MHC-II expression. The MHC-II response of

microglia to intraneuronal pSyn aggregates is heterogeneous, limited to a subpopulation of microglia within the immediate vicinity of the SNpc inclusions. The heterogeneity of inflammatory responses within individual microglia (Masuda et al., 2020) and between different brain regions (Tan et al., 2020) has been well-documented. The number of MHC-IIir microglia positively correlates to the number of pSyn immunoreactive SNpc neurons and is near absent during the nigral degeneration phase (Duffy et al., 2018). In the present study, microglial depletion with PLX3397B attenuated the increase in microglia associated with aggregation and degeneration in the SNpc, as would be expected, but the soma size of the remaining microglia increased in rats that received PLX3397B for 6 months. Further, the MHC-IIir microglial subpopulation was not impacted by microglial depletion. We had initially hypothesized that with ~40% depletion of microglia we may observe ~40% reduction in MHC-IIir microglia with PLX3397B. The maintenance of the pSyn inclusion associated MHC-IIir microglia subpopulation, despite significant microglial depletion, suggests that the remaining, depleted microglia maintain the capacity to mount an identical proinflammatory response. This finding is not unique to this study, as other microglia depletion studies have shown similar maintenance or an increase in inflammatory responses when general microglial populations are depleted (Bennett et al., 2018; Elmore et al., 2014, 2018) along with increases in adaptive immune cells within the brain (Yan et al., 2021). The approach of microglia repopulation as a therapeutic strategy in order to “reset” microglia has been recently proposed with the goal of exchanging dysfunctional with functional microglia. However, the results from repopulation studies vary (Barnett et al., 2021; Han et al., 2019) and suggest that repopulation comes from the remaining

microglia. Our data suggests a microglia repopulation strategy would not be beneficial, and that the inflammatory response will be maintained.

Our study is unique in that the microglial depletion was sustained for a period of 6 months, whereas most previous microglial depletion studies use much shorter depletion intervals (7-28 days (Barnett et al., 2021; Elmore et al., 2018; Fu et al., 2020; Han et al., 2019; Yang et al., 2018). We observed evidence of a more pronounced inflammatory state in our 6-month microglial depletion study compared to our 2-month microglial depletion study. Specifically, after 6 months of microglial depletion, microglia soma size was increased, even within control PBS injected rats. Further, after 6 months of microglial depletion we observed MHC-II immunoreactive cells in multiple brain regions, also in control rats. Normally, except for border associated macrophages (Utz & Greter, 2019; van Hove et al., 2019), MHC-II immunoreactive cells are not often observed in uninjured brain regions in control rats. Our data suggests an imbalance of the innate immune system associated with long term microglial depletion, a phenomenon that should be examined in a more rigorous, quantitative manner in future studies.

Inflammatory microglia may contribute to PD progression; however, the present study suggests that partial microglia depletion may not be an effective, disease modifying therapeutic approach. One limitation of the present study is that the response of other cell types (peripheral macrophages, astrocytes, adaptive immune cells) to microglial depletion was not examined. Previous studies have indicated that near complete microglia depletion can impact astrocytes and adaptive immune cells (Bennett et al., 2018). Another limitation of the present study is that microglial depletion was not to the magnitude (~90%) that has previously been

achieved in mouse studies (Elmore et al., 2014; Han et al., 2019; Oh et al., 2020). It is possible that greater levels of depletion (along with repopulation) may have a different effect in the α-syn PFF than what we observed under the present experimental parameters (Han et al., 2019a, 2019b). Of particular relevance to this dissertation, our findings point to a need to understand the full phenotype of the MHC-IIir microglial subpopulation that is maintained despite significant general microglial depletion. In experiments described in the next chapter of this dissertation we investigate the **a**-synuclein **a**ggregate associated **m**icroglial (a-SAM) phenotype. Further understanding of this a-SAM phenotype could potentially identify specific inflammatory processes to target to attenuate neuroinflammation in PD.

APPENDIX

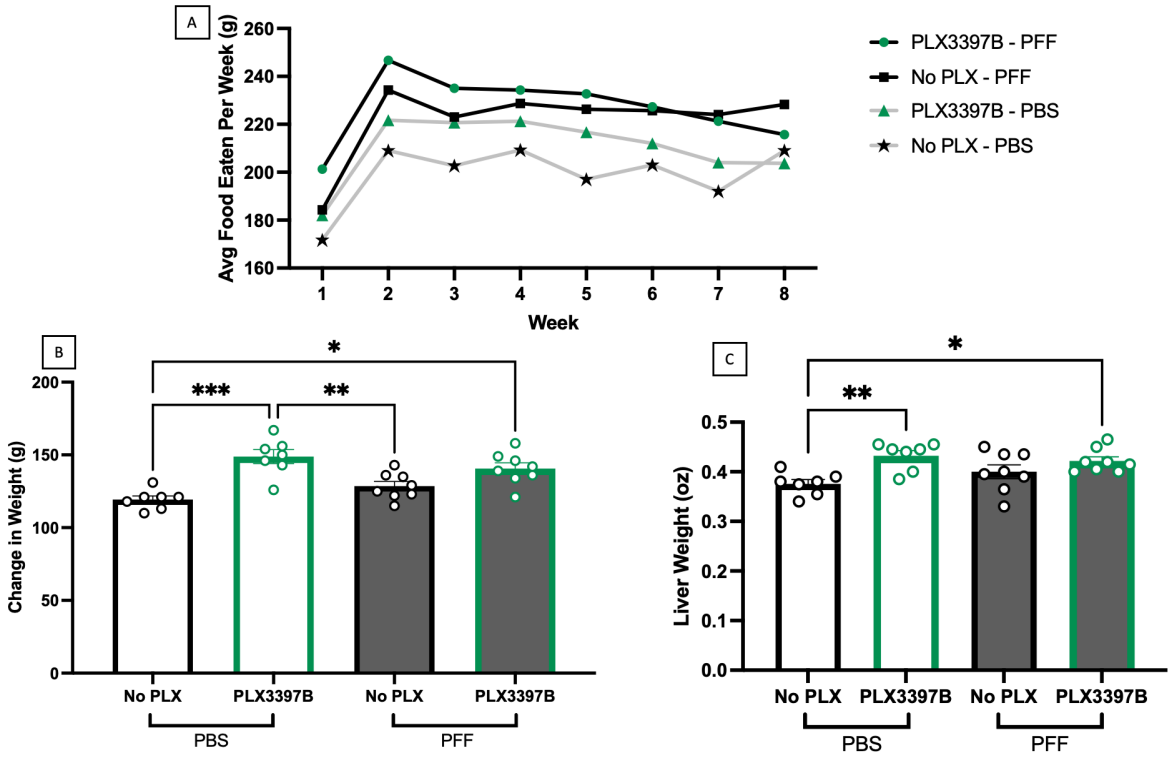

Figure A3.8. Chow consumption, rat weight change and liver weights after 2-months of PLX3397B treatment. A: Food consumption each week in all 4 treatment groups over 2-months post-surgery. **B:** Average change in weights seen in all treatment groups. **C:** Liver weights at euthanasia.

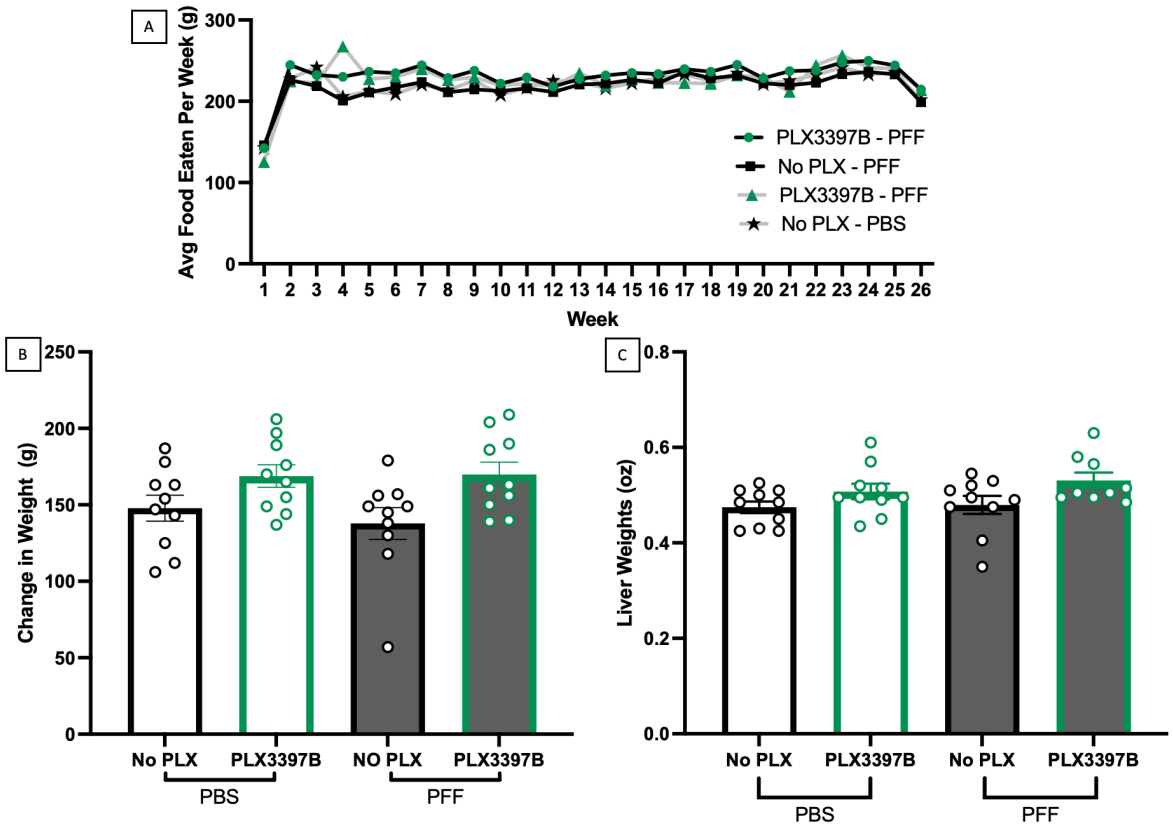

Figure A3.9. Chow consumption, rat weight change and liver weights after 6-months of PLX3397B treatment. **A:** Food consumption each week in all 4 treatment groups over 6-months post-surgery. **B:** Average change in weights seen in all treatment groups. **C:** Liver weights at euthanasia.

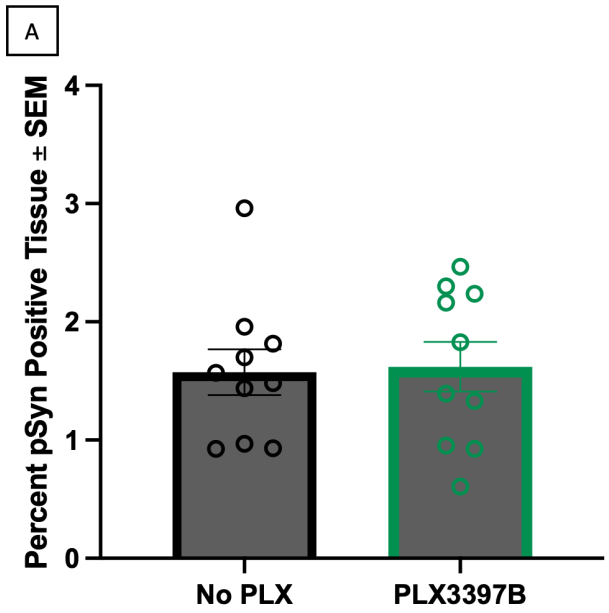

Figure A3.10. Partial microglial depletion does not impact accumulation of striatal pSyn 6 months after α-syn PFF injection.

A: Quantification of pSyn accumulation in the striatum in α-syn PFF PLX3397B rats compared to α-syn PFF rats that were fed control chow. No significant difference was seen in striatal pSyn load between treatment groups at six months post α-syn injection.

Abbreviations: α-syn PFF= alpha-synuclein pre-formed fibrils; PBS= phosphate buffered saline; pSyn= alpha-synuclein phosphorylated at serine 129.

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Chapter 4: The alpha-synuclein inclusion associated microglia (a-SAM) phenotype suggests a multidimensional immune response

Abstract

Parkinson's disease (PD) is characterized by alpha-synuclein (α-syn) inclusions, the progressive loss of dopamine neurons in the substantia nigra pars compacta (SNpc) and neuroinflammation. These pathological features can be recapitulated *in vivo* using the alpha-synuclein preformed fibril (PFF) model. Specifically, in association with accumulation of phosphorylated α-syn (pSyn) inclusions in the SNpc, microglia increase soma size and MHC-II expression. This microglial response parallels pSyn inclusion formation, peaking at 2 months following intrastriatal PFF injection, months prior to the SNpc degeneration observed in the model. In a previous study in our laboratory RNA-Seq was conducted to analyze transcript levels in the SNpc 2 months following PFF injection. This dataset identified upregulation of multiple transcripts associated with innate and adaptive immune response pathways. In the present study we sought to validate and expand these findings, taking two different approaches. Three-month-old male and female Fischer 344 rats received intrastriatal injections of either α-syn PFFs, PBS, or no injection. At 2-months post α-syn PFF injection fluorescent in situ hybridization (FISH) or droplet digital PCR (ddPCR) was conducted. Our FISH results identify an **a**-syn **a**ggregate associated **m**icroglial (a-SAM) phenotype that is characterized by upregulation of *Cd74, Cxcl10, Rt-1a2, Grn, Csf1r, Tyrobp, C3, C1qa* and *Fcer1g*. Our ddPCR results identify additional neuroinflammatory genes, including *Cd4, Stat1, Casp1, Axl* and *Il18*, all of which are significantly upregulated in inclusion bearing nigral tissue. Collectively these findings implicate that the deposition of pathological α-syn inclusions in the SNpc is associated with perturbations in immune functions related to complement, inflammasome and T cell activation, phagocytosis, and interferon gamma signaling. Our results identify a specific, reproducible phenotype of

microglia associated with pSyn inclusion inclusions in the SNpc. This comprehensive understanding of the multidimensional response of microglia to pathological α-syn aggregates may help to uncover novel therapeutic targets that could facilitate future anti-inflammatory, disease-modifying strategies for PD.

Introduction

Parkinson's Disease, the second most common neurodegenerative disease, affects around 1 million people in the USA with 60,000 newly diagnosed people each year (Marras et al., 2018) . Pathologically, PD is characterized by the presence of proteinaceous alpha-synuclein (α-syn) inclusions (Lewy bodies) and the progressive loss of the nigrostriatal dopamine (DA) neurons. While the exact cause of PD is still unknown, mounting evidence has suggested that neuroinflammation, mediated by microglia, may play a significant role in PD progression and neuropathology (Mc GEER et al., 1993; Tansey et al., 2022; Tansey & Romero-Ramos, 2019). These pathological features can be recapitulated *in vivo* using the alpha-synuclein preformed fibril (PFF) model. Specifically, the accumulation of phosphorylated α-syn (pSyn) inclusions in the substantia nigra pars compacta (SNpc) peaks at 2 months following intrastriatal PFF injection, accompanied by a similar peak in reactive microglia and astrocytes, with SNpc degeneration observed at 5-6 months (Duffy et al., 2018; Harms et al., 2013; J. R. Patterson et al., 2019).

Our laboratory has previously demonstrated that the accumulation of α-syn aggregates in the SNpc following intrastriatal PFF injection is associated with a localized increase in microglial number and reactive morphology, along with increased expression of MHC-II by a specific subpopulation of microglia in the immediate vicinity of inclusions (Duffy et al., 2018; Miller et al., 2021). Microglia continually survey the brain environment and are the first responders to insults. Microglial polarization states have been considered to be either a "resting" state (general surveillance state) or activated (M1: pro-inflammatory or M2: anti-inflammatory). However, increasing evidence suggests that microglial activity is more

multidimensional rather than linear. The spectrum of microglial "activation" is based on three basic microglial functions- Host defense, wound healing, and immune regulations - with each function able to overlap with the others (Mosser & Edwards, 2008).

Recently our laboratory used an unbiased, refined bulk RNA-Seq approach to analyze transcript levels in the SNpc 2 months following PFF injection, during the early peak of pSyn inclusion formation and associated microglial activation (Patterson et al., 2020). This dataset identified upregulation of multiple transcripts associated with innate and adaptive immune response pathways (i.e antigen presentation, natural killer cell mediated cytotoxicity, complement pathway, T-cell activation, inflammasome activation). In the present study we sought to validate and expand these findings, taking two different approaches. First, we investigated whether microglia or MHC-II immunoreactive microglia were the cellular source of some of the upregulated transcripts previously identified using RNA-Seq. For this experiment we used an *in-situ* hybridization approach using inclusion seeded SNpc tissue two months following PFF injections. Second, we investigated expression changes of additional immune related genes previously identified in PD or Alzheimer's disease (AD) or preclinical models (Barnum & Tansey, 2010; Bettcher et al., 2021; Boche & Gordon, 2022; Deczkowska et al., 2018; Dubbelaar et al., 2018; Heidari et al., 2022; Keren-Shaul et al., 2017; Morgan & Mielke, 2021; Sanchez-Guajardo et al., 2013; Tansey & Romero-Ramos, 2019), independent of our previous RNA-Seq findings. For this experiment we used droplet digital PCR (ddPCR) in inclusion seeded SNpc tissue two-months following α-syn PFF injections. The goal of these two complementary approaches was to identify a reproducible neuroinflammatory gene expression signature associated with α-syn inclusions in the SNpc. A more comprehensive understanding of the

multidimensional response of microglia to pathological α-syn aggregates could facilitate future investigation of anti-inflammatory, disease-modifying approaches for PD

Using our *in-situ* hybridization approach we identified an **a**-syn **a**ggregate associated **m**icroglial (a-SAM) phenotype that is characterized by upregulation of *Cd74, Cxcl10, Rt1-a2, Grn, Csf1r, Tyrobp, C3, C1qa* and *Fcer1g*. This a-SAM phenotype implicates the involvement of multiple immune pathways in the microglial response to α-syn aggregation in the SNpc, prior to degeneration. Using our ddPCR approach we have identified additional neuroinflammatory genes, including *Cd4, Stat1, Casp1, Axl* and *IL18*, all of which are significantly upregulated in inclusion bearing nigral tissue. Collectively these findings implicate that the deposition of pathological α-syn inclusions in the SNpc is associated with perturbations in immune functions related to complement, inflammasome and T cell activation, phagocytosis, and interferon gamma signaling. A more comprehensive understanding of the multidimensional response of microglia to pathological α-syn aggregates may help to uncover novel therapeutic targets that could facilitate future anti-inflammatory, disease-modifying strategies for PD.

Methods

Experimental Overview

Experiment 1: Cellular identity of α-syn inclusion associated innate immune transcripts. We investigated whether microglia or MHC-II immunoreactive microglia were the cellular source of some of the upregulated transcripts previously identified using RNA-Seq in association with SNpc inclusion deposition (J. Patterson et al., 2020). Rats were unilaterally injected with either α-syn preformed fibrils or PBS and euthanized at 60 days post injection (peak SNpc α-syn aggregation following PFF injection) and brain tissue was analyzed using i*n situ* hybridization (RNAscope™). Figure. 4.1A illustrates the experimental design.

Experiment 2: Measurement of PD and AD associated neuroinflammatory genes using ddPCR**.** We investigated expression levels of immune related genes previously identified in PD or Alzheimer's disease literature (Boche & Gordon, 2022; Deczkowska et al., 2018; Dubbelaar et al., 2018; Keren-Shaul et al., 2017; Morgan & Mielke, 2021; Srinivasan et al., 2016). Rats were unilaterally injected with either α-syn preformed fibrils or PBS and euthanized at 60 days post injection (peak SNpc α-syn aggregation following PFF injection). An additional cohort of naive rats also was included and ddPCR was performed on inclusion-containing and control SNpc. Figure 4.1B illustrates the experimental design.

Animals

Three-month old, male or female Fischer 344 rats (Charles River; Experiment 1 n=10; Experiment 2 n=30) were housed, 2-3 per cage, at the Grand Rapids Research Center which is fully approved through the Association for Assessment and Accreditation of Laboratory Animal

Care (AAALAC). Rats were in a room with a 12-hour light/dark cycle and provided food and water *ad libitum.* All procedures were done in accordance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University.

α-syn PFF Preparation and Fibril Size Verification

Preformed fibrils were generated from wild-type-full length, recombinant mouse α-syn monomers as previously described (Luk et al., 2012; Polinski et al., 2018; Volpicelli-Daley et al., 2011, 2014). Quality control was completed on full length fibrils to ensure fibril formation (transmission electron microscopy), amyloid structure (thioflavin T assay), pelletability as compared to monomers (sedimentation assay), and low endotoxin contamination (*Limulus* amebocyte lysate assay; <0.5 endotoxin unites/mg of total protein) (All quality control except transmission electron microscopy done in the Luk lab at the university of Pennsylvania). On surgery day, PFFs were thawed to room temperature and diluted to 4ug/µl in sterile Dulbecco's phosphate buffered saline and sonicated with an ultrasonic homogenizer (300 VT; Biologics, Inc.) for 60- 1s pulses with pulser at 20% and power output at 30%. An aliquot of sonicated PFFs was analyzed using transmission electron microscopy. Aliquoted PFFs were prepared on Formvar/carbon-coated copper grids (EMSDIASUM, FCF300-Cu). Grids were twice washed on drops of ddH2O. grids were then incubated in 10µl of sonicated PFFs dilutes 1:50 in sterile PBS for 1 min followed by a 1-minute incubation in 10µl of aqueous 2% uranyl acetate and then allowed to dry. Grids were then imaged with a JEOL JEM-1400+ transmission electron microscope. Post-surgery, the fibril lengths were measured using ImageJ 1.53K (Wayne Rasband and contributors, National Institutes of Health, USA). The mean length for the FISH surgical

cohort was 37.82 ± 0.67 nm and for the ddPCR surgical cohort was 39.97 ± 0.76 nm (Fig. 4.1C, D). Fibril length < 50 nm is required for successful seeding of endogenous α-syn inclusions (Tarutani et al., 2016).

Stereotaxic Injections

Unilateral intrastriatal α-syn PFF injections were conducted as previously described (Patterson et al., 2019). Rats were anesthetized with isoflurane (5% induction and 1.5% maintenance) and received unilateral intrastriatal injections to the left hemisphere (2 x 2 µl, AP +1.6, ML +2.0, DV -4.0; AP +0.1, ML +4.2, DV -5.0, AP and ML coordinates relative to Bregma, DV coordinates relative to dura). PFFs (4 ug/µl; 16 ug total) or an equal volume of PBS were injected at a rate of 0.5 µl/min with a pulled glass capillary tube attached to a 10 µl Hamilton syringe. After each injection the needle was left in place for 1 minute, retracted 0.5 mm and left for 2 minutes to avoid PFF displacement, and then fully retracted. All animals received analgesic (1.2 mg/kg of sustained release buprenorphine) after surgery and were monitored until euthanasia.

Euthanasia - Experiment 1

Animals were euthanized at 60 days post injection based on the time course of the model described previously (Duffy et al., 2018; J. R. Patterson et al., 2019). Rats were given a 30mg/kg pentobarbital i.p. injection (Euthanasia-III Solution, MED-PHARMEX Incorporated) and perfused intracardially with heparinized 0.9% saline. Brains were removed and post-fixed in 4% paraformaldehyde (PFA) for one week and then transferred to 30% sucrose in 0.1M phosphate

buffer until sunk. Brains were frozen with dry ice and sectioned at 40µm (1:6 series of sections) on a sliding microtome and stored in cryoprotectant (30% sucrose, 30% ethylene glycol, in 0.1M Phosphate Buffer (PB), pH 7.3) at -20°C.

RNAscope™ HiPlex Fluorescent in situ Hybridization Combined with Immunofluorescence

RNAscope™ HiPlex Fluorescent *in situ* hybridization was used in nigral tissue sections to determine cellular localization of a subset of genes previously identified to be significantly upregulated in association with α-syn inclusions (J. Patterson et al., 2020). In the previous study, 176 upregulated transcripts associated with immune related pathways and with known expression in microglia (A. Saunders et al., 2018) were upregulated in both male and female rats. Transcripts per million (TPM) values > 15 in PFF injected male rats were used to select genes expected to be easily visualized with *in situ* hybridization (Table 4.1). Free floating sections were washed 4x 10minutes in 0.1M TBS containing 0.5% Triton-X100 (TBS-Tx) and then quenched in ACD Bio Hydrogen Peroxide (Advanced Cell Diagnostics, 322335) for 1 hour. Tissue was then washed 4 x 10minutes in TBS-Tx and then washed 2 x 10 in 1:4 diluted TBS-Tx in ultra-pure water. Tissue was mounted on HistoBond+ slides (VWR VistaVision, 16004-406) in diluted TBS-Tx and placed on a slide warmer at 60°C overnight. The slides were then incubated in an ACD RNAscope™ Target Retrieval buffer (1:10 dilution; Advanced Cell Diagnostics, 322001) warmed to 99°C for 10 minutes and then quickly washed 2x in ultra-pure H_2O. Tissue was then outlined with a PapPen (INFO) and 3 drops of ACD protease III (Advanced Cell Diagnostics; 322337) was added and incubated in the Hybez ™II oven at 40.0°C (Advanced Cell Diagnostics) for 30 minutes. Slides where then quickly washed 2x and diluted ACD probes (1:50; See Table

Table A4.4) for detailed probe information) were added to the tissue and incubated in the Hybez ™II oven for 2 hours. 3 amplification steps were done with ACD amplification buffers #1,2, &3 (RNAscope™HiPlex12 Detection Reagents (488,550,650) v2; Advanced Cell Diagnostics, 324410) alternated with 2 quick washes in RNAscope™Wash Buffer (1:500 Dilution; Advanced Cell Diagnostics, 310091) in between each 30-minute amplification incubation in the Hybez ™II oven. Following the 3rd amplification incubation slides were quickly washed 2x and incubated for 15 minutes in the hybrex oven with the appropriate ACD fluorophores for the tails on the probes (RNAscope™ HiPlex12 Detection Reagents (488,550,650) v2; Advanced Cell Diagnostics, 324410). Slides were quickly washed 2x and blocked in 10% normal goat serum (NGS) in TBX-Tx for 1 hour at room temperature. Sections were incubated with IF primary (mouse anti-pSyn (1:100; Abcam, AB184674); rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1) (1:100; Wako, 019-09741); rabbit anti tyrosine hydroxylase (TH) (1:400, MIllipore, AB152) in 1% NGS/TBS-Tx overnight at room temperature. Slides were quickly washed 2x in TBS-Tx and incubated in Alexa Fluor Secondaries (pSyn- 568 goat anti mouse (1:250; Invitrogen A11011) Iba1 and TH-488 goat anti rabbit (1:250; Invitrogen, A11034 for 2 hours at room temperature. Slides were quickly washed 2x and a drop of RNAscope™HiPlex DAPI (Advanced cell Diagnostics; 324420) was added and left for 1 minute. Excess DAPI was removed, and slides were cover slipped with ProLong™ Gold antifade reagent (Invitrogen, P36930). Images were taken using Nikon Eclipse Ni-U microscope with CFI60 infinity optical system (Nikon Instruments Inc.) using the 20x and 40x objectives.

Euthanasia - Experiment 2

Animals were euthanized at 60 days post injection. Rats were given a 30mg/kg pentobarbital i.p. injection (Euthanasia-III Solution, MED-PHARMEX Incorporated) and perfused intracardially with heparinized 0.9% saline. Brains were removed and flash frozen in 2-methylbutane on dry ice and stored at −80 °C. Frozen brains were mounted in OTC, mounted on a cryostat, and sectioned to the level of the SN. The ipsilateral SNpc was microdissected (1mm (tall) x 2mm (wide) oval punch) at −15 °C. Tissue was collected in DNase/RNase free microcentrifuge tubes containing 100µl TRIzol reagent Invitrogen 26696026), homogenized with a disposable pestle, volume of TRIzol was brought to 1mL, mixed, and samples were frozen on dry ice and stored at −80 °C.

RNA Isolated for Droplet Digital PCR

RNA isolation was conducted as described previously (Patterson et al., 2022). Samples were thawed on ice and briefly centrifuged. Phasemaker microcentrifuge tubes (Invitrogen, A33248) were prepared according to manufacturer specifications. Samples were transferred to Phasemaker tubes and incubated at RT for 5 mins, followed by an addition of 200µl of chloroform and incubated at RT for 10min. The tubes were centrifuged for 5 min at 16,000xg at 4 °C, the aqueous phase was transferred to an RNase free tube and an equal volume of 100% ethanol was added and vortexed. A column-based nucleic acid purification kit (Zymo Research, R1016) was utilized. 600µl of sample was added to the column, at a time, and centrifuged for 1 min at 12,000xg until the entire sample has been added. All wash and prep buffer steps were performed by adding the buffer to the column and centrifugation for 1 min at 12,000xg.

Samples were washed with 400µl of RNA was buffer followed by the addition of 1X DNase I cocktail (DNase I from Thermo Scientific FEREN0521; Reaction buffer with MgCl2 from Thermo Scientific FERB43) and incubated at RT for 15 min followed by centrifugation. 400µl of RNA prep buffer was then added to the column and centrifuged. Columns were washed 2x with RNA wash buffer (700µl then 400µl), an extra centrifugation step was done for 2min to dry the column. 15µl of DNase/RNase free water was added to the column and incubated for 1 min then eluted by centrifugation at 10,000g for 1 min. Quality and quantity of RNA was assessed with Agilent 2100 Bioanalyzer using an Agilent RNA 6000 Pico Kit (5067-1513). RNA was diluted to 1 ng/µL with DNase/RNase free water, aliquoted and stored at −80 °C.

Droplet Digital PCR (ddPCR)

RNA was thawed, on ice, and 2-10ng was mixed (based on optimization) with iScript Reverse Transcription Supermix for cDNA synthesis (Bio-Rad, 1708841). Thermocycler setting for cDNA synthesis: 5 min at 25 °C, 20 min at 46 °C, 1 min at 95 °C, hold at 4 °C (constant lid temperature of 105 °C). cDNA was diluted with 2X cDNA storage buffer (equal parts 10 mM Tris HCl (pH 7.5) and 0.1 mM EDTA pH (8.0)) and stored at −20 °C. Probes used for rats were Applied Biosystems #4331182 and the probes spanned across an exon-exon junction (See Table A4.5 for probe details). Equal parts cDNA and master mix were added to tubes, mixed, briefly centrifuged, and 20 µL added to the sample wells of DG8 droplet generator cartridges (Bio-Rad, 1864008). 70 µL of droplet generation oil (Bio-Rad, 1863005) was added to the oil wells in the cartridge. A rubber gasket (Bio-Rad, 1863009) was secured over the cartridge, and a QX droplet generator (Bio-Rad, 186-4002) was used to produce RNA containing droplets. 40 µL of droplets

are transferred to a 96-well plate (Bio-Rad, 12001925) and sealed with pierceable foil (Bio-Rad, 181-4040) by a plate sealer (Bio-Rad, 181-4000). Plates were transferred to a thermocycler (Bio-Rad, C1000), with the following settings: 10 min at 95 °C, 39 cycles (30 s at 94 °C, 1 min at 60 °C), 10 min at 98 °C, hold at 12 °C (constant lid temperature of 105 °C). Plates were then transferred to the QX200 droplet reader (Bio-Rad, 1864003), and results analyzed with QuantaSoft software. For all samples, the gene of interest was normalized to the reference gene, *Rpl13*.

Statistical Analysis

All statistical tests of the results were completed using GraphPad Prism software (version 9, GraphPad, La Jolla, CA). Outliers were assessed with the absolute deviation from the median method (Leys et al., 2013) utilizing the very conservative difference of 2.5X median absolute deviation as the exclusion criteria. Statistical significance was set to $\alpha \leq 0.05$. Comparisons were made across all groups using one-way ANOVA with a *post-hoc* Tukey test.

Results

Experiment 1: Cellular identity of α-syn inclusion associated innate immune transcripts

Microglia within the pSyn containing SNpc express Cd74

Unilateral intrastriatal injection of mouse α-syn PFFs results in peak intraneuronal phosphorylated α-syn (pSyn) aggregation in the SNpc 2 months post injection (Duffy et al., 2018; J. R. Patterson et al., 2019). In all rats in which fluorescent *in situ* hybridization (FISH) was conducted seeding of pSyn inclusions in tyrosine hydroxylase immunoreactive (THir) SNpc neurons ipsilateral to the injected striatal hemisphere was confirmed using immunofluorescence (IF) (Figure 4.2A, B). Tissue sections adjacent to the pSyn seeded SNpc were examined for *Cd74* and *Iba1* mRNA expression. *Cd74* mRNA positive cells were relatively absent in the midbrain with the exception of the ipsilateral, pSyn seeded SNpc and the region of the ventral meninges (Figure 4.2C). CD74 mediates assembly and subcellular trafficking of MHC-II (Schröder, 2016) and is normally expressed by border associated macrophages (BAMs; (Utz & Greter, 2019; van Hove et al., 2019) Figure 4.2C) in both α-syn PFF and PBS injected animals. *Cd74* mRNA positive cells within the SNpc were abundantly present in the ipsilateral SNpc of PFF injected rats and rarely observed in the ipsilateral SNpc of PBS control rats (data not shown). All *Cd74* mRNA positive cells in the ipsilateral inclusion bearing SNpc colocalized with Iba1 mRNA (Figure 4.2D-F). These data suggest that a subset of microglia in close proximity to pSyn containing SNpc neurons express *Cd74*. This finding is consistent with our earlier results demonstrating that a subset of microglia within the pSyn seeded SNpc express MHC-II (Duffy et al., 2018; Miller et al., 2021; Chapter 3- figure 3.4)

Microglia within the pSyn containing SNpc upregulate multiple transcripts associated with innate immune response pathways

We next sought to determine whether microglia are the cellular source of the upregulation in some of the innate immune response pathway associated transcripts previously observed in the earlier RNA-Seq data set. We examined the inclusion bearing ipsilateral SNpc of PFF injected rats using FISH to label specific genes of interest (Table 4.1) combined with IF for Iba1 (Figure 4.3 A-H). mRNA for *Cxcl10, Rt1-a2, Grn, Tyrobp, Fger1g, Csf1r, C3* and *C1qa* was specifically upregulated in the ipsilateral SNpc of PFF injected rats. All selected genes colocalized to Iba1 immunofluorescent cells within the inclusion bearing SNpc (Figure 4.3A *Cxcl10*; Figure 4.3B *Rt1-a2*; Figure 4.3C *Grn*; Figure 4.3D *Tyrobp*; Figure 4.3E *Fcer1g*; Figure 4.3F CSF1r; Figure 4.3G *C3*; Figure 4.3H *C1qa*). Soma size of Iba1 immunofluorescent microglia appeared variable and no relationship between soma size and specific mRNA expression of the individual genes was appreciable. These data suggest that, in addition to *Cd74*, a subset of microglia within the pSyn seeded SNpc upregulate *Cxcl10, Rt1-a2, Grn, Tyrobp, Fger1g, CSF1R, C3* and *C1qa.*

Microglia within the pSyn containing SNpc that upregulate innate immune transcripts are Cd74^{+}

CD74 mediates assembly and subcellular trafficking of MHC-II. In previous studies a significant positive association is observed between pSyn inclusion load and number of MHC-II immunoreactive microglia in the SNpc (Duffy et al., 2018). Thus, in order to determine whether the microglial subpopulation in which innate immune genes are upregulated is the same MHC-II immunoreactive subpopulation that correlates with inclusion load, we used *Cd74* FISH as a

surrogate marker of MHC-II to examine our panel of innate immune response pathway associated transcripts. All selected genes colocalized with *Cd74* mRNA positive cells within the inclusion bearing SNpc at 2-months post α-syn injection (Figure 4.4A *Cxcl10*; Figure 4.4B *Rt1-a2*; Figure 4.4C *Grn*; Figure 4.4D*Tyrobp*; Figure 4.4E *Fcer1g*; Figure 4.4F CSF1r; Figure 4.4G *C3*). These data, combined with earlier findings (Duffy et al., 2018), suggest that a subset of *Cd74*/MHC-II positive microglia within the pSyn seeded SNpc upregulate *Cxcl10, Rt1-a2, Grn, Tyrobp, Fger1g, CSF1R, C3* and that the magnitude of the upregulation of these genes may be directly proportional to the amount of pathological pSyn inclusions.

Additional cell types in the pSyn containing SNpc upregulate Cxcl10 and Rt1-a2

Two of the genes we examined, *Cxcl10* and *Rt1-a2*, colocalized with Iba-1 immunofluorescent microglia (Figure 4.3A, B) and *Cd74*+ microglia (Figure 4.4A, B) in the pSyn containing SNpc, as well as in additional cells that were devoid of Iba1 and *Cd74* (Figure 4.5A, B). This suggests that other cell types beyond microglia, presumably neurons and/or astrocytes (Cebrián et al., 2014; Cebrian et al., 2014; McKimmie & Michlmayr, 2014), may also upregulate *Cxcl10* and *Rt1-a2*in association with α-syn inclusion deposition in the SNpc. Further studies are required to determine the cellular identity of the cells other than microglia that are observed to upregulate *Cxcl10* and *Rt1-a2.*

Experiment 2: Measurement of PD and AD associated neuroinflammatory genes using ddPCR

C3, Cd4, Stat1, Casp1, Axl, and IL18 mRNA are upregulated in pSyn containing SNpc

In a separate experiment we also examined the expression levels of multiple genes previously associated with neuroinflammation in either AD or PD (Boche & Gordon, 2022; Deczkowska et al., 2018; Dubbelaar et al., 2018; Keren-Shaul et al., 2017; Morgan & Mielke, 2021; Sanchez-Guajardo et al., 2013; Srinivasan et al., 2016; Tansey & Romero-Ramos, 2019). The specific genes examined, *C3, Cd4, Stat1, Casp1, Axl, IL18, TLR4, P2Ry12, Lyz2, Il23a, Lpl* and *Irf3,* included genes representing multiple immune pathways (i.e., phagocytosis, T-cell activation, microglial regulation, and cell proliferation, (Table 4.2). Some of these genes were found to be significantly upregulated in the inclusion bearing SNpc in the RNA-Seq dataset (*C3, STAT1*), some were observed to be upregulated in only one sex in the RNA-Seq dataset (*Casp1, TLR4, Lyz2*), some were upregulated but did not pass the false discovery test (*Axl, Il18, P2Ry12, Lpl, Il23a*), while others showed no changes in the RNA-Seq dataset or did not appear (*CD4*). mRNA expression was quantified using ddPCR on microdissected ipsilateral SNpc from naive, PBS control and PFF injected female rats at the 2-month time point. ddPCR analysis shows significant increases in 6 of the 12 genes examined in inclusion bearing SNpc compared to PBS SNpc. Specifically, mRNA expression of *C3* (complement pathway, $p<0.0001$), *CD4* (T cell activation, $p<0.0001$), *Stat1* (JAK/STAT pathway, $p<0.001$), *Casp1* (inflammasome, ***$p<0.001$), *Axl* (phagocytosis, $p<0.001$), and *IL18* (interferon gamma production and inflammasome, **$p<0.01$) was significantly increased in the ipsilateral SNpc of α-syn PFF injected rats compared to PBS controls (Figure 4.6). Of these, *C3, Cd4, Stat1 and Casp1* displayed a 50% or greater upregulation with *C3* demonstrating the highest, four-fold upregulation. No significant changes

were observed in mRNA expression levels of *TLR4, P2Ry12, Lyz2, Il23a, Lpl, Irf3* were observed (Figure 4.6, $p>0.05$). This additional ddPCR data set illustrates that upregulation of multiple immune related genes can be detected in the SNpc during peak α-syn aggregation using a ddPCR approach, including detection of changes not identified using a RNA-Seq approach. Further, some gene expression changes previously associated with neuroinflammation in PD or AD tissue (i.e., P2Ry12, Lyz2, Lpl) are not observed in the inclusion bearing SNpc.

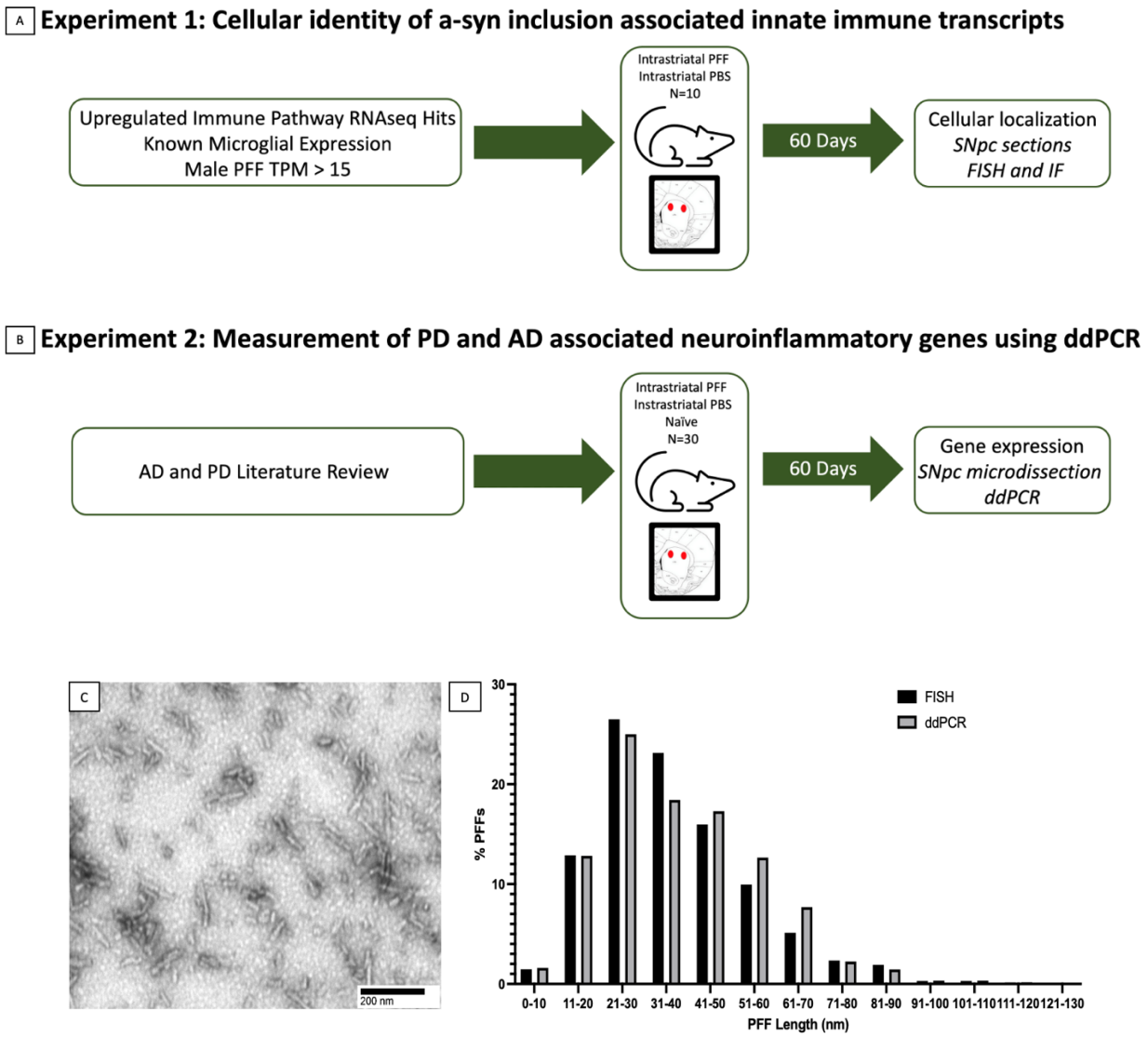

Figure 4.1. Overview of Experimental Design and PFF Size Distribution.

A: Experiment 1: Cellular identity of α-syn inclusion associated innate immune transcripts. Immune related transcripts upregulated in association with α-syn inclusion deposition in the SNpc in a previous RNA-Seq study (J. Patterson et al., 2020) in which transcript per million (TPM) values were > 15 in a-synuclein preformed fibril (α-syn PFF) injected male rats were selected for investigation of cellular source. Young, 3-month-old male Fischer 344 rats received two intrastriatal injections of sonicated α-syn PFFs or an equal volume of phosphate buffered saline (PBS). Rats were euthanized at 2-months post injection and brains were collected for

Figure 4.1. (Cont'd)

immunofluorescence (IF) and fluorescent *in situ* hybridization (FISH) within the SNpc. **B.** Experiment 2: Measurement of PD and AD associated neuroinflammatory genes using ddPCR**.** Expression levels of immune related genes previously identified in the PD or AD literature were examined in a separate cohort of rats. Young, 3-month-old male Fischer 344 rats received two intrastriatal injections of sonicated α-syn PFFs or an equal volume of PBS. Rats were euthanized at 60 days post injection. An additional cohort of naive rats also was included. Gene expression in the ipsilateral microdissected SNpc was quantified using (ddPCR). **C:** Representative electron micrograph of sonicated α-syn fibrils. **D:** Measurement distribution of ~650 sonicated fibrils prior to injection (mean fibril size- FISH surgical cohort: 37.82 ± 0.67 nm, ddPCR surgical cohort: 39.97 ± 0.76 nm). Abbreviations: TPM= transcript per million; PD= Parkinson's disease; AD= Alzheimer's disease; α-syn PFF= alpha-synuclein pre-formed fibrils; PBS= phosphate buffered saline; ddPCR= droplet digital PCR; IF= immunofluorescence; FISH= fluorescent *in situ* hybridization SNpc= substantia nigra pars compacta.

Gene	Protein Name	Control Mean TPM (Male)	PFF Mean TPM (Male)	Estimated Fold Change (Male)
Cxcl10	C-X-C Motif Chemokine Ligand 10	0.437	15.192	2.432
Rt1-a2	RT1 class Ia, locus A2	7.476	16.737	1.105
Grn	Progranulin	8.799	19.255	0.481
Tyrobp	Transmembrane Immune Signaling Adaptor TYROBP	20.215	47.249	0.677
Fcer1g	Fragment of IgE Receptor Ig	47.845	103.891	0.498
Csf1r	Colony Stimulating Factor 1 Receptor	21.616	34.651	0.475
C3	Complement component 3	6.311	26.378	1.783
C1qa	Complement C1q A Chain	26.583	59.431	0.891
Cd74	CD74 Antigen	7.246	65.076	2.236
Cd74	CD74 Antigen	2.336	20.060	2.130

Table 4.1. Transcripts selected for FISH localization in Experiment 1.

CXCL10*:* Chemoattractant for CXCR3+ cells: CD4+ and CD8+ T cells, NK cells, subset of B cells (McKimmie & Michlmayr, 2014). *RT1-A2:* Antigen processing and presentation of endogenous peptide antigen via MHC class I (Hewitt, 2003). *Grn*: Progranulin; Regulation of lysosomal function and microglial responses in the CNS; Neurotrophic and anti-inflammatory properties (Paushter et al., 2018). *Tyrobp*: Enhances phagocytosis; Inflammatory response suppression (Griciuc & Tanzi, 2021) . *Fcer1g*: Couples humoral and cellular immunity; Directs interactions between immune complexes and effectors cells (Bodea et al., 2014). *CSF1R*: Colony stimulating factor 1 receptor; Required for activation, proliferation, and differentiation of microglia (Elmore et al., 2014). *C3*: Complement pathway (Carpanini et al., 2019) . *C1qa*: First component of the serum complement system (Reid, 2018). *CD74*: Mediates assembly and subcellular trafficking of

Table 4.1. (Cont'd)

the MHCII (Schröder, 2016). Abbreviations: TPM= transcript per million; PD= Parkinson's disease; AD= Alzheimer's disease; α-syn PFF= alpha-synuclein pre-formed fibrils; PBS= phosphate buffered saline; ddPCR= droplet digital PCR; IF= immunofluorescence; FISH= fluorescent *in situ* hybridization SNpc= substantia nigra pars compacta; *Cxcl10*= C-X-C Motif Chemokine Ligand 10; *Rt1-a2*= RT1 class Ia, locus A2; *Grn* = Progranulin; *Tyrobp* = Transmembrane Immune Signaling Adaptor TYROBP; *Fcer1g* = Fc Fragment Of IgE Receptor Ig; CSF1R= Colony Stimulating Factor 1 Receptor; C3= complement component 3; *C1qa* = Complement C1q A Chain; *Cd74*= CD74 Antigen (Invariant Polypeptide Of Major Histocompatibility Complex, Class II Antigen-Associated.

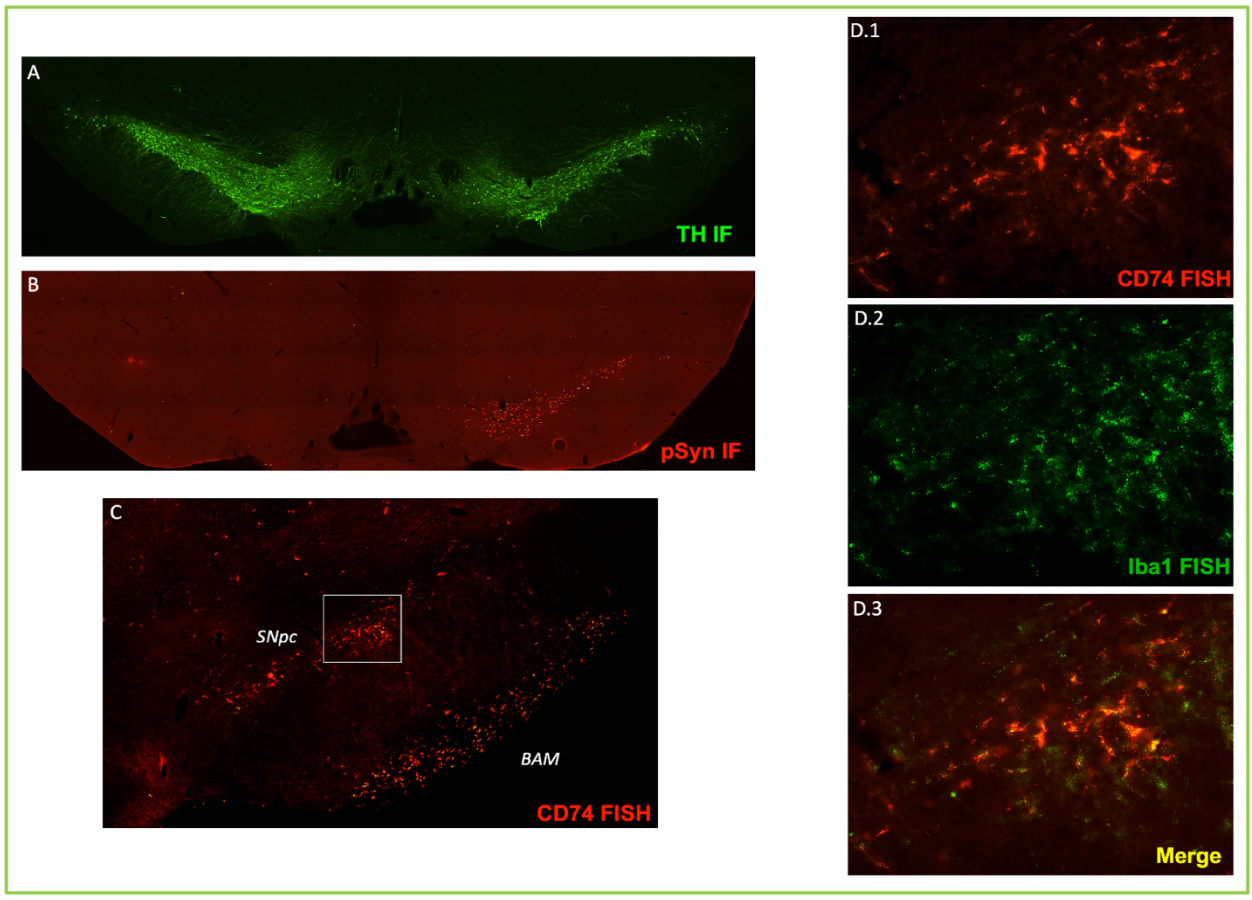

Figure 4.2. **Microglia within the pSyn containing SNpc express *Cd74*.**

A: Representative immunofluorescence (IF) image of tyrosine hydroxylase immunoreactive (THir) neurons within the substantia nigra pars compacta (SNpc). **B**: Representative adjacent IF image revealing phosphorylated alpha-synuclein (pSyn) deposition in the SNpc ipsilateral to PFF injection at 2-months. **C:** Fluorescent *in situ* hybridization (FISH) reveals *Cd74* expression in border associated macrophages (BAMS) and within the ipsilateral SNpc. **D**. *Cd74*mRNA (red) colocalized with Iba1 mRNA (Green) within the α-syn aggregate containing SNpc.

Abbreviations: IF= Immunofluorescence; TH= Tyrosine hydroxylase; pSyn= alpha-synuclein phosphorylated at serine 129; SNpc= substantia nigra pars compacta; BAM= Border associated macrophages; FISH= fluorescent in situ hybridization; *Cd74*= *Cd74* Antigen (Invariant

Figure 4.2. (Cont'd)

Polypeptide of Major Histocompatibility Complex, Class II Antigen-Associated; Iba1= Ionized calcium-binding adaptor molecule 1.

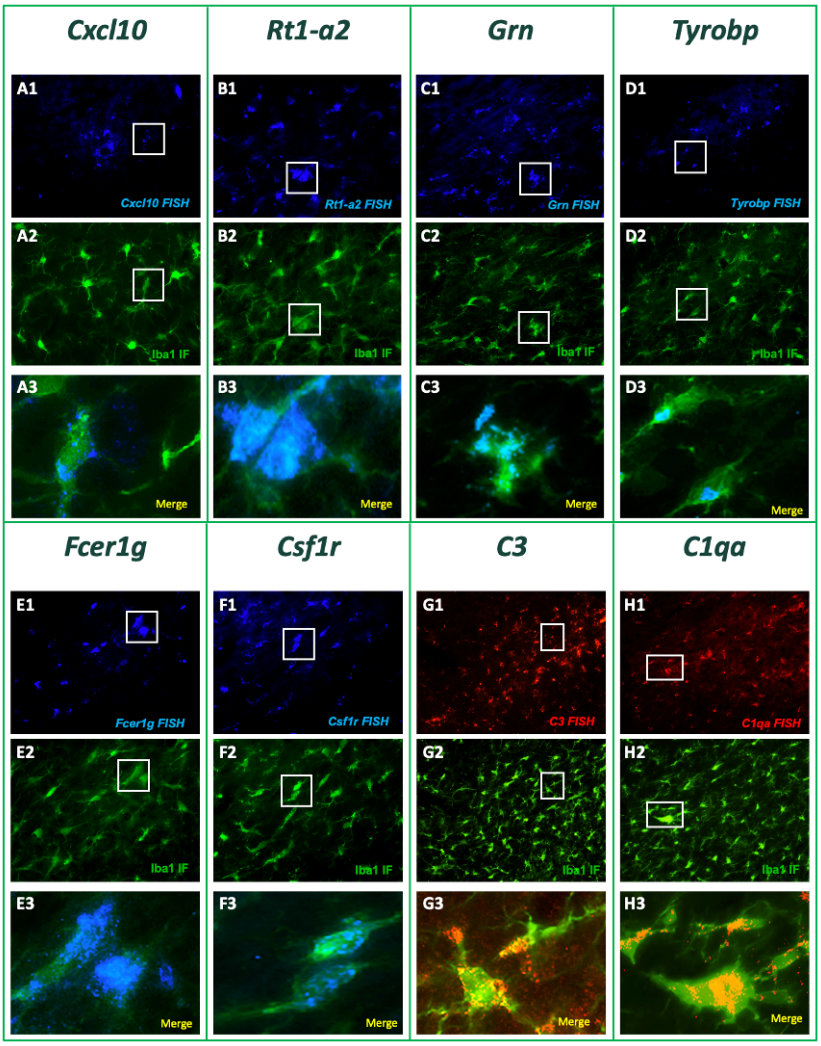

Figure 4.3. Microglia within the pSyn containing SNpc upregulate multiple transcripts associated with innate immune response pathways.

Fluorescent *in situ* hybridization (FISH) for specific target mRNA was combined with immunofluorescence (IF) for ionized calcium binding adaptor molecule 1 (Iba1) to investigate the cellular localization of immune related transcripts upregulated in association with α-syn inclusion deposition in the SNpc in a previous RNA-Seq study (J. Patterson et al., 2020). mRNA for *Cxcl10, Rt1-a2, Grn, Tyrobp, Fger1g, CSF1R, C3* and *C1qa* was specifically upregulated in the ipsilateral SNpc of alpha-synuclein preformed fibril (α-syn PFF) injected rats in which

Figure 4.3. (Cont'd)

phosphorylated α-syn inclusions were present. All selected genes colocalized to Iba1 IF cells within the inclusion bearing SNpc. All images taken in the ipsilateral SNpc. **A:** Representative images of *Cxcl10* FISH (Blue) and Iba1 IF (Green). **B:** Representative images of *Rt1-a2* FISH (Blue) and Iba1 IF (Green). **C:** Representative images of *Grn* FISH (Blue) and Iba1 IF (Green). **D:** Representative images of *Tyrobp* FISH (Blue) and Iba1 IF (Green). **E:** Representative images of *Fcer1g* FISH (Blue) and Iba1 IF (Green). **F**: Representative images of CSF1R FISH (Blue) and Iba1 IF (Green). **G:** Representative images of C3 FISH (Blue) and Iba1 IF (Green). **H:** Representative images of *C1qa* FISH (Blue) and Iba1 IF (Green). Abbreviations: FISH= fluorescence in situ hybridization; IF= immunofluorescence; Ipsilateral= ipsilateral hemisphere relative to α-syn PFF injection; SNpc=substantia nigra pars compacta; Iba1= Ionized calcium-binding adaptor molecule 1; *Cxcl10*= C-X-C Motif Chemokine Ligand 10; *Rt1-a2*= RT1 class Ia, locus A2; *Grn* = Progranulin; *Tyrobp* = Transmembrane Immune Signaling Adaptor TYROBP; *Fcer1g* = Fc Fragment Of IgE Receptor Ig; CSF1R= Colony Stimulating Factor 1 Receptor; C3= complement component 3; C1QA= Complement C1q A Chain; *Cd74*= *Cd74* Antigen (Invariant Polypeptide Of Major Histocompatibility Complex, Class II Antigen- Associated.

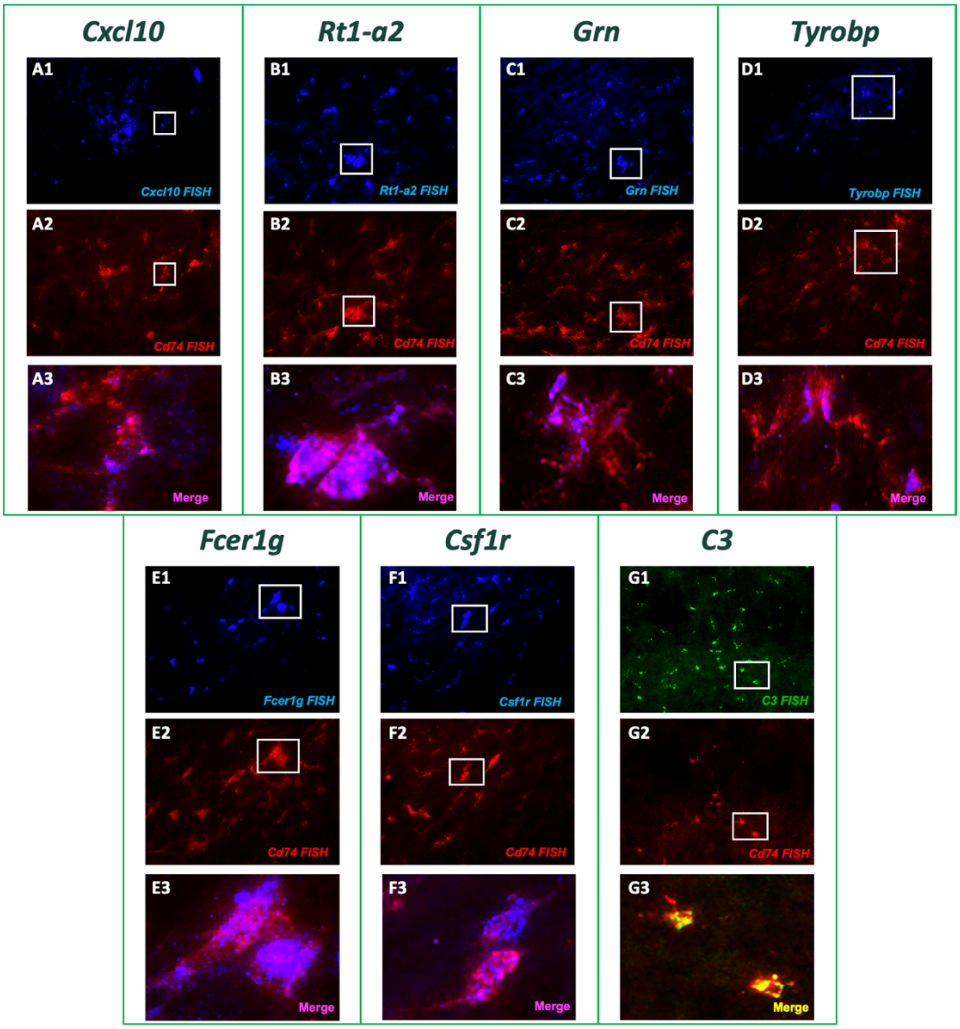

Figure 4.4. Microglia within the pSyn containing SNpc that upregulate innate immune transcripts are *Cd74*$^{+}$.

Fluorescent *in situ* hybridization (FISH) for specific target mRNA was combined with FISH for *Cd74*. a-SAM analysis shows colocalization of selected genes with *Cd74*$^{+}$ microglia. All selected genes colocalized with *Cd74* mRNA positive cells within the inclusion bearing SNpc at 2-months post α-syn injection. All images taken in the ipsilateral SNpc. **A:** Representative images of *Cxcl10* FISH (Blue) and *Cd74* FISH (red). **B:** Representative images of *Rt1-a2*FISH (Blue) and *Cd74* FISH (red). **C:** Representative images of *Grn* FISH (Blue) and *Cd74* FISH (red). **D:** Representative

Figure 4.4. (Cont'd)

images of *Tyrobp* FISH (Blue) and *Cd74* FISH (red). **E:** Representative images of *Fcer1g* FISH (Blue) and *Cd74* FISH (red). **F**: Representative images of CSF1R FISH (Blue) and *Cd74* FISH (red). **G:** Representative images of C3 FISH (Green) and *Cd74* FISH (red).

Abbreviations: FISH= fluorescence in situ hybridization; IF= immunofluorescence; Ipsilateral= ipsilateral hemisphere relative to α-syn PFF injection; SNpc=substantia nigra pars compacta; Iba1= Ionized calcium-binding adaptor molecule 1; *Cxcl10*= C-X-C Motif Chemokine Ligand 10; *Rt1-a2*= RT1 class Ia, locus A2; *Grn* = Progranulin; *Tyrobp* = Transmembrane Immune Signaling Adaptor TYROBP; *Fcer1g* = Fc Fragment Of IgE Receptor Ig; CSF1R= Colony Stimulating Factor 1 Receptor; C3= complement component 3; *C1qa* = Complement C1q A Chain; *Cd74*= *Cd74* Antigen (Invariant Polypeptide Of Major Histocompatibility Complex, Class II Antigen-Associated.

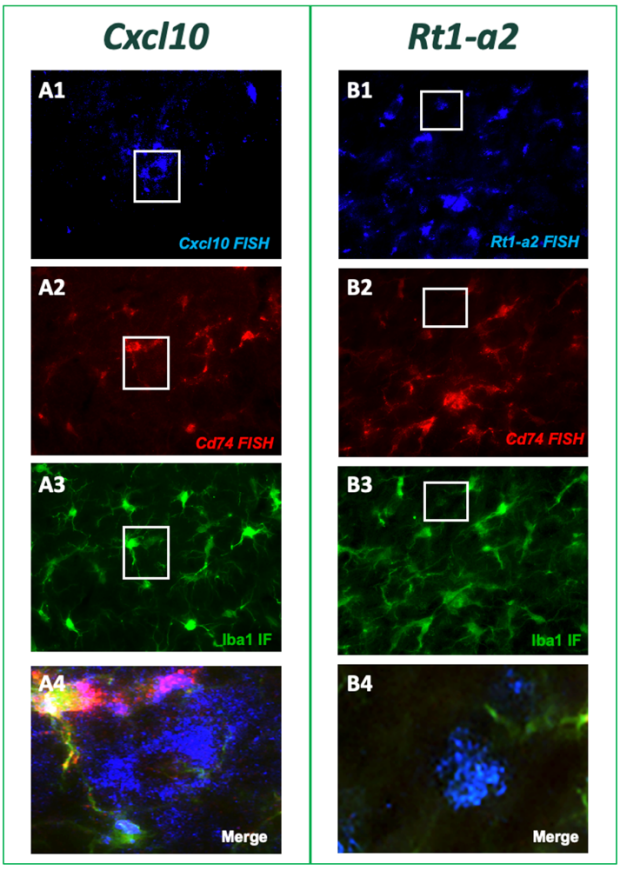

Figure 4.5. **Additional cell types in the pSyn containing SNpc upregulate *Cxcl10* and *Rt1-a2*.** Fluorescent *in situ* hybridization (FISH) for *Cxcl10* or *Rt1-a2* combined with immunofluorescence (IF) for ionized calcium binding adaptor molecule 1 (Iba1) or FISH for *Cd74* reveals upregulation in other cell types in the alpha-synuclein inclusion bearing substantia nigra pars compacta (SNpc), in addition to microglia. All images taken in the ipsilateral SNpc. **A**: Representative images showing *Cxcl10* FISH (Blue) in the absence of either *Cd74* FISH (Red) or Iba1 IF (Green), suggesting expression of *Cxcl10* by another cell type. **B**: Representative images showing *Rt1-a2* FISH (Blue) in the absence of either *Cd74* FISH (Red), or Iba1 IF (Green) suggesting expression of *Rt1-a2* by another cell type.

Figure 4.5. (Cont'd)

Abbreviations: FISH= fluorescence in situ hybridization; IF= immunofluorescence; Ipsilateral= ipsilateral hemisphere relative to α-syn PFF injection; SNpc=substantia nigra pars compacta; Iba1= Ionized calcium-binding adaptor molecule 1; *Cxcl10*= C-X-C Motif Chemokine Ligand 10; *Rt1-a2*= RT1 class Ia, locus A2; *Cd74*= *Cd74* Antigen (Invariant Polypeptide of Major Histocompatibility Complex, Class II Antigen- Associated.

Gene	Protein Name
C3	Complement component 3
Cd4	T-Cell Surface Glycoprotein CD4
Stat1	Signal Transducer and Activator of Transcription 1
Casp1	Caspase 1
Axl	AXL Receptor Tyrosine Kinase
Il18	Interleukin 18
Tlr4	Toll Like Receptor 4
P2ry12	Purinergic Receptor P2Y, G-Protein Coupled, 12
Lyz2	lysozyme 2
Il23a	Interleukin 23 Subunit Alpha
Lpl	Lipoprotein Lipase
Irf3	Interferon Regulatory Factor 3

Table 4.2. Transcripts selected for Droplet Digital PCR analysis in Experiment 2.

C3: Complement Pathway (Carpanini et al., 2019). CD4: T- cell activation through antigen presenting cells (Luckheeram et al., 2012). STAT1: JAK/STAT Pathway, Transcription factor activated by interferons and interleukins (Seif et al., 2017; Tiwari & Pal, 2017). Casp1: Inflammasome activation leading to IL1beta and IL18 activation (Lee et al., 2015). Axl: Microglial phagocytosis responses (Tondo et al., 2019). IL18: Interferon gamma inducing factor in NK cells and T cells (Alboni et al., 2010). TLR4: Pattern recognition receptor (Heidari et al., 2022; Yang et al., 2018). P2Ry12: Microglial regulatory function(Gómez Morillas et al., 2021; Walker et al., 2020) . Lyz2: Lysosomal activation (Deczkowska et al., 2018; Keren-Shaul et al., 2017). IL23a: T cell activation; differentiation and expansion of Th17 cells

Table 4.2. (Cont'd)

(Deczkowska et al., 2018; Nitsch et al., 2021). Lpl: Lipid metabolism (Deczkowska et al., 2018; Keren-Shaul et al., 2017). Irf3: regulator of type I interferon dependent immune responses (Fryer et al., 2021).

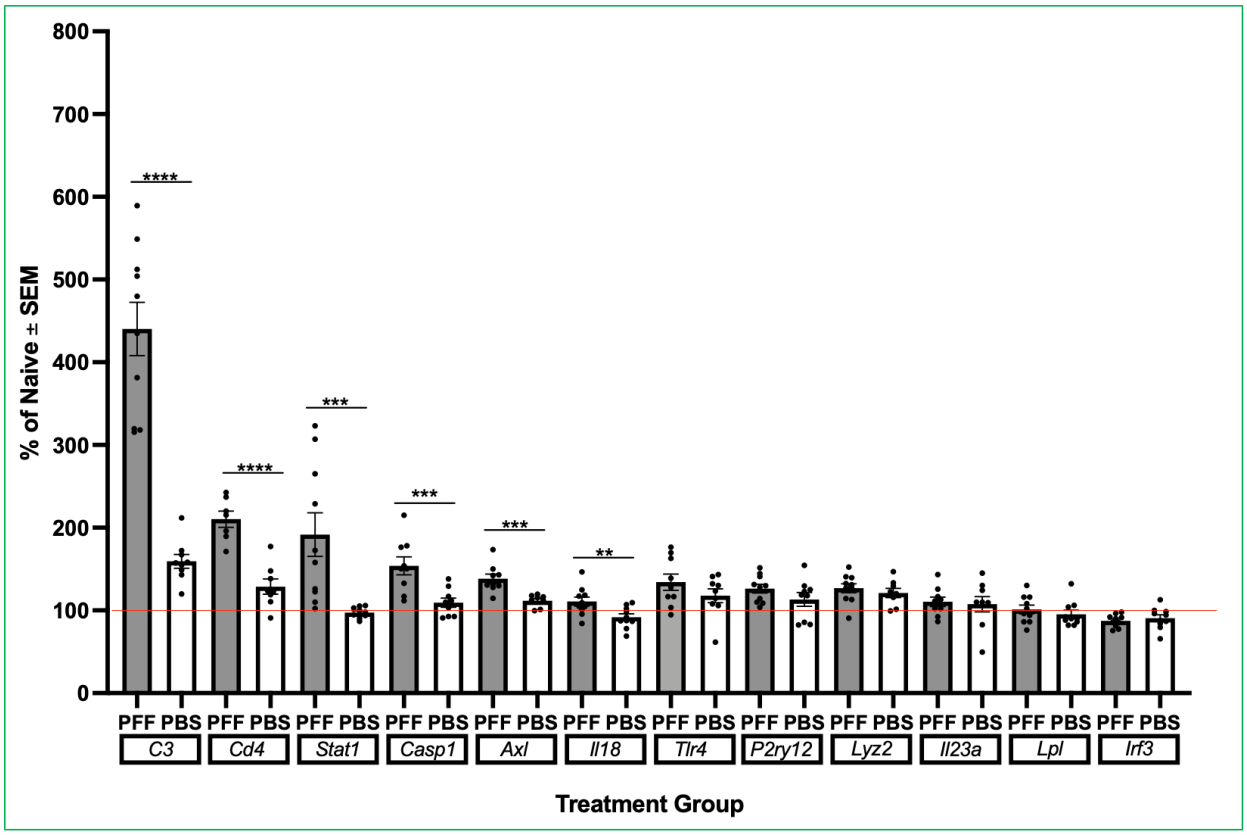

Figure 4.6. Droplet Digital PCR (ddPCR) reveals upregulation of *C3, Cd4, Stat1, Casp1, Axl*, and *IL18* mRNA in the pSyn containing SNpc.

Expression levels of multiple disease associated microglial (DAMS) were quantified in the ipsilateral substantia nigra pars compacta (SNpc) of naive, PBS control and α-syn PFF injected rats at the 2-month time point using ddPCR. Significant increases were observed in *C3, CD4, Stat1, Casp1, Axl* and *IL18* in inclusion bearing SNpc (gray bars) compared to PBS SNpc (white bars). No significant changes were observed for *TLR4, P2Ry12, Lyz2, Il23a, Lpl or Irf3.* The gene of interest was normalized to the reference gene, *Rpl13.* ****p<0.0001, ***p<0.001, **p<0.01. Values represent percent of naive SNpc ± SEM.

Abbreviations: DAMs= Disease associated macrophages; SNpc= substantia nigra pars compacta; PBS= Phosphate Buffered saline; α-syn PFF= alpha-synuclein pre-formed fibrils; ddPCR= droplet

Figure 4.6. (Cont'd)

digital PCR; C3= complement component 3; CD4= T-Cell Surface Glycoprotein CD4; Stat1= Signal Transducer and Activator of Transcription 1; Casp1= Caspase 1; Axl= AXL Receptor Tyrosine Kinase; Il18=Interleukin 18; TLR4= Toll Like Receptor 4; P2Ry12= Purinergic Receptor P2Y, G-Protein Coupled, 12; Lyz2= lysozyme 2; IL23a= Interleukin 23 Subunit Alpha; Lpl= Lipoprotein Lipase; Irf3= Interferon Regulatory Factor 3.

Discussion

Under homeostatic conditions microglia are dynamic cells that continuously survey the microenvironment (Nimmerjahn et al., 2005). Upon activation, microglia undergo morphological changes including cell body enlargement and thickening of ramified processes. Both morphological changes and MHC-II expression are associated with neurodegeneration, however these changes alone cannot provide definitive information regarding the genetic phenotype that is being expressed by these cells (Cartier et al., 2014; Walker & Lue, 2015). In the present study, evaluation beyond microglial soma size and MHC-II expression was conducted to increase our understanding of the gene expression changes of microglia responding to chronic α-syn aggregation and degeneration insults. Immune associated transcripts, previously identified using an unbiased RNA-Seq approach to exhibit the greatest magnitude of upregulation and expression in the α-syn inclusion bearing SNpc (Patterson et al., 2020), were analyzed to identify their cellular localization. Our results demonstrate that a subset of microglia in immediate proximity to pSyn inclusions upregulate *Cd74, Cxcl10, Rt1-a2, Grn, Tyrobp, Fger1g, CSF1R, C3* and *C1qa.* Our FISH approach identifies an **a**-**s**yn **a**ggregate associated **m**icroglial (a-SAM) phenotype.

The a-SAM phenotype includes expression of multiple proinflammatory genes, including *Rt1-a2, Fcer1g, C3, C1qa,* and *Cd74*. *Rt1-a2*encodes for MHC-I expression to display intracellular proteins to cytotoxic CD8+ T-cells (Hobson & Sulzer, 2022) and *Cd74* is an MHC class II chaperone, MHC-II displays extracellular proteins to cytotoxic CD4+ T-cells (Stumptner-Cuvelette & Benaroch, 2002). *C3* and *C1qa* are critical components of the complement pathway that tag foreign pathogens for elimination by phagocytosis (Bodea et al., 2014) and *Fcer1g*

mediates efficient phagocytosis (Bodea et al., 2014; Sierksma et al., 2020). However, some of the a-SAMs that we identified are also associated with anti-inflammatory effects. Signaling of TREM2 (Triggering Receptor Expressed on Myeloid Cells 2) through TYROBP has been shown to suppress proinflammatory signals (Griciuc & Tanzi, 2021). *G*RN regulates lysosomal function and proinflammatory signals (Paushter et al., 2018). Whereas CXCL10 is a chemoattractant for CXCR3+, CD4+ T cells, CD8+ T cells, NK cells, and a subset of B cells (McKimmie & Michlmayr, 2014) it also has also been shown to be inhibit proinflammatory cytokine synthesis (Arimoto et al., 2007). Figure 4.7 illustrates an overall schematic of the a-SAMs we have identified. Many of the proteins encoded by the a-SAM genes are interconnected in their functions (Figure A4.8). These findings lend credence to the concept of a heterogeneous immune response, illustrating a dynamic interplay to maintain immune response balance (De la Fuente et al., 2012; Dubbelaar et al., 2018). The inherent genetic program of the brain's innate immune system initially evolved to protect against bacteria, viruses, and parasites. Only very recently in evolution was the immune system required to respond to chronic human neurodegenerative conditions of aging, perhaps explaining the “hodgepodge” of immune responses we observe in our study (Morgan & Mielke, 2021).

While not providing specific cellular localization, our ddPCR results identified additional neuroinflammatory genes that are upregulated in inclusion bearing nigral tissue, including *Cd4, Stat1, Casp 1, Axl* and *IL18.* These genes are associated with phagocytosis (Axl, (Burstyn-Cohen & Hochberg, 2021; Tondo et al., 2019), interferon production pathways (Stat1, (Barcia et al., 2011; Paul et al., 2021), inflammasome activation (Casp1 and Il18; (Gordon et al., 2018; Wang et al., 2016; Xu et al., 2011), and the adaptive immune system (CD4;(Brochard et al., 2009;

Mittal et al., 2019; J. A. H. Saunders et al., 2012)). Interestingly, CD4+ cells, as well as CD8+ cells have been observed in postmortem PD brains (Brochard et al., 2009).

Based on the literature, there were some genes that we expected to see changes in but did not. Two of these genes are P2Ry12 and TLR4. Purinergic Receptor P2RY12 has mainly been considered a marker of resting microglia (Boche & Gordon, 2022; Gómez Morillas et al., 2021; Heidari et al., 2022) that is required for microglial environmental surveillance and decreases expression in activated, responding microglia (Boche & Gordon, 2022). However, our data shows no change in P2Ry12 in the α-syn inclusion containing SNpc as compared to the controls. Walker et al (2020) showed a similar lack of decrease in P2Ry12 in microglia surrounding diffuse plaques within postmortem AD brains (Walker et al., 2020). We also did not observe any changes in TLR4 during the aggregation phase of the PFF model. Toll-like receptors are innate immune receptors that recognize pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs), endogenous substances released during tissue damage (Heidari et al., 2022). PD patients have elevated serum levels of TLR4 proteins which correlate with disease stage (Yang et al., 2018). It is likely that alterations in P2Ry12 and TLR4, as well as many other inflammation-associated genes, are disease-state dependent. Indeed, inflammatory markers differs between different PD stages as well as between PD and Incidental Lewy Body Disease (ILBD, α-syn aggregation in the absence of degeneration) (Dijkstra et al., 2015; Walker et al., 2016). One limitation of the present study is that analysis of neuroinflammation was conducted only during the aggregation stage, understanding inflammatory gene expression changes associated with the neurodegeneration stage of the α-syn PFF model would be informative. Overall, our results demonstrate the heterogeneity of the

inflammatory response to α-syn aggregates within the SNpc. Validation of the a-SAM microglial phenotype is required in PD and iLBD tissue to confirm the same gene and pathway changes. A more comprehensive understanding of the gene expression signature of microglia associated with pathological a-syn aggregates may facilitate future investigation of anti-inflammatory, disease-modifying approaches for PD.

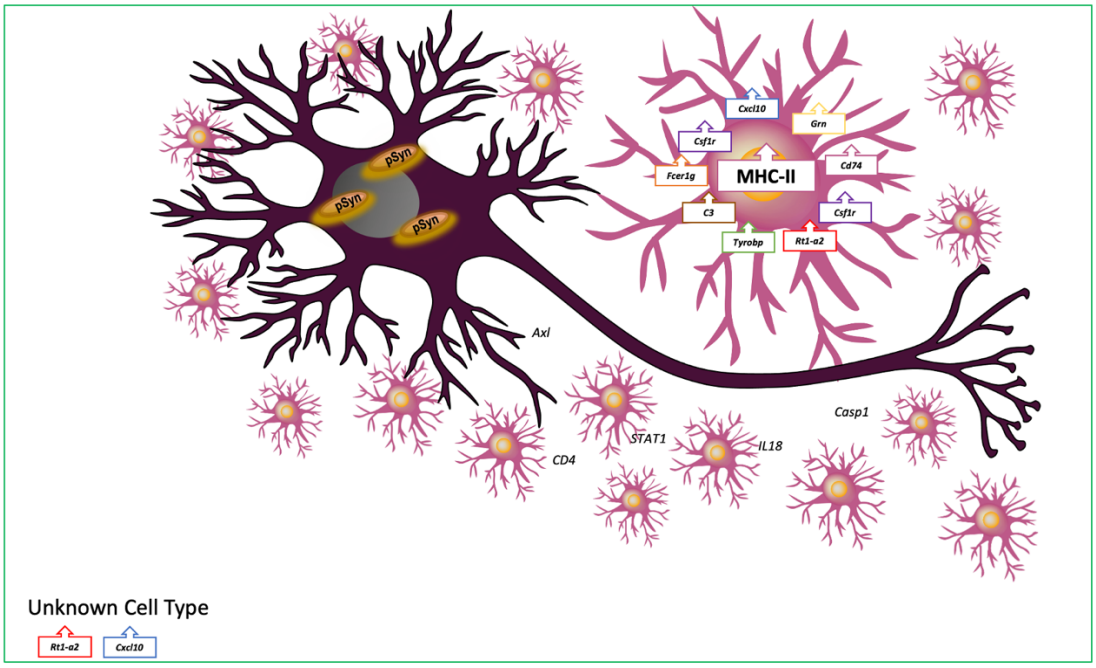

Figure 4.7. **Overview schematic of immune related genes upregulated in association with pSyn accumulation in the SNpc**.

A subset of microglia within the immediate proximity of alpha-synuclein bearing ipsilateral SNpc neurons upregulate *Cd74, Cxcl10, Rt1-a2, Grn, CSF1R, C3,* and *C1qa*. This **a**-synuclein **a**ggregate associated **m**icroglial (a-SAM) phenotype implicates the involvement of multiple immune pathways in the microglial response to a-syn aggregation in the SNpc, prior to degeneration. In addition, *Rt1-a2* and *Cxcl10* are upregulated by additional cell types. Using our ddPCR approach

Figure 4.7. (Cont'd)

we have identified additional neuroinflammatory genes, including *Cd4, Stat1, Casp1, Axl* and *IL18*, all of which are significantly upregulated in inclusion bearing nigral tissue. Findings in the present study suggest that the deposition of pathological a-syn inclusions in the SNpc is associated with perturbations in immune functions related to complement, inflammasome and T cell activation, phagocytosis, and interferon gamma production.

Abbreviations: Ipsilateral= ipsilateral hemisphere relative to a-syn PFF injection; SNpc=substantia nigra pars compacta; Iba1= Ionized calcium-binding adaptor molecule 1; *Cxcl10*= C-X-C Motif Chemokine Ligand 10; *Rt1-a2*= RT1 class Ia, locus A2; *Grn* = Progranulin; *Tyrobp* = Transmembrane Immune Signaling Adaptor TYROBP; *Fcer1g* = Fc Fragment Of IgE Receptor Ig; CSF1R= Colony Stimulating Factor 1 Receptor; C3= complement component 3; C1QA= Complement C1q A Chain; *Cd74*= *Cd74* Antigen (Invariant Polypeptide Of Major Histocompatibility Complex, Class II Antigen- Associated; CD4= T-Cell Surface Glycoprotein CD4; Stat1= Signal Transducer And Activator Of Transcription 1; Axl= AXL Receptor Tyrosine Kinase; Il18=Interleukin 18.

APPENDIX

Gene	Control Mean TPM (Female)	PFF Mean TPM (Female)	Estimated Fold Change (Female)
Cxcl10	0.175	2.049	3.176
Rt1-a2	4.502	13.104	0.863
Grn	8.968	14.573	0.765
Tyrobp	18.831	38.099	0.831
Fcer1g	44.388	74.207	0.740
Csf1r	18.752	30.145	0.459
C3	4.199	26.023	1.553
C1qa	20.704	51.263	0.755
Cd74	6.101	61.511	2.291
Cd74	2.369	20.648	2.168

Table A4.3. Transcripts selected for FISH localization female TPM and estimated fold changes.

Abbreviations: TPM= transcript per million; PD= Parkinson's disease; AD= Alzheimer's disease; α-syn PFF= alpha-synuclein pre-formed fibrils; PBS= phosphate buffered saline; ddPCR= droplet digital PCR; IF= immunofluorescence; FISH= fluorescent *in situ* hybridization SNpc= substantia nigra pars compacta; *Cxcl10*= C-X-C Motif Chemokine Ligand 10; *Rt1-a2*= RT1 class Ia, locus A2; *Grn* = Progranulin; *Tyrobp* = Transmembrane Immune Signaling Adaptor TYROBP; *Fcer1g* = Fc Fragment Of IgE Receptor Ig; CSF1R= Colony Stimulating Factor 1 Receptor; C3= complement component 3; C1QA= Complement C1q A Chain; *Cd74*= *Cd74* Antigen (Invariant Polypeptide Of Major Histocompatibility Complex, Class II Antigen-Associated.

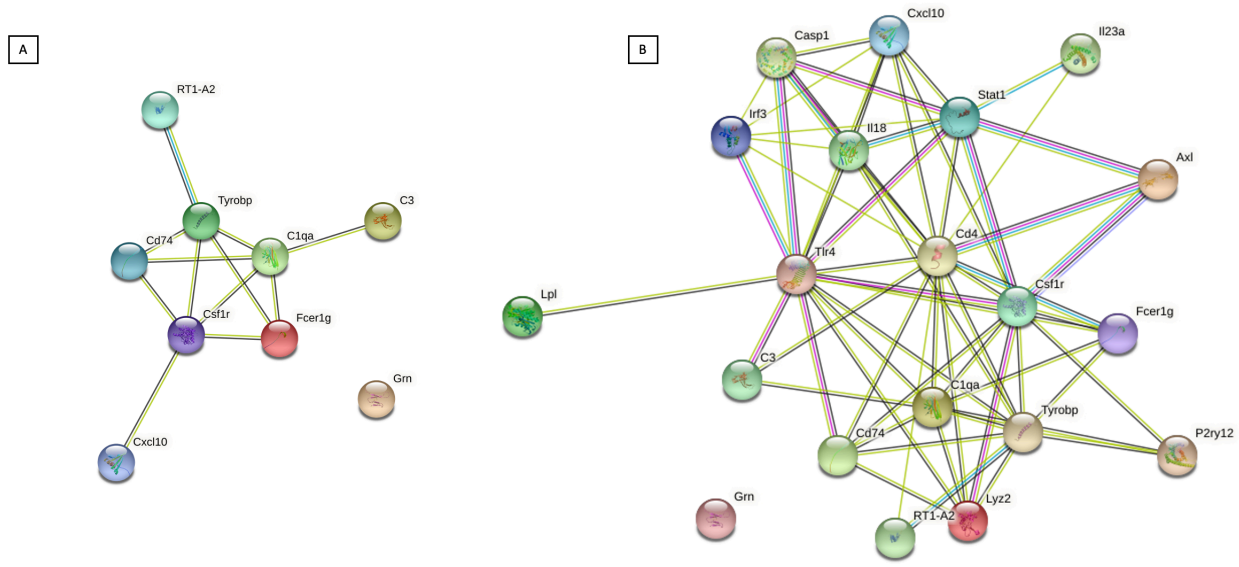

Figure A4.8. a-SAM genes show interconnected functional pathways.

A: a-SAM genes STRING protein function network. **B**: All Protein (a-SAM and ddPCR) STRING protein function network.

Abbreviations: *Cxcl10*= C-X-C Motif Chemokine Ligand 10; *Rt1-a2*= RT1 class Ia, locus A2; *Grn* = Progranulin; *Tyrobp* = Transmembrane Immune Signaling Adaptor TYROBP; *Fcer1g* = Fc Fragment Of IgE Receptor Ig; CSF1R= Colony Stimulating Factor 1 Receptor; C3= complement component 3; C1QA= Complement C1q A Chain; *Cd74*= *Cd74* Antigen (Invariant Polypeptide Of Major Histocompatibility Complex, Class II Antigen- Associated; Aif1=allograft inflammatory factor 1 (Iba1=Ionized calcium binding adaptor molecule 1); CD4= T-Cell Surface Glycoprotein CD4; Stat1= Signal Transducer And Activator Of Transcription 1; Axl= AXL Receptor Tyrosine Kinase; Il18=Interleukin 18; TLR4= Toll Like Receptor 4; P2Ry12= Purinergic Receptor P2Y, G-Protein Coupled, 12; Lyz2= lysozyme 2; IL23a= Interleukin 23 Subunit Alpha; Lpl= Lipoprotein Lipase; Irf3= Interferon Regulatory Factor 3

Gene	Probe Reference #
C3	866601
Aif1	457731-C2
Cxcl10	553191-T8
Rt1-a2	1165371-T8
Tyrobp	1165381-T8
Grn	862741-T8
Fcer1g	1165391-T8
Csf1r	485771-T8
Cd74	489711-T9

Table A4.4. FISH probe details.

All probes designed and purchased from Advanced Call Diagnostics Incorporated.

Abbreviations: *Cxcl10*= C-X-C Motif Chemokine Ligand 10; *Rt1-a2*= RT1 class Ia, locus A2; *Grn* = Progranulin; *Tyrobp* = Transmembrane Immune Signaling Adaptor TYROBP; *Fcer1g* = Fc Fragment Of IgE Receptor Ig; CSF1R= Colony Stimulating Factor 1 Receptor; C3= complement component 3; C1QA= Complement C1q A Chain; *Cd74*= *Cd74* Antigen (Invariant Polypeptide Of Major Histocompatibility Complex, Class II Antigen- Associated; Aif1=allograft inflammatory factor 1 (Iba1=Ionized calcium binding adaptor molecule 1).

Gene	Probe Cat #
C3	Rn00566466_m1
Cd4	Rn005622856_m1
Stat1	Rn00583505_m1
Casp1	Rn00562724_m1
Axl	Rn00627285_m1
Il18	Rn01422083_m1
Tlr4	Rn00569848_m1
P2ry12	Rn00575653_m1
Lyz2	Rn00562794_m1
Il23a	Rn00590334_g1
Lpl	Rn00561482_m1
Irf3	Rn01764369_m1
Rpl13	Rn00821258_g1

Table A4.5. ddPCR probe details.

All Probes purchased from Thermo Fischer Scientific.

Abbreviations: ddPCR= droplet digital PCR; C3= complement component 3; CD4= T-Cell Surface Glycoprotein CD4; Stat1= Signal Transducer and Activator of Transcription 1; Axl= AXL Receptor Tyrosine Kinase; Il18=Interleukin 18; TLR4= Toll Like Receptor 4; P2Ry12= Purinergic Receptor P2Y, G-Protein Coupled, 12; Lyz2= lysozyme 2; IL23a= Interleukin 23 Subunit Alpha; Lpl= Lipoprotein Lipase; Irf3= Interferon Regulatory Factor 3; Rpl13= Ribosomal Protein L1.

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Chapter 5: General Conclusions and Remaining Questions

General Conclusions

The presented work has accomplished several goals. Chapter 1 provides an overview of the clinical and pathological aspects of Parkinson's disease (PD), the current laboratory animal models, and current treatment strategies. It further focused on the current understanding of the role neuroinflammation has in the etiology and progression of PD. Chapter 2 summarizes the literature surrounding the use of the α-syn PFF model of synucleinopathy to study inflammation. This chapter describes the ability to utilize the two distinct phases, the aggregation phase, and the neurodegeneration phase, in order to understand the inflammatory response to each.

Chapter 3 investigates the therapeutic potential of microglia depletion within the α-syn PFF model of synucleinopathy. This study demonstrated that partial microglial depletion does not prevent α-syn aggregation in the SNpc or the striatum, attenuate the inflammatory response to aggregation or degeneration, or prevent nigral degeneration following intrastriatal PFF injection. Microglial based inflammation has been under investigation in the hope to identify a therapeutic target in order to dampen down the inflammation seen in PD. However, this study suggests that partial microglia depletion may not be an effective, disease modifying therapeutic approach. Indeed, partial microglial depletion may induce a heightened proinflammatory state in remaining microglia.

Chapter 4 investigates the **a**-**s**yn **a**ggregate associated **m**icroglial (a-SAM) phenotype and associated immune response pathways. Collectively these findings implicate that the deposition of pathological α-syn inclusions in the SNpc is associated with perturbations in immune functions related to complement, inflammasome and T cell activation, phagocytosis, and

interferon gamma signaling. The activation of not only inflammatory pathways but also anti-inflammatory pathways provide a look into the immune system's ability to balance its responses to an insult. A more comprehensive understanding of the multidimensional response of microglia to pathological α-syn aggregates may help to uncover novel therapeutic targets that could facilitate future anti-inflammatory, disease-modifying strategies for PD.

Based on the results from the experiments in this dissertation some immediate follow up investigations can be envisioned.

How does partial depletion affect astrocytes (and other cell types)?

While microglia have been the main cell of study, and the focus of this dissertation, astrocytes are, in fact, the most abundant glial cell type of the brain (Matejuk & Ransohoff, 2020). Markers of activated astrocytes have been observed in postmortem PD brains (Booth et al., 2017; Rostami et al., 2020). Further studies have shown the ability for microglia to control astrocyte function (Liddelow et al., 2017, 2020; Yun et al., 2018). Previous studies have demonstrated that complete microglial depletion (~99%) leads to an increase in astrocytic mRNA markers, GFAP and S100 (Elmore et al., 2014). However, to our knowledge, the effect of partial depletion upon astrocytes has yet to be examined within a PD model of insult. As such, analysis of astrocyte morphology and gene expression changes would shed further light on the interplay between microglia and astrocytes in response to α-syn aggregation and neurodegeneration. In addition, cell types beyond astrocytes also contribute to the inflammation response and communicate with microglia, including peripheral macrophages,

adaptive immune cells. Further examination of these cell types and their responses to microglia depletion should be analyzed.

Does microglial repopulation change the degeneration cascade?

Repopulation of microglia following depletion is suggested to lead to the replacement of pro-inflammatory activated microglia allow for new naive microglia to fill the niche and protect from long term inflammation (Barnett et al., 2021; Coleman et al., 2020). While partial microglial depletion did not lead to a change in the inflammatory response to a-syn aggregates or degeneration, previous studies that have achieved near complete microglial depletion have mixed results as to whether repopulation is beneficial (Elmore et al., 2014; Han et al., 2019a) . To better understand the possible therapeutic potential of microglial repopulation within the a-syn PFF model a greater magnitude of microglial depletion will be needed (i.e., increase Pexidartinib dosage or a genetic depletion method (Han et al., 2017)). Depletion throughout the aggregation phase followed by repopulation during the degeneration phase, through the removal of the depletion method, may provide insight as to whether microglial repopulation can lead to a change in the degeneration cascade.

What drives the widespread MHC-II response that is occurring with long term partial depletion?

Long term partial microglial depletion shows a widespread MHC-II-ir inflammatory response regardless of the presence of a-syn aggregates or degeneration. While it is plausible to assume that remaining microglia upregulate MHC-II expression, some evidence exists to suggest that astrocytic expression of MHC-II can occur (Rostami et al., 2020). Further analysis

into the identity of the widespread inflammatory cells through colocalization of MHC-II with microglial or astrocytic markers will allow for the better understanding of the effects that long term partial microglial depletion has on the immune environment.

How does partial depletion affect the a-SAM phenotype?

In Aim 2 we endeavored to characterize the a-SAM phenotype in the α-syn PFF model. Microglia have been considered a possible therapeutic target due to the early microglial response seen in PD (Barnett et al., 2021; Bennett et al., 2018; Han et al., 2019b; Tansey & Romero-Ramos, 2019) and Aim 1 sought to understand whether depletion of microglia could attenuate disease progression. The response of microglia to the pathological events in the PFF model persisted, specifically increased soma size and MHC-II expression, despite significant microglial depletion. What remains to be understood is whether the a-SAM phenotype is similarly maintained under conditions of microglia depletion. Ongoing studies are attempting to answer this question through analysis of a-SAM gene expression within the SNpc following months of Pexidartnib treatment.

Are all the a-SAM genes expressed by the same microglia or do microglial subpopulations exist?

While our results strongly suggest that the a-SAM genes colocalize with CD74+ microglia within the SNpc, however confirmation with confocal microscopy and z-stack analysis is still required for definitively demonstrate co-expression. Further, it remains unclear whether all a-SAM genes are expressed by all CD74+ cells or if there is a heterogeneity in the expression of

the genes. To examine this issue further *in-situ* hybridization analysis could be conducted in a multiplex approach to examine all a-SAM genes simultaneously.

How does the a-SAM phenotype change with disease progression?

Previous work has shown differences in the pattern of inflammation in postmortem tissue from PD and iLBD patients (Dijkstra et al., 2015; Walker et al., 2016). In Aim 2 we have made progress in characterizing the a-SAM phenotype and investigating the different immune pathways that respond to α-syn aggregation. Follow up experiments, looking at additional phases of the α-syn PFF model, in particular the neurodegeneration phase, would increase our understanding of disease-stage specific immune system responses. Once a degeneration associated microglial phenotype was identified in the PFF model, these findings would require validation in PD and iLBD tissue.

Questions Still to Answer: The Bigger Picture

What is the spatial transcriptomic pattern of the microglial response to α-syn *aggregation?*

Microglia have been shown to have unique gene expression profiles within different regions of the brain (Mastroeni et al., 2018) . In our present approach we targeted specific a-SAM genes based on TPMs from bulk RNAseq data and/or a literature search followed by cellular localization analysis with *in-situ* hybridization. However, with newer modern technology an unbiased spatial analysis of gene expression could be accomplished using a spatial transcriptomic analysis. A spatial transcriptomic approach would allow for analysis of gene expression as well as cellular localization with greater sensitivity that is not limited by the TPMs

needed for *in-situ* hybridization. Utilizing tissue sections at 2-months post a-syn PFF injection that has been immunohistochemically labeled for Iba1 (pan microglia marker) the tissue can be hybridized to the spatial transcriptomic chip containing an RNA array allowing for the expression pattern to be localized and analyzed. This method would allow for the transcriptomic analysis of the entire microglial population within a multitude of brain structures, with and without a-syn aggregates (i.e., ipsilateral versus contralateral SNpc).

Does microglial activation/neuroinflammation contribute to degeneration?

This work demonstrates the heterogeneity and multidimensional aspects of the immune system response to α-syn aggregation. One question that remains is whether inflammation is truly a contributor to disease progression or merely a byproduct. In vitro, in the absence of microglia and other immune cells, neurons seeded with α-syn aggregates will die (Volpicelli-Daley et al., 2014). This demonstrates that α-syn aggregation triggered cell death can be a cell autonomous phenomenon. We provide evidence that upregulation of a multitude of immune pathways are associated with α-syn pathology, and that this occurs prior to degeneration in the vivo PFF model system, however the actual contribution of the immune system to disease progression remains unanswered. Our microglial depletion experiment described in Chapter 3 initially sought to address this question, however depletion of the microglial "army" did not impact the localized microglial "special forces", and thus we were unsuccessful in providing evidence to support or refute the role of microglia in neurodegeneration. However, a future study with the ability for complete microglial depletion (Han et al., 2017) can help to further the understanding of the role microglia may play in neurodegeneration.

What specifically triggers activation and MHC-II expression on inclusion-associated microglia?

Inflammation research focuses on microglia due to the MHC-IIir expression on microglia found in the vicinity of Lewy pathology (Imamura et al., 2003; McGeer et al., 1988). However, the exact cause of microglial activation remains unknown. The presence of MHC-II complex suggests that microglia have internalized something from the extracellular milieu and are presenting it to the adaptive immune system (CD4+ T cells). The exact epitope that is being presented is unknown, although it has been hypothesized that the MHC-II complex might be presenting a form of α-syn, or a protein that is induced by α-syn aggregation (Harms et al., 2013), or neuronal debris (Beyer et al., 2000).

To identify the MHCII bound peptide a liquid chromatography- tandem mass spectrometry (LC-MS/MS) and quantification analysis may be able to be done (Bozzacco & Yu, 2013; Gerber et al., 2003). This protocol calls for the isolation of the MHCII- peptide complex through immunoprecipitation followed by LC-MS/MS and quantification. Utilizing 2-month post a-syn PFF injected tissue, the MHCII complex can be isolated from the ipsilateral SNpc and run through the LC/MS-MS for identification and quantification of MHCII peptides from endogenous proteins. Identification of the epitope may allow for a more targeted approach to prevent inflammation rather than attenuate it. Alternatively, perhaps microglial response to inflammation is not the earliest neuroinflammatory event. MHC-I expression on dopamine neurons in the PD brain has been observed (Cebrián et al., 2014; Cebrian et al., 2014), raising the possibility that neurons may initiate the neuroinflammatory response.

What role does phagocytosis play?

When neurons are in distress, as is presumed to be the case when α-syn aggregates are present, they can send out "eat me" or "do not eat me signals" to microglia as to whether phagocytosis should ensue. Phagocytosis of neurons by microglia can occur within hours (Brown & Neher, 2014). A large inflammatory response is seen in response to α-syn aggregation, that includes genes related to phagocytosis, however neurons are not absent (if phagocytosed by microglia) until months later in the model. In this context, what is the tipping point in Lewy-body containing neurons that results in "eat me" signals. Do aggregate-containing neurons release "do not eat me" signals for a period and then switch to an "Eat me" signal later on? Is there an "eat me" signal but phagocytotic processes fail? Does MHC-II expression result due to failed phagocytosis? Further analysis of the "eat me" signals (i.e., milk fat globule EGF factor 8 (MFG-E8), microglial vitronectin receptors (VNRs) and Annexin A1) along with "do not eat me" signal (i.e., CD47, sialic acid-binding immunoglobulin-like lectin-11 (SIGLEC-11), and plasminogen activator inhibitor type 1 (PAI1) can help elucidate whether phagocytosis is occurring within the SNpc in the presence of a-syn aggregates during phase 1 of the a-syn PFF model (Brown & Neher, 2014).

Answers to any of the aforementioned questions would significantly advance our understanding of the immune system's role and contribution to disease progression in PD.

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