

Investigating microglial and neuronal cellular responses to alpha-synuclein preformed fibril and adeno-associated viral vector delivery to the rat substantia nigra and to primary cortical rat cell cultures



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Dedication

To Kevin and Helena. May this research be a ripple in the wave of your work.

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university. This body of work is entirely my own, and was conducted with the help of Dr. Danka Kozareva, Dr. Teresa Burke-Mullaly, Matthew McAuslan, Rebecca Maher, Myles Corrigan, and Daniel McLoone.

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March, 2025

Disclosures

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Summary

The aetiology of synucleinopathic disease is stochastic. Through a culmination of age, genetic predisposition, and environmental insult, changes in glial and neuronal cell physiology can drive the onset and progression of symptoms associated with Parkinson's disease (PD), Parkinson's disease with dementia (PDD), pure autonomic failure (PAF), multiple system atrophy (MSA), or Lewy body dementia.

These neurodegenerative conditions are characterised by the aggregation of alpha-synuclein (α Syn) into Lewy bodies, proteinaceous intracytoplasmic inclusions which drive dysfunction and degeneration of neuronal and glial cells in which they accumulate. Chronic microglial activation has been well regarded as a detrimental consequence of α Syn aggregation in prodromal stages of synucleinopathy, and is thought to precede Lewy body accumulation and neurodegeneration. In PD, this is evident in the substantia nigra, a midbrain nucleus within the basal ganglia, which plays an important role in motor function. α Syn-mediated microglial activation leads to the upregulation of soluble pro-inflammatory signalling mediators, which provoke damage to vulnerable neurons within the degenerating substantia nigra. α Syn may also prime microglia to elicit an enhanced and prolonged pro-inflammatory response upon subsequent recognition of otherwise amenable immune challenges. Persistent microglial stimulation drives an inflammatory response, which is cytotoxic to neurons. While mid-to-late-stage PD symptomatology is predominantly directed by intraneuronal α Syn aggregation, reactive microglial-associated neuronal death within the substantia nigra likely plays a significant role in driving symptoms of early-stage disease. As such, targeting microglial activation to alleviate inflammation may be an effective means for ameliorating α Syn-associated neurodegeneration and associated motor dysfunction.

The locus coeruleus (LC), a neuronal nucleus of the dorsolateral pontine tegmentum, is the primary hub of noradrenergic cell bodies and the principal source from which noradrenaline is supplied to nearly all regions of the brain and spinal cord. A catecholaminergic neurotransmitter, noradrenaline is the endogenous agonist for G-protein coupled alpha- and beta-adrenergic receptors, which are expressed on neuronal and glial cell membranes. Noradrenaline excites potent anti-inflammatory and neurotrophic properties in the brain through interaction with the beta2 adrenergic receptor (β_2 AR). Both noradrenaline and β_2 AR agonists can suppress the proliferation of microglia and moderate the expression of pro-inflammatory markers. There is evidence in support of a neuroprotective role for noradrenaline and β_2 AR agonists in the α Syn-burdened brain, where, in addition to mitigating chronic microglial activation and cytokine production, β_2 AR stimulation can regulate α Syn gene and protein expression, and promote astrocytic neurotrophin production.

This thesis describes experimental approaches to develop a preclinical model in which the effect of α Syn on microglial activation and early-stage PD-like neurodegeneration in adult rats may be assessed. The model was further employed to assess the efficacy of a long-acting brain penetrable pharmacological β_2 AR agonist to regulate microglia and provide neuroprotection in the rat substantia nigra. Microglial and neuronal interaction was subsequently assessed in α Syn-treated primary rat glial and neuronal cell cultures, to assess the potential for α Syn to prime microglial reactivity, and to reduce neuronal complexity, a key feature of early-stage neurodegeneration.

Human recombinant wild-type (WT) α Syn preformed fibrils (PFFs), or A53T-mutant α Syn PFFs (A53T PFFs) were unilaterally delivered, either alone, or in combination with wild-type or A53T-mutant α Syn-expressing adeno-associated viral (AAV) vector, via stereotactic surgical injection into the substantia nigra of adult male and female Wistar rats. To assess the potential for β_2 AR activation to alleviate pathology induced by α Syn administration, animals were further treated with the long-

acting brain penetrable β_2 AR agonist formoterol two weeks following α Syn delivery. At four and five weeks post-delivery, skilled motor function and limb-use asymmetry were assessed in the stepping and cylinder tests, respectively. Rotational behaviour in the amphetamine-induced rotations test was undertaken to functionally evaluate the presence of a unilateral nigral lesion. At six weeks-post-delivery, animals were euthanised by sodium pentobarbital overdose, and their brains fixed for post-mortem analyses through transcardial perfusion and fixation. Cryopreserved brain tissue was serially sectioned in preparation for immunofluorescence and histochemical analyses of α Syn burden, microglial reactivity, and nigral neuronal integrity. α Syn burden was assessed through immunofluorescence staining of α Syn phosphorylated at serine residue 129 (pSer129), a post-translational modification of the α Syn protein associated with cytotoxic aggregation. Microglial reactivity was assessed through histological staining of ionized calcium binding adapter molecule 1 (Iba1), a microglial specific calcium binding protein and marker of microglial activation. Nigral dopaminergic neuronal cell counts were assessed through histological staining for tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine biosynthesis and phenotypic marker of dopaminergic neurons.

WT PFF alone, drove a reduction in use of the limb contralateral to the injection site for skilled motor behaviours, promoted phosphorylation of endogenous α Syn, and stimulated an increase in Iba1+ microglial reactivity in the ipsilateral substantia nigra six weeks after unilateral intranigral injection, without incurring changes to nigral TH+ dopaminergic neuronal cell counts. Intranigral delivery of WT PFF, in combination with WT α Syn-expressing AAV (WT PFF+AAV), drove a further reduction in contralateral limb-use, increase in α Syn phosphorylation, and rise in Iba1+ microglial cell counts in ipsilateral the substantia nigra, where nigral TH+ dopaminergic neuronal cell counts remained unchanged. Intranigral delivery of A53T PFF alone, also reduced use of the limb contralateral to the injection site for skilled motor behaviours, but failed to affect α Syn phosphorylation, or stimulate changes in Iba1+ or TH+ immunoreactivity in the nigra. In contrast, unilateral intranigral delivery of A53T PFF, in combination with AAV expressing human A53T-mutant α Syn (A53T PFF+AAV), increased α Syn phosphorylation, increased Iba1+ immunoreactivity, and reduced TH+ dopaminergic neuronal cell counts in the nigra six weeks post-delivery. Cellular changes induced by A53T PFF+AAV delivery were coupled with reduced contralateral limb-use in tests of skilled motor behaviour. Formoterol (0.1mg/kg, i.p., once daily for 7 days) treatment failed to alter pSer129+ or Iba1+ immunoreactivity, but appeared to mitigate the severity of TH+ neuronal cell body loss in the ipsilateral substantia nigra six weeks post A53T PFF+AAV delivery, compared to vehicle-treated controls. Behaviourally, treatment with formoterol may partially prevent the reduction in contralateral limb-use in spontaneous motor tasks associated with intranigral A53T PFF+AAV delivery.

Clinically, pharmacological agents which target the effects of α Syn on microglial-neuronal interaction in early stages of neurodegeneration may prove useful for the treatment of neurodegenerative synucleinopathic conditions. The development of clinically relevant preclinical models in which new or existing drug candidates can be assessed will be essential for identifying disease-modifying therapies. In addition to targeting β_2 AR activation to influence α Syn expression or microglial activation, alternative targets which may afford protection against α Syn-associated neuronal cell loss must be assessed for disease intervention.

In vitro cell culture models can be used to elucidate underlying disease mechanisms, identify promising drugs targets, and to inform experiments in animal models of synucleinopathy and early-stage neurodegeneration. Rat primary mixed glial, enriched microglial, and mature cortical neuronal cultures were employed to assess a role for glial cells as mediators of α Syn-induced neuronal atrophy, and explore variant forms of α Syn in producing these effects *in vitro*. Primary mixed glial

and enriched microglial cultures were prepared from neonatal rat cortices, grown to maturity, and treated with human recombinant WT PFF, A53T PFF, dopamine-stabilised oligomers (DSO), epigallocatechin gallate-stabilised oligomers (EGCGO), or soluble filaments (FILL). Chemical stabilisation of oligomeric α Syn by either dopamine or epigallocatechin gallate is required to stabilise the protein in its oligomeric form and prevent spontaneous fibrillation during experimental use.

Rat interferon gamma (IFN γ) was employed as a treatment to stimulate glial cells in culture, to assess the response of α Syn-primed microglia to subsequent stimulation. Release of lactate dehydrogenase (LDH) from cells into culture media was measured to assess cell viability. Iba1+ immunocytochemistry was conducted to assess microglial morphology, and quantitative real-time polymerase chain reaction (qRT-PCR) was employed to assess mRNA expression of target interleukins (IL) and tumour necrosis factors (TNF) characteristic of stimulated microglia (IL-1 β , IL-1 α , IL-6, IL-10, and TNF α). Glial conditioned media was collected and applied to mature primary cortical neuronal cultures. LDH release was further measured to assess neuronal cell viability. Immunocytochemical staining of microtubule association protein 2 (MAP2), a neuronal cytoskeletal protein enriched in neuronal dendrites, was conducted to trace neuronal outgrowth and complexity.

Neither filaments, oligomers, nor fibrils induced overt changes to microglial morphology or mRNA expression of target cytokines. Transfer of conditioned media from α Syn-treated glial cultures did not drive changes in primary neurite outgrowth, neuritic length, or branch extension. Application of IFN γ to α Syn-primed glia pre-treated with PFFs also induced negligible changes to mRNA expression of target cytokines and microglial morphology. Glial conditioned media from α Syn-primed glial cultures subsequently stimulated with IFN γ had no effect on neuronal complexity. With the aim of enhancing the glia response to known immunogenic stimuli in culture, cell culture protocols were amended to increase the quantity of microglial cells in both mixed and enriched glial cultures. Nonetheless, glial response to treatment with PFF and/or IFN γ remained negligible, as did the effects of conditioned media transfer on primary neuronal complexity. Alternatives to primary cultures, including immortalised monocytic cell lines or induced pluripotent stem cell (iPSC)-derived glial cultures may allow for future exploration of the molecular mechanisms through which α Syn mediates reactive-glial-associated neuronal atrophy, and for the assessment of neuroprotective treatment strategies.

Primary rat glia and neurons in culture proved only mildly reactive to α Syn PFFs, which showed a propensity to increase Iba1+ cells in the rat substantia nigra *in vivo*. Differential outputs following the use of these constructs in animal models compared to in cell culture models raise questions about the glial stimulatory properties and/or aggregatory abilities which α Syn PFFs exhibit when applied *in vivo* versus *in vitro*.

Table of Contents

Dedication	2
Declaration	3
Disclosures	4
Acknowledgements	5
Summary	6
Table of Contents	9
Abstract.....	16
List of abbreviations	17
List of figures	22
Chapter 1: Introduction	30
1.1. Parkinson's disease.....	30
1.1.1. Parkinson's disease aetiology.....	30
1.1.2. Vulnerability of dopaminergic neurons in the midbrain in Parkinson's disease.....	31
1.1.3. Insight from neurotoxin models of Parkinson's disease.....	31
1.2. Alpha-synuclein: the primary structural component of Lewy bodies	32
1.2.1. Synucleinopathic diseases.....	32
1.2.2. Alpha-synuclein structure, function, distribution, and aggregation.....	35
1.2.3. Exploiting the molecular features of fibrillar alpha-synuclein for the development of disease-relevant experimental models of synucleinopathy	39
1.2.4. Adeno-associated viral vector tools for increasing alpha-synuclein expression in experimental models of synucleinopathy	39
1.2.5. Combination of alpha-synuclein preformed fibrils and adeno-associated viral vector-mediated alpha-synuclein expression to recapitulate synucleinopathy in a rodent model.....	45
1.3. Inflammation at the wheel of disease progression in the alpha-synuclein-burdened brain	46
1.3.1. A role for microglia in the healthy brain	46
1.3.2. Overactive microglia impair neuronal function.....	47
1.3.3. Inflammatory models of synucleinopathy.....	50
1.3.4. Primed microglia exacerbate neurodegeneration in the alpha-synuclein-burdened brain.....	52
1.4. Noradrenaline: an innate anti-inflammatory neurotransmitter.....	54
1.4.1. Noradrenergic vulnerability in the synucleinopathic brain	54

1.4.2. Reconciling alpha-synuclein aggregation, inflammation, and dopaminergic degeneration by enhancing the noradrenergic tone	56
1.4.2.1. Beta2 adrenergic receptor activation regulates alpha-synuclein gene expression and translation to protein	56
1.4.2.2. Beta2 adrenergic receptor activation suppresses microglial activation and cytokine expression	57
1.4.2.3. Beta2 adrenergic receptor activation promotes astrocytic production of neurotrophic factors	58
1.4.2.4. Beta2 adrenergic receptor activation confers neuroprotection in animal models of neurodegeneration	58
1.5. Evaluating the potential of formoterol in the treatment of alpha-synuclein-associated inflammation in preclinical models of neurodegenerative disease.....	59
1.6. Aims and objectives of this thesis	60
Chapter 2: Materials and Methods.....	62
2.1. Materials	62
2.2. <i>In vivo</i> methods.....	67
2.2.1. Animals	67
2.2.2. Preformed fibril preparation for use <i>in vivo</i>	67
2.2.3. Adeno-associated viral vector preparation for use <i>in vivo</i>	67
2.2.4. Stereotactic surgery.....	67
2.2.5. Formoterol preparation	68
2.2.6. Behavioural Assessments.....	68
2.2.6.1. Cylinder test of limb-use asymmetry.....	68
2.2.6.2. Stepping test of forelimb akinesia	69
2.2.6.3. Amphetamine-induced rotations test	69
2.2.7. Transcardial perfusion and tissue cryopreservation	71
2.2.8. Tissue processing	71
2.2.9. pSer129 immunofluorescence (with TH co-stain).....	71
2.2.10. Imaging and analysis of pSer129+ alpha-synuclein immunofluorescence	71
2.2.11. Iba1 immunohistochemistry	72
2.2.12. Imaging and analysis of Iba1+ microglia	72
2.2.13. TH immunohistochemistry	73
2.2.14. Image and analysis of TH+ neurons.....	73
2.3. <i>In vitro</i> methods.....	73
2.3.1. Aseptic technique	73
2.3.2. Preparation of primary cortical neuronal cultures.....	73
2.3.3. Preparation of primary cortical mixed glial cultures	74
2.3.4. Preparation of primary cortical enriched microglial cultures	74
2.3.5. Preparation of alpha-synuclein species for treatment of primary neuronal and glial cultures	75
2.3.6. Collection of mixed glial and enriched microglial conditioned media.....	75
2.3.7. Lactate dehydrogenase cytotoxicity assay	75

2.3.8. Iba1 immunocytochemistry	75
2.3.9. Imaging and analysis of Iba1+ microglia.....	76
2.3.10. MAP2 immunocytochemistry	78
2.3.11. Imaging and analysis of MAP2+ mature neurons	78
2.3.12. Quantitative real time polymerase chain reaction (qRT-PCR)	80
2.3.12.1. Cell lysis	80
2.3.12.2. RNA isolation	80
2.3.12.3. cDNA manufacture	80
2.3.12.4. Polymerase chain reaction	80
2.4. Data analysis and graphical presentation	81

Chapter 3: An assessment of PD-related pathologies six weeks following unilateral delivery of wild-type or A53T-mutant human alpha-synuclein preformed fibrils to the rat substantia nigra 82

3.1. Introduction	82
3.2. Aims and objectives.....	85
3.3. Experimental design	86
3.4. Methods	87
3.5. Results	88
3.5.1. Cylinder test.....	88
3.5.2. Stepping test	92
3.5.3. Amphetamine-induced rotations test.....	96
3.5.4. pSer129 immunofluorescence	97
3.5.5. Iba1 immunohistochemistry	99
3.5.6. TH immunohistochemistry.....	102
3.6. Discussion	105
3.7. Chapter conclusion & next steps.....	112

Chapter 4: An assessment of PD-related pathology in rats six weeks following unilateral intranigral delivery of human alpha-synuclein preformed fibrils in combination with human alpha-synuclein-expressing adeno-associated viral vector 113

4.1. Introduction	113
4.2. Aims and objectives.....	119
4.3. Experimental design	120
4.4. Methods	122
4.5. Results	123
4.5.1. Cylinder test.....	123
4.5.2. Stepping test	126
4.5.3. Amphetamine-induced rotations test.....	129

4.5.4. pSer129 immunofluorescence.....	130
4.5.5. Iba1 immunohistochemistry	132
4.5.6. TH Immunohistochemistry	134
4.6. Discussion.....	136
4.7. Chapter conclusion and next steps	141

Chapter 5: An investigation of the effects of the beta2 adrenergic receptor agonist, formoterol, on PD-related pathology in rats following intranigral delivery of A53T-mutant human alpha-synuclein preformed fibrils in combination with alpha-synuclein-expressing adeno-associated viral vector

142

5.1. Introduction.....	142
5.2. Aims and objectives	147
5.3. Experimental design.....	148
5.4. Methods.....	149
5.5. Results.....	150
5.5.1. Cylinder test.....	150
5.5.2. Stepping test.....	154
5.5.3. Amphetamine-induced rotations test.....	158
5.5.4. pSer129 immunofluorescence.....	159
5.5.5. Iba1 immunohistochemistry	161
5.5.6. TH Immunohistochemistry	164
5.5.7. Iba1, TH, pSer129 correlation	166
5.1. Discussion.....	168
5.2. Concluding remarks and next steps	178

Chapter 6: Utilising alpha-synuclein filaments, oligomers, and preformed fibrils to develop an *in vitro* model of PD-related inflammation and neurodegeneration

179

6.1. Introduction.....	179
6.2. Aims and objectives	186
6.3. Experimental design.....	187
6.4. Methods.....	188
6.5. Results.....	192
6.5.1. Mixed glial response to 24-hour treatment with α Syn filaments, oligomers, or PFFs (4 μ g/ml).....	192
6.5.3. Mixed glial response to treatment with WT PFFs (4 μ g/ml) for 24-96 hours.....	197
6.5.4. Neuronal response to treatment with conditioned media transferred from mixed glial cultures treated with WT PFFs (4 μ g/ml) for 24-96 hours.	202
6.5.5. Mixed glial response to 24-hour treatment with IFN γ	204

6.5.6. Neuronal response to treatment with conditioned media from mixed glia treated for 24 hours with IFN γ .	207
6.5.7. Mixed glial response to pre-treatment with A53T PFFs for 24 hours prior to treatment with IFN γ .	209
6.5.8. Neuronal response to treatment with conditioned media transferred from mixed glia pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ .	211
6.5.9. Mixed glial response to pre-treatment with IFN γ for 24 hours prior to treatment with A53T PFF.	213
6.5.10. Neuronal response to treatment with conditioned media transferred from mixed glia pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF.	215
6.5.11. Enriched microglial response to treatment with IFN γ for 24 hours.	217
6.5.12. Neuronal response to treatment with conditioned media transferred from enriched microglial cultures treated for 24 hours with IFN γ .	220
6.5.13. Neuronal response to treatment with conditioned media transferred from enriched microglia pre-treated with A53T PFF for 24 hours prior to treatment with IFN γ .	222
6.5.14. Neuronal response to treatment with conditioned media transferred from enriched microglia treated with IFN γ in DMEM.	223
6.6. Discussion	224
Chapter 7: Discussion	233
7.1. Summary of findings	233
7.2. The prion hypothesis: using preformed fibrils as seeds for accelerating prion-like pathology in models of synucleinopathy.	234
7.3. Considerations for intranigral injection protocols used for the development of unilateral rodent models of synucleinopathy	236
7.4. Inclusion of appropriate controls in <i>in vitro</i> and <i>in vivo</i> investigations of alpha-synuclein preformed fibril and/or adeno-associated viral vector associated synucleinopathy	237
7.5. Optimization of <i>in vitro</i> models to further assess glial-neuronal interactions associated with the application of alpha-synuclein to glia and neurons in culture	240
7.6. Differences in preformed fibril origin, generation, and purification protocols may account for variation in cellular response	243
7.7. Astrocytic involvement in synucleopathic disease	245
7.8. Sex differences in rodent models of synucleinopathy.	246
7.9. Considerations for the use of non-steroidal anti-inflammatory drugs as post-surgical analgesics in animal models of neuroinflammation	248
7.10. Prospects of repurposing beta2 adrenergic receptor agonists for the treatment of Parkinson's disease and other synucleinopathies	249
Appendix I: An assessment of PD-related pathologies in the rat substantia nigra six weeks following intranigral delivery of A53T-mutant alpha-synuclein-expressing adeno-associated viral vector.	254

1.1. Background (introduction, aims, objectives, and experimental design)	254
1.2. Results	256
1.2.1. pSer129+ Immunofluorescence	256
1.2.2. Iba1+ Immunohistochemistry	256
1.2.3. TH+ Immunohistochemistry	256
1.3. Discussion and conclusion	261
 Appendix II: An assessment of the impact of formoterol on PD-related pathologies six weeks following unilateral intranigral delivery of wild-type alpha-synuclein preformed fibrils	
263	
2.1. Background (introduction, aims, objectives, and experimental design)	263
2.2. Results	266
2.2.1. Cylinder test	266
2.2.2. Stepping test	270
2.2.3. Rotarod test	275
2.2.4. pSer129 immunofluorescence	276
2.2.5. Iba1 immunohistochemistry	277
2.2.6. TH immunohistochemistry	278
2.3. Brief conclusion	279
 Appendix III: An assessment of the effect of formoterol on neuronal and glial cellular responses to a systemic lipopolysaccharide challenge following unilateral delivery of wild-type alpha-synuclein preformed fibrils to the rat substantia nigra	
280	
3.1. Background (introduction, aims, objectives, and experimental design)	280
3.2. Results	282
3.2.1. Cylinder test	282
3.2.2. Stepping test	286
3.2.3. Rotarod test	291
3.2.4. pSer129 immunofluorescence	292
3.2.5. Iba1 immunohistochemistry	293
3.2.6. TH immunohistochemistry	294
3.3. Brief conclusion	295
 Appendix IV: Immunofluorescence and histochemical approaches to confirm the presence of alpha-synuclein in WT PFF-treated animals	
296	
4.1. Fluorescence immunohistochemistry	296
4.2. DAB immunohistochemistry	299
 Appendix V: Dissemination	
302	

Publications.....	302
Poster Presentations	302
Outreach.....	303
Bibliography	306

Abstract

Intracytoplasmic accumulation of the alpha-synuclein (α Syn) protein in the brain is a pathogenic hallmark of Parkinson's disease and other synucleinopathic diseases. In the diseased brain, α Syn exploits its innate ability to recruit naïve monomers for nucleation, polymerisation, and elongation into larger order polymers of fibrillar structure. α Syn fibrils obstruct physiological cellular processing by propagating throughout neuronal networks and aggregating intracellularly into proteinaceous inclusions called Lewy bodies. Prior to Lewy body accumulation and neuronal dysfunction, fibrillar α Syn triggers chronic microglial activation and production of pro-inflammatory cytokines, which inflict cytotoxic damage to vulnerable neuronal populations. The A53T mutation, an autosomal dominant point mutation in the gene encoding α Syn, is thought to accelerate the capacity for α Syn to fibrillise, and to promote microglial activation towards a pro-inflammatory phenotype in the brain. Activation of the glial expressed beta2 adrenergic receptor (β_2 AR) drives anti-inflammatory signalling in the brain, conferring protection against neurodegeneration associated with microglial activation. As such, pharmacologically targeting β_2 ARs may prove efficacious in alleviating α Syn-induced inflammation and neurodegeneration observed in patients with synucleinopathy. Development of clinically relevant animal models of synucleinopathy are warranted to provide a means to assess mechanisms of α Syn-associated microglial activation and neurodegeneration, and to evaluate the potential of combative treatment strategies targeting underlying disease mechanisms. The present investigation involves the development of a novel rat model of synucleinopathy, comprising unilateral intranigral fibrillar deposition and genetic overexpression of α Syn to drive microglial activation, neurodegeneration, and motor dysfunction representative of neuropathology observed in human synucleinopathic conditions. Unilateral stereotactic delivery of A53T-mutant human recombinant α Syn preformed fibrils (PFF), in combination with adeno-associated viral (AAV) vector-mediated expression of A53T-mutant human α Syn, into the substantia nigra, induced phosphorylation of endogenous rat α Syn, stimulated microglial activation, and reduced dopaminergic neuronal cell bodies in the substantia nigra ipsilateral to the injection. Cellular changes were coupled with reduced contralateral limb-use, six weeks post-delivery. Treatment with the long-acting and brain penetrable β_2 AR agonist formoterol, previously reported to reduce microglial activation in an inflammatory rat model of Parkinson's disease, proved ineffective in altering the distribution of phosphorylated α Syn or prominence of microglial reactivity. Formoterol treatment appeared to mitigate the unilateral loss of nigral dopaminergic neuronal cell bodies, and partially alleviate motor behavioural changes associated with intranigral A53T PFF+AAV. These results suggest a possible neuroprotective role for β_2 AR activation in synucleinopathic conditions. Further experiments, which assessed α Syn-induced changes in rat primary microglial cultures, and related changes in neuronal complexity following the transfer of glial conditioned media to primary neuronal cultures, were inconclusive regarding the possible underlying α Syn-associated glial-neuronal interactions contributing to mechanisms of neurodegeneration. Treatment of mixed and enriched microglial cultures with α Syn PFFs failed to trigger microglial activation, or glial-associated changes in neuronal complexity. These outcomes highlighted the limitations of the present *in vitro* approaches for modelling glial-neuronal interactions in neurodegenerative disease states, and/or evaluating the potential of pharmacological agents for disease modifying approaches. Further research is required to optimise models and to elucidate glial-neuronal interactions underlying neurodegeneration in synucleinopathic diseases.

List of abbreviations

AADC	Aromatic amino acid decarboxylase
AAP	Assembly activating protein
AAV	Adeno-associated virus
ABC	Avidin biotin complex
ADHD	Attention deficit hyperactivity disorder
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analyses of variance
ARRIVE	Animal Research: Reporting of <i>In vivo</i> Experiments
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
A30P	Alanine to proline substitution at 30 th amino acid of SNCA protein
A53T	Alanine to threonine substitution at 53 rd amino acid of SNCA protein
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
β_2AR	Beta2 adrenergic receptor
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CBA	Chicken β -actin
CBD	Corticobasal degeneration
CBE	Conduritol β -epoxide
CCL2	Chemokine ligand 2
CD	Cluster of differentiation
cDNA	Complementary DNA
CDNF	Cerebral dopamine neurotrophic factor
CMV	Cytomegalovirus
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CREB	cAMP-response element binding
CSF	Cerebrospinal fluid
CXCL10	Chemokine ligand 10
DAB	3,3-diaminobenzidine
DAMP	Damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole

DAT	Dopamine transporter
DDC	DOPA decarboxylase
DIV	Days <i>in vitro</i>
DLB	Dementia with Lewy bodies
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPA	3, 4-dihydroxyphenylalanine
DSO	Dopamine-stabilised oligomers
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EGCGO	Epigallocatechin gallate-stabilised oligomers
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assays
engDMEM	Enriched glial DMEM
ER	Endoplasmic reticulum
EtOH	Ethanol
E35K	Glutamic acid to lysine substitution at 35th amino acid of SNCA protein
E46K	Glutamic acid to lysine substitution at 46th amino acid of SNCA protein
E57K	Glutamic acid to lysine substitution at 57th amino acid of SNCA protein
FILL	Filaments
fNBM	Feeding NBM
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCase	Glucocerebrosidase
GDNF	Glial cell line-derived neurotrophic factor
Gc	Genome copies
GCI	Glial cell inclusion
GFAP	Glial fibrillary acid protein
gmCSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G-protein-coupled receptor
H3K27	Histone 3 lysine 27
IFN	Interferon
IL	Interleukin
ImiD	Immunomodulatory drug

iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal (injection)
iPSC	Induced pluripotent stem cell
iRBD	iREM behaviour disorder
iREM	Isolated rapid eye movement
ITR	Inverted terminal repeats
IκBα	NFκB inhibitor protein
JAK/STAT	Janus kinase (JAK) signal transducer and activator of transcription (STAT)
JNK	cJun NH2-terminal kinase
K⁺	Potassium
LAMP	Lysosome-associated membrane protein
LB	Lewy body
LC	Locus coeruleus
LDH	Lactate dehydrogenase
L-DOPA	Levodopa
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAO-B	Monoamine oxidase-B
MAP2	Microtubule associated protein 2
MAPK	Mitogen-activated protein kinase
MetOH	Methanol
MHC	Major histocompatibility complex
MPP⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine
mRNA	Messenger ribonucleic acid
MSA	Multiple system atrophy
mxgDMEM	Mixed glial DMEM
Na⁺	Sodium
NAC	Non-amyloid-β component
NaCl	Saline
NAD⁺	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide + hydrogen
NAT	Noradrenaline transporter
NBM	Neurobasal media

NFκB	Nuclear factor-κ B
NGF	Nerve growth factor
NGS	Normal goat serum
NHS	Normal horse serum
NKA	Na ⁺ /K ⁺ activated ATPase
NLRP	NOD-like receptor protein
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NRI	Noradrenaline reuptake inhibitor
NSAID	Non-steroidal anti inflammatory
OPCA	Olivopontocerebellar atrophy
p	Promoter
pA	Poly-adenosine
PAF	Pure autonomic failure
Pam3	Pam3Csk4
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-T	PBS with 0.1% Triton-X
PD	Parkinson's disease
PDD	Parkinson's disease with dementia
PET	Positron emission tomography
PFF	Preformed fibrils
PGP	P-glycoprotein
PI3K	Phosphatidyl inositol-3 kinase
PINK1	Phosphatase and tensin homolog-induced putative kinase 1
PKA	Protein kinase A
pNBM	Plating NBM
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern-recognition receptors
pSer129	Phosphorylation of αSyn Ser129
PSP	Progressive supranuclear palsy
pαSyn	Phosphorylated αSyn
qRT-PCR	Quantitative real-time polymerase chain reaction
rAAV	Recombinant AAV
RNA	Ribonucleic acid

RT	Room temperature
SEM	Standard error of the mean
Ser129	α Syn serine residue 129
SNARE	Soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein receptor
SNCA	Synuclein alpha gene
SND	Striatonigral degeneration
SNRI	Serotonin and noradrenaline reuptake inhibitor
SR	Scavenger receptor
ssRNA	Single stranded ribonucleic acid
s.c.	Subcutaneous (injection)
TEM	Transmission electron microscopy
TGF	Transformation growth factor
TH	Tyrosine hydroxylase
THB	Tetrahydrobiopterin
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF Receptor
Trk	Tyrosine protein kinase
TXA₂	Thromboxane A ₂
T_{1/2}	Half-life
Ub	Ubiquitin
vg	Viral genomes
VMAT2	Vesicular monoamine transporter
WT	Wild-type
3-OMD	3-O-methyl-DOPA
3R's	Replacement, Reduction and Refinement
6-OHDA	6-hydroxy-dopamine
$\Delta\Delta$CT	Comparative CT
αSyn	Alpha-synuclein

List of figures

- Figure 1.1 Schematic overview of the distinct pathological features of α Synucleinopathies.
- Figure 1.2 The primary structure of α Synuclein.
- Figure 1.3 α Synuclein fibril formation pathway.
- Figure 1.4 Generating α Synuclein preformed fibrils from α Synuclein monomers.
- Figure 1.5 Engineering recombinant adeno-associated viral vectors for systemic delivery of a gene of interest.
- Figure 1.6 Adeno-associated viral vector transduction pathway.
- Figure 1.7 Cyclic relationship between chronic neuroinflammation and continuous neurodegeneration in α Synucleinopathies.
- Figure 2.1 Schematic representation of behavioural tests.
- Figure 2.2 Imaging and analysis of fluorescently stained Iba1+ microglia using ImageJ.
- Figure 2.3 Imaging and analysis of fluorescently stained MAP2+ primary neurons using Sholl analysis.
- Figure 3.1. WT PFF and A53T PFF reduce placements made with the forelimb contralateral to the injection four weeks after intranigral delivery.
- Figure 3.2. WT PFF and A53T PFF reduce wall placements made with the forelimb contralateral to the injection four weeks after intranigral delivery.
- Figure 3.3. Intranigral delivery of WT PFF reduces contralateral forelimb-use in the cylinder test four weeks after intranigral delivery.
- Figure 3.4. WT and A53T PFF appear to reduce contralateral forelimb-use in the cylinder test four weeks after intranigral delivery when number of placements are expressed as % of baseline.
- Figure 3.5. Reduced stepping with the limb contralateral to the injected hemisphere in all treatment groups.
- Figure 3.6. Reduced forward stepping with the limb contralateral to the injected hemisphere.
- Figure 3.7. Intranigral delivery of WT or A53T PFF do not effect stepping behaviour five weeks after delivery.

- Figure 3.8. Forelimb-use asymmetry is not observed in the stepping test five weeks after intranigral delivery of WT or A53T PFFs.
- Figure 3.9. WT or A53T PFFs do not affect amphetamine-induced rotations six weeks after intranigral injection.
- Figure 3.10. WT PFF increases pSer129+ α Syn immunoreactivity in the ipsilateral hemisphere six weeks after intranigral delivery.
- Figure 3.11. pSer129+ α Syn immunofluorescence is increased in the ipsilateral substantia nigra six weeks after intranigral delivery of WT PFF.
- Figure 3.12. WT PFFs increase the number of Iba1+ microglia in the ipsilateral and contralateral hemispheres six weeks after intranigral delivery.
- Figure 3.13. Iba1+ microglial cells are increased in the substantia nigra six weeks after intranigral delivery of WT PFF.
- Figure 3.14. Iba1+ microglial cells are increased in the substantia nigra six weeks after intranigral delivery of WT PFF.
- Figure 3.15. Neither WT nor A53T PFFs affect the number of TH+ cell bodies in the substantia nigra six weeks after intranigral delivery.
- Figure 3.16. TH+ dopaminergic neuronal cell bodies in the substantia nigra remain unaffected six weeks after unilateral intranigral delivery of WT PFF or A53T PFF.
- Figure 3.17. TH+ dopaminergic neuronal cell bodies in the substantia nigra remain unaffected six weeks after unilateral intranigral delivery of WT PFF or A53T PFF.
- Figure 4.1. WT PFF+AAV and A53T PFF+AAV reduce the number of placements made with the forelimb contralateral to the injection four weeks after intranigral delivery.
- Figure 4.2. WT PFF+AAV and A53T PFF+AAV reduce the number of wall placements made with the forelimb contralateral to the injection four weeks after intranigral delivery.
- Figure 4.3. WT PFF+AAV and A53T PFF+AAV reduce contralateral forelimb-use in the cylinder test four weeks after intranigral delivery.
- Figure 4.4. WT PFF+AAV and A53T PFF+AAV reduce the number of steps made with the forelimb contralateral to the injection five weeks after intranigral delivery.
- Figure 4.5. WT PFF+AAV and A53T PFF+AAV reduce the number of forwards steps made with the forelimb contralateral to the injection five weeks after intranigral delivery.

- Figure 4.6. Reduced combined contralateral steps in A53T PFF+AAV-treated animals and reduced forward contralateral steps in WT PFF+AAV-treated animals when steps are presented as a % of baseline.
- Figure 4.7. A53T PFF+AAV increases total rotations and rotations in the direction contralateral to the lesion in the amphetamine-induced rotations test six weeks after intranigral delivery.
- Figure 4.8. WT PFF+AAV and A53T PFF+AAV increases pSer129+ α Syn immunoreactivity in the ipsilateral hemisphere six weeks after intranigral delivery.
- Figure 4.9. pSer129+ α Syn immunofluorescence is increased in the ipsilateral substantia nigra six weeks after intranigral delivery of WT PFF+AAV and A53T PFF+AAV.
- Figure 4.10. A53T PFF+ AAV increases the number of Iba1+ microglia in the ipsilateral hemisphere six weeks after intranigral delivery.
- Figure 4.11. Iba1+ microglial cells are increased in the ipsilateral substantia nigra six weeks after intranigral delivery of A53T PFF+AAV.
- Figure 4.12. A53T PFF+AAV reduces TH+ cell bodies in the ipsilateral substantia nigra six weeks after intranigral delivery.
- Figure 4.13. TH+ dopaminergic neuronal cell bodies are reduced in the ipsilateral substantia nigra six weeks after intranigral delivery of A53T PFF+AAV.
- Figure 5.1. Formoterol does not alleviate the reduction in combined placements observed in the cylinder test four weeks after intranigral delivery of A53T PFF+AAV.
- Figure 5.2. Formoterol partially mitigates the reduction in wall placements observed in the cylinder test four weeks after intranigral delivery of A53T PFF+AAV.
- Figure 5.3. Formoterol-treated animals display increased wall placements made with the limb contralateral to the injection (% of baseline) compared to vehicle-treated controls.
- Figure 5.4. Formoterol does not impact combined placements or wall placements made with the limb contralateral to the injection four weeks after intranigral delivery of A53T PFF+AAV.
- Figure 5.5. Formoterol does not affect combined stepping test performance observed five weeks post-A53T PFF+AAV delivery to the substantia nigra.
- Figure 5.6. Formoterol does not affect forward stepping test performance observed five weeks post-A53T PFF+AAV delivery to the substantia nigra.

- Figure 5.7. Formoterol does not impact stepping test performance four weeks after intranigral delivery of A53T PFF+AAV.
- Figure 5.8. Formoterol does not impact stepping test performance five weeks post-intranigral delivery of A53T PFF+AAV.
- Figure 5.9. Formoterol has no effect on rotational behaviours in the amphetamine-induced rotations test six weeks post-intranigral delivery of A53T PFF+AAV.
- Figure 5.10. Formoterol does not influence pSer129+ α Syn immunofluorescence observed in the ipsilateral hemisphere six weeks post-intranigral delivery of A53T PFF+AAV.
- Figure 5.11. pSer129+ α Syn immunofluorescence in the ipsilateral and contralateral substantia nigra six weeks post-intranigral delivery of A53T PFF+AAV.
- Figure 5.12. Formoterol does not influence the number of Iba1+ microglia in the ipsilateral hemisphere six weeks post-intranigral delivery of A53T PFF+AAV.
- Figure 5.13. Formoterol does not influence the number of Iba1+ microglia in the ipsilateral hemisphere six weeks post-intranigral delivery of A53T PFF+AAV.
- Figure 5.14. Delivery of A53T PFF+AAV into the substantia nigra triggers microglial mobilisation beyond the injection site.
- Figure 5.15. Formoterol partially mitigates the reduction in TH+ cell bodies observed in the ipsilateral hemisphere six weeks post-intranigral delivery of A53T PFF+AAV.
- Figure 5.16. TH+ cell bodies in the ipsilateral and contralateral hemisphere six weeks post-intranigral delivery of A53T PFF+AAV.
- Figure 5.17. Correlation analysis between pSer129+ immunofluorescence, Iba1+ microglial cells counts, and TH+ neuronal cell counts.
- Figure 6.1. Iba1+ primary microglia in primary mixed glial cells.
- Figure 6.2. Iba1+ primary microglia in primary enriched glial cells.
- Figure 6.3. MAP2+ primary neurons treated with conditioned media transferred from primary mixed glial and enriched microglial cultures.
- Figure 6.4. MAP2+ primary neurons treated with conditioned media transferred from primary mixed glial and enriched microglial cultures.
- Figure 6.5. Proportion of Iba1+ cells and LDH activity in primary mixed glial cultures treated with α Syn filaments, oligomers, or PFFs for 24 hours.

- Figure 6.6. Morphology of Iba1+ microglia in primary mixed glial cultures treated with α Syn filaments, oligomers, or PFFs (4 μ g/ml) for 24 hours.
- Figure 6.7. mRNA expression of target cytokines in mixed glial cells treated with α Syn filaments, oligomers, or PFF (4 μ g/ml) for 24 hours.
- Figure 6.8. LDH activity in primary neuronal cultures treated for 24 hours with conditioned media from mixed glial cells treated with α Syn filaments, oligomers, or PFF (4 μ g/ml) for 24 hours.
- Figure 6.9. Sholl profile of primary neurons treated for 24 hours with conditioned media from mixed glial cells treated with α Syn filaments, oligomers, or PFFs (4 μ g/ml), for 24 hours.
- Figure 6.10. Proportion of Iba1+ cells and LDH activity in primary mixed glial cultures treated with WT PFFs for 24-96 hours.
- Figure 6.11. Morphology of Iba1+ microglia in primary mixed glial cultures treated with WT PFF for 24-96 hours.
- Figure 6.12. mRNA expression of target cytokines in mixed glial cells treated with WT PFF for 24-144 hours.
- Figure 6.13. LDH activity in primary neuronal cultures treated for 24 hours with conditioned media transferred from mixed glial cells treated with WT PFFs for 24-96 hours.
- Figure 6.14. Sholl profile of primary neuronal neurons treated for 24 hours with conditioned media transferred from mixed glial cells treated with WT PFF for 24-96 hours.
- Figure 6.15. Proportion of Iba1+ cells and LDH activity in primary mixed glial cultures treated with 0-500ng/ml IFN γ for 24 hours.
- Figure 6.16. Morphology of Iba1+ microglia in primary mixed glial cultures treated with 0-500ng/ml IFN γ for 24 hours.
- Figure 6.17. mRNA expression of target cytokines in mixed glial cells treated with 0-500ng/ml IFN γ for 24 hours.
- Figure 6.18. LDH activity in primary neuronal cultures treated for 24 hours with conditioned media transferred from mixed glial cells treated with 0-500ng/ml IFN γ for 24 hours.
- Figure 6.19. Sholl profile of MAP2+ primary neurons treated for 24 hours with conditioned media transferred from mixed glial cells treated with 0-500ng/ml IFN γ for 24 hours.

- Figure 6.20. Proportion of Iba1+ cells and LDH activity in primary mixed glial cultures pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ .
- Figure 6.21. mRNA expression of target cytokines in mixed glial cells pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ .
- Figure 6.22. LDH activity in primary neuronal cultures treated with conditioned media from mixed glial cells pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ .
- Figure 6.23. Sholl profile of MAP2+ primary neurons treated with conditioned media transferred from mixed glial cells pre-treated with A53T PFFs for 24 for hours prior to treatment with IFN γ .
- Figure 6.24. Proportion of Iba1+ cells and LDH activity in mixed glial cultures pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF.
- Figure 6.25. mRNA expression of target cytokines in mixed glial cells pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF.
- Figure 6.26. LDH activity in primary neuronal cultures treated with conditioned media from mixed glial cultures pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF.
- Figure 6.27. Sholl profile of MAP2+ primary neurons treated with conditioned media from mixed glial cultures pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF.
- Figure 6.28. Proportions of Iba1+ cells and LDH activity in primary enriched microglial cultures treated with 0-50ng/ml IFN γ for 24 hours.
- Figure 6.29. Morphology of Iba1+ microglia in primary enriched microglial cultures treated with 0-50ng/ml IFN γ for 24 hours.
- Figure 6.30. mRNA expression of target cytokines in enriched microglial cells treated with 0-50ng/ml IFN γ for 24 hours.
- Figure 6.31. LDH activity in primary neuronal cultures treated with conditioned media transferred from enriched microglial cells treated for 24 hours with 0-50ng/ml IFN γ .
- Figure 6.32. Sholl profile of MAP2+ primary neurons treated for 24 hours with culture media from enriched microglial cells conditioned with 0-50ng/ml IFN γ for 24 hours.
- Figure 6.33. Sholl profile of MAP2+ primary neurons treated with conditioned media transferred from enriched microglial cultures pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ .

- Figure 6.34 Sholl profile of MAP2+ primary neurons treated with conditioned media transferred from enriched microglial cultures treated with IFN γ in DMEM.
- Figure A1.1. AAV-mediated expression of human A53T-mutant α Syn increased pSer129+ immunoreactivity and Iba1+ microglial cell counts in the ipsilateral substantia nigra six weeks following intranigral delivery to the rat.
- Figure A1.2 α Syn immunofluorescence is increased in the ipsilateral substantia nigra six weeks after intranigral delivery of AAV A53T.
- Figure A1.3 Increased Iba1+ microglial cell counts in the ipsilateral substantia nigra six weeks after intranigral delivery of AAV A53T.
- Figure A1.4 TH+ dopaminergic neuronal cells in the substantia nigra six weeks after intranigral delivery of AAV A53T.
- Figure A2.1. Effect of formoterol on combined cylinder test scores five weeks-post intranigral delivery of WT PFF.
- Figure A2.2. Effect of formoterol on wall placement scores in the cylinder test five weeks-post intranigral delivery of WT PFF.
- Figure A2.3. Effect of formoterol on cylinder test scores five weeks-post intranigral delivery of WT PFF.
- Figure A2.4. Effect of formoterol on combined stepping test scores five weeks-post intranigral delivery of WT PFF.
- Figure A2.5. Effect of formoterol on forward stepping test scores five weeks-post intranigral delivery of WT PFF.
- Figure A2.6. Effects of formoterol on stepping test scores five weeks-post intranigral delivery of WT PFF.
- Figure A2.7. Effect of formoterol on rotarod scores six weeks-post intranigral delivery of WT PFF.
- Figure A2.8. pSer129+ α Syn is undetected in the substantia nigra six weeks post intranigral delivery of WT PFF.
- Figure A2.9. Increased Iba1+ microglial cell counts are observed in the ipsilateral substantia nigra six weeks post intranigral delivery of WT PFF.
- Figure A2.10 Neither WT PFFs nor treatment with formoterol affect the numbers of TH+ dopaminergic neuronal cell counts in the ipsilateral substantia nigra six weeks post intranigral delivery of WT PFF.

- Figure A3.1. Effect of formoterol on combined cylinder test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF.
- Figure A3.2. Effect of formoterol on wall placement scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF.
- Figure A3.3. Effect of formoterol on cylinder test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF.
- Figure A3.4. Effect of formoterol on combined stepping test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF.
- Figure A3.5. Effect of formoterol on forward stepping test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF.
- Figure A3.6. Effects of formoterol on stepping test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF.
- Figure A3.7. Effect of formoterol on rotarod scores of LPS-challenged animals six weeks-post intranigral delivery of WT PFF.
- Figure A3.8. pSer129+ α Syn is undetected in the ipsilateral substantia nigra six weeks post intranigral delivery of WT PFF.
- Figure A3.9. Effect of formoterol on Iba1+ microglial cell counts in LPS-challenged animals six weeks post intranigral delivery of WT PFF.
- Figure A3.10 Effect of formoterol on TH+ dopaminergic neuronal cell counts in the substantia nigra of LPS-challenged animals six weeks post intranigral delivery of WT PFF.

Chapter 1: Introduction

1.1. Parkinson's disease

1.1.1. Parkinson's disease aetiology

Parkinson's disease (PD) is a progressive and incurable neurodegenerative disorder affecting 0.3% of people within the general global population and 1% of people over the age of 60. Globally, an estimated 6.1 million people worldwide live with PD (de Lau & Breteler, 2006) (Tysnes & Storstein, 2017). In Ireland, the incidence is 0.1% in the general population and 1% in those over the age of 80, amounting to an estimated 12,000 people in Ireland (Cassidy et al., 2022). Of these documented cases, about 5% are classified as familial (Ascherio & Schwarzschild, 2016). In familial PD, disease onset can be explained in large by mutations in genetic code, which drive PD-related pathology and symptomatology. A subset of six genes has been linked with the onset of familial PD. These include 1) the *SNCA* gene (*PARK 1*; *PARK 4*) on chromosome 4, which encodes for alpha-synuclein (α Syn), 2) the *LRRK2* gene (*PARK 8*) on chromosome 12, which encodes for leucine-rich repeat kinase 2 (*LRRK2*), 3) the *PRKN* gene (*PARK 2*) on chromosome 6, which encodes for parkin, 4) the *PINK1* gene (*PARK 6*) on chromosome 1, which encodes for phosphatase and tensin homolog-induced putative kinase 1 (*PINK1*), 5) the *DJ-1* gene (*PARK 7*) on chromosome 1, which encodes for protein deglycase DJ-1, and 6) the *APT13A2* gene (*PARK 9*) on chromosome 1, which encodes for adenosine triphosphatase (ATPase) 13A2 (Nuytemans et al., 2010). The other 95% of PD cases can be classified as sporadic or idiopathic, the pathogenesis of which result from a variety of inconclusive environmental, toxin-related and/or genetic factors (de Lau & Breteler, 2006). Age, however, is the number one risk factor associated with all forms of the disease.

Whether familial or sporadic, PD symptomatology is most frequently characterised by the definitive motor symptoms which arise in patients over time. These include a resting tremor, postural instability, muscle rigidity, and bradykinesia. PD-associated motor deficits result from the hallmark loss of dopaminergic neurons in the substantia nigra, a midbrain nucleus which plays a critical role in mediating voluntary motor function. As part of the basal ganglia circuitry, the substantia nigra is a highly interconnected nucleus. Damage to its neuronal population has a detrimental domino effect on downstream nuclei to which nigral fibres project. Indeed, as neuronal damage and neurochemical imbalance progress throughout additional regions the brain, non-motor-associated cognitive symptoms, which reflect the function of the brain region disrupted, can manifest. Depletion of nigral dopaminergic input into the striatum, for example, impedes decision-making, reward processing, and execution of simple motor tasks. Cholinergic depletion in the forebrain contributes to symptoms of dementia, hallucinations, psychosis, dysphagia, impaired stereopsis, and other symptoms of severe cognitive impairment (Braak et al., 2004). Loss of noradrenergic neurons in the locus coeruleus (LC) and serotonergic neurons in the raphe nuclei typically present as symptoms of depression, drowsiness, anxiety, sleep disturbances, and pain (Del Tredici & Braak, 2013; Noyce et al., 2012; Postuma et al., 2012; Wang et al., 2023). The prodromal symptoms of PD, however, are seldom recognized as indicators of early-stage disease onset as they are so closely associated with normal patterns of ageing. As an unfortunate result, the stages at which disease-specific symptoms are recognized and diagnosed are typically underscored by cellular disarray which has covertly progressed over time (Massano & Bhatia, 2012). By the time patients present to the clinic with PD-like motor symptoms, it is estimated that 50% of midbrain dopaminergic neurons have been destroyed (German et al., 1989), which is accompanied by an 80% depletion of striatal dopamine

content (Gibb & Lees, 1988). This highlights the critical need for early-stage-disease detection mechanisms.

1.1.2. Vulnerability of dopaminergic neurons in the midbrain in Parkinson's disease

The biosynthesis of dopamine takes place in the cytosol of catecholaminergic neurons. The metabolic pathway begins with the hydroxylation of phenylalanine by the enzyme phenylalanine hydroxylase, producing L-tyrosine, which gets further hydroxylated by tyrosine hydroxylase (TH) to yield 3,4-dihydroxyphenylethyl amine (DOPA). Its oxidation is dependent on the metabolic cofactor tetrahydrobiopterin (THB). DOPA is then decarboxylated to dopamine by aromatic amino acid decarboxylase (AADC), also known as DOPA decarboxylase (DDC). Further metabolism of dopamine to noradrenaline occurs within cellular storage vesicles of noradrenergic neurons. As dopamine is unable to cross the blood brain barrier, dopamine intake has no therapeutic value in PD. Levodopa (L-DOPA), the precursor to dopamine, can cross the blood brain barrier. Upon systemic administration, L-DOPA can enter the central nervous system (CNS), synthesize dopamine, and supplement centrally depleted pools within the CNS of PD patients (Latif et al., 2021). Although no effective cure has been developed for PD, synthetic L-DOPA has remained the gold-standard for treatment since its first report of success in the early 1960's (Birkmayer & Hornykiewicz, 1962; Gretaarsdottir et al., 2021). In more recent years, its functional success has been boosted by co-administration of pharmacological agent carbidopa, a peripheral DDC inhibitor, which prolongs the effect of L-DOPA by inhibiting its breakdown before reaching the blood brain barrier. In a similar fashion, pharmacological inhibition of catechol-O-methyltransferase (COMT) prevents the early unwanted enzymatic degradation of peripheral L-DOPA to 3-O-methyl-DOPA (3-OMD). Monoamine oxidase B (MAO-B) is an enzyme which can catalyse the deamination of monoamines including dopamine. MAO-B inhibitors, including selegiline and rasagiline, are widely prescribed in conjunction with L-DOPA to prevent further enzymatic depletion of newly synthesised dopamine (Regensburger et al., 2023). Dopamine receptor agonists and dopamine transporter (DAT) blockers are additional commonly used medications which, through boosting dopamine availability in the neuronal cleft, provide symptomatic relief for PD patients (Latif et al., 2021). Dopamine-targeting medications which treat symptoms of PD have been hugely successful in enhancing the quality of life of patients, but fail to slow or stop progression of the disease. Limitations in the currently available dopamine-targeting treatments highlight the critical need for exploring the cellular and molecular mechanisms which underscore or precede dopaminergic depletion. Targeting these mechanisms pharmacologically may prove more efficacious as disease-altering therapeutics.

The α Syn protein is central in driving PD aetiology. Accumulation and deposition of α Syn is considered a hallmark pathogenic process that leads to the formation of Lewy bodies. Lewy bodies are small intracytoplasmic inclusions in the brain, spinal cord, and peripheral nervous system, which are present in both pre-motor and motor stages of the disease. Pathologically, PD is characterized by the buildup of intra-neuronal Lewy body inclusions, comprised predominantly of fibrillar α Syn. These hallmark proteinaceous deposits are accompanied by inflammation in the brain, a toxic process which is known to threaten the integrity of nigrostriatal dopaminergic neurons in the PD brain (Kettenmann et al., 2011; Qin et al., 2004; Saijo et al., 2013).

1.1.3. Insight from neurotoxin models of Parkinson's disease

Toxin-based animal models of PD have been developed, the most classic examples being the 6-hydroxy-dopamine (6-OHDA) model and the 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP)

model. 6-OHDA is structurally similar to dopamine, allowing it to bind to membrane-bound catecholamine transporters and be selectively taken up by dopaminergic and noradrenergic neurons. Once internalized, 6-OHDA is oxidized, and its highly cytotoxic derivatives, hydrogen peroxide and paraquinone, induce mitochondrial dysfunction, oxidative stress, and eventually neuronal death (Penttinen et al., 2016). Injection of 6-OHDA into the rodent midbrain triggers acute dopaminergic degeneration of striatal nerve terminals and loss of nigral cell bodies. Though not through progressive patterns characteristic of human PD, 6-OHDA can drive dopaminergic destruction of the entire nigrostriatal pathway, which can be seen as early as 42 hours post-delivery. While the 6-OHDA model has been commonly used to model PD in animals, it is highly limited in that it fails to reproduce Lewy body-like pathology which is central to PD pathogenesis (Meredith et al., 2008). Repeated systemic injection of MPTP, a byproduct of synthetic pethidine, has also led to development of an animal model of PD. Its lipophilicity allows it to cross the blood brain barrier, where it is taken up by astrocytes and converted to 1-methyl-4-phenylpyridinium (MPP⁺), a mitochondrial complex 1 inhibitor. Uptake into dopaminergic neurons ultimately results in cell death and activation of pro-inflammatory microglia. This model, applicable mainly in mice and non-human primates, generates reliable and reproducible lesions of the nigrostriatal dopaminergic system, which, depending on the dose, can deplete striatal dopamine content by 40% (14 mg/kg i.p. x 4 times for 1 day) to 90% (20 mg/kg i.p. x 4 times in 1 day) in 7 days (Jackson-Lewis & Przedborski, 2007). Mitochondrial damage, nigral TH loss, inflammation, and motor deficits are also key features of this model, but its failure to demonstrate α Syn inclusions limit its use as a disease-relevant model for treatment development (Gao et al., 2023; Meredith et al., 2008).

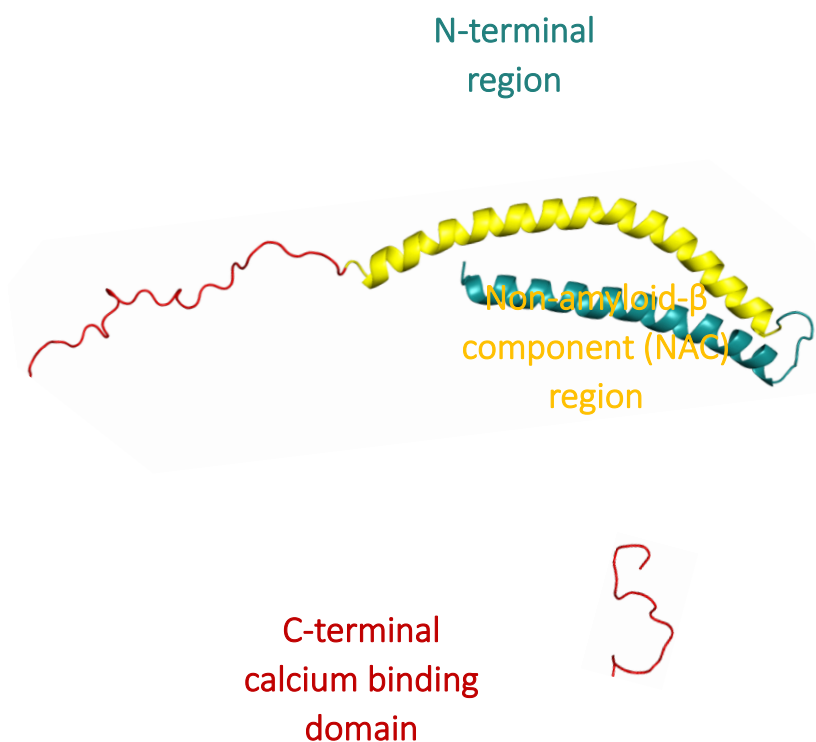
1.2. Alpha-synuclein: the primary structural component of Lewy bodies

1.2.1. Synucleinopathic diseases

Synucleinopathies describe a group of neurodegenerative diseases in which abnormal aggregation of α Syn in the CNS drives disease pathology. PD, Lewy body dementia, Parkinson's disease with dementia (PDD), and pure autonomic failure (PAF) are syndromes in which toxic α Syn constructs accumulate inside neurons. α Syn accumulation can also persist in non-neuronal cells, as is the aetiology of multiple system atrophy (MSA), a synucleopathic disease which features abnormal α Syn deposition in glial cells (Lamotte & Singer, 2023). Cardinal symptoms which feature in neuronal synucleinopathies include cognitive fluctuations, gastrointestinal disturbances, visual hallucinations, and dementia, which, depending on the disease, exist on a background of overt or latent parkinsonism. Despite unifying under one umbrella, the varying synucleinopathies exhibit distinct clinical and pathological presentations. In the clinic, PD is diagnosed following the clear onset of classic parkinsonian motor dysfunction representative of nigral dopaminergic dysfunction. PDD presentation can manifest as the development of cognitive impairment and memory loss on top of an established case of PD (Emre et al., 2007; McKeith et al., 2017). In contrast, patients with Lewy body dementia typically exhibit cognitive impairment for approximately two years before the appearance of parkinsonian motor symptoms. In 25% of cases of Lewy body dementia, parkinsonian symptoms never materialize (Ferman et al., 2011; Kim et al., 2014). Indeed, the clinical and pathological overlap between certain synucleinopathies, namely Lewy body dementia and PDD, has led to widespread debate over whether different diagnoses reflect one disease which manifests along a synucleinopathy spectrum (Friedman, 2018; Goldmann Gross et al., 2008; Jellinger & Korszyn, 2018), or, whether various synucleinopathies are fact distinct diseases with distinguishable

pathology, genetic predispositions and/or environmental risk factors (Boeve et al., 2016; Gatt et al., 2013; Postuma et al., 2015) (Figure 1.1).

The variability between pathology of these disease states can likely be explained by different spatial and temporal distribution patterns which α Syn displays as it accumulates intracellularly and spreads throughout the brain. The Braak-staging hypothesis describes a directional sequence through which



α -Synuclein primary structure

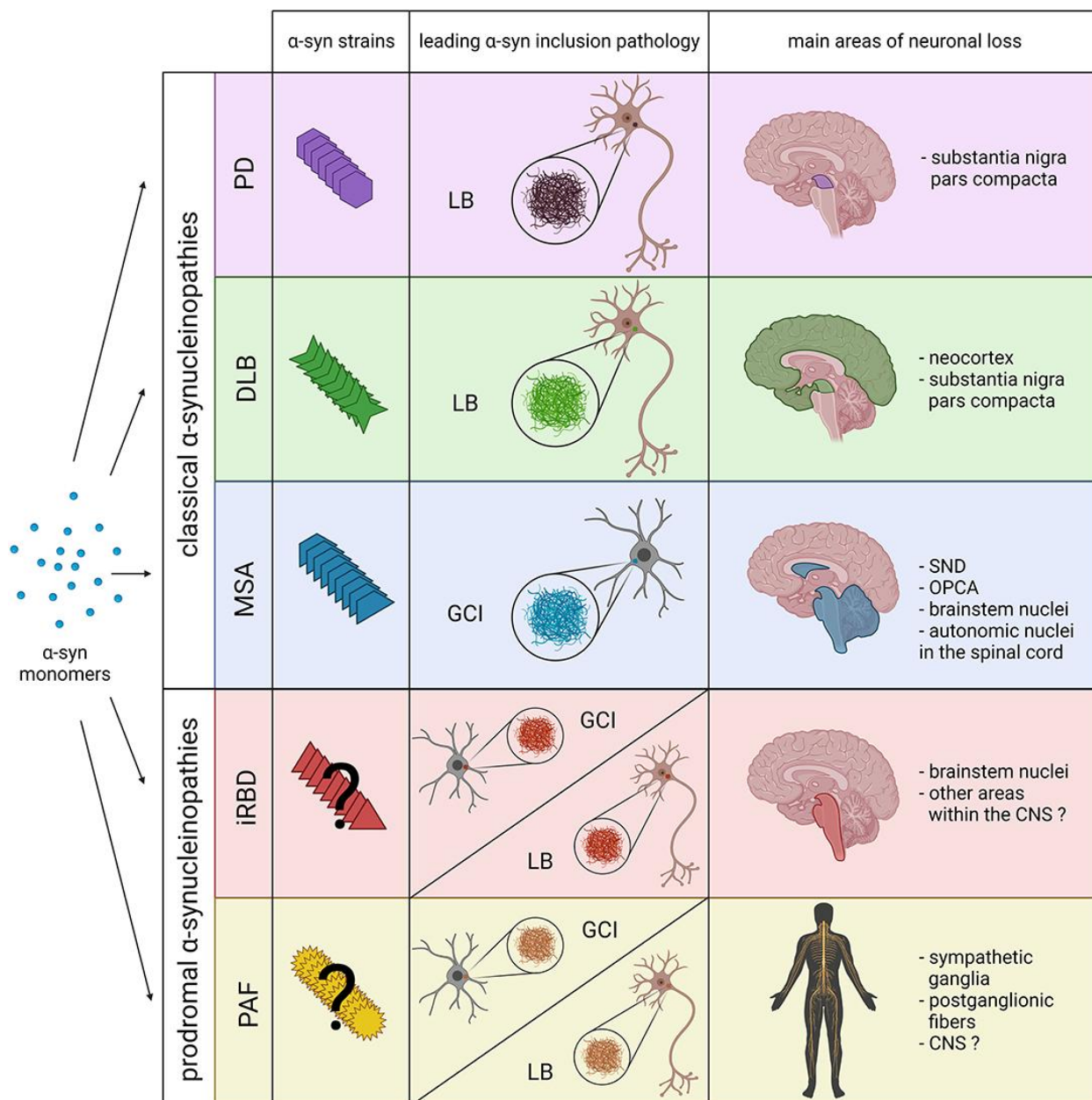


Figure 1.1. Schematic overview of the distinct pathological features of αSynucleinopathies. In its native, unfolded state, αSynuclein (αSyn) exists in a compact monomeric α-helical conformation. Under pathological conditions, αSyn shows remarkable structural plasticity, and can exist in different conformational states within a cell. Novel structural and biochemical analyses of the main pathological features of synucleinopathies have demonstrated the existence of different αSyn strains across classical αSynucleinopathies including Parkinson's disease (PD), Dementia with Lewy bodies (DLB), and Multiple system atrophy (MSA), and across prodromal αSynucleinopathies including Pure autonomic failure (PAF) and Isolated rapid eye movement (iREM) sleep behaviour disorder (iRBD). Recent experimental data also indicate that distinct αSyn strains might be associated with the affected cell type and type of αSyn inclusion (neuronal Lewy bodies (LB), glial cell inclusions (GCI), etc.), in addition to neurodegeneration pattern and disease severity. It is still unclear whether distinct αSyn strains are also associated with prodromal αSynucleinopathies, or if they only constitute early phases of the classical cohort. It is therefore still unknown whether buildup of αSyn in brains of patients with PAF, iRBD, or other non-classical αSynucleinopathies could lead to the formation of LBs, GCIs and/or neuronal loss in other areas within the CNS. Striatonigral degeneration (SND), Olivopontocerebellar atrophy (OPCA). Figure adapted from Malfertheiner et al., 2021, *Front. Neurol.*

α Syn aggregates are likely to spread throughout the brain, thus giving rise to predictable patterns of symptomatology associated with regional dysfunction (Braak et al., 2004). In PD, the topographical spread of α Syn and progressive accumulation of Lewy body plaques are thought to begin in the dorsal motor nuclei of the Vagus nerve and anterior olfactory structures, and predictably propagate through the midbrain and limbic regions, until finally reaching the neocortex (Braak et al., 2005). The directional distribution through which Lewy bodies accumulate in non-PD synucleinopathies does not strictly postulate the same stepwise progression. As such, utilising Braak staging for diagnosis of PDD, Lewy body dementia, or other synucleinopathies, may be less valid. More informed criteria have since been developed to distinguish between brainstem-predominant, limbic-predominant, and diffuse-neocortical variants of synucleinopathy (McKeith et al., 2017). In addition to variations in sequential α Syn propagation, heterogeneity between synucleinopathies can likely be explained by different conformers which α Syn can display. With a fluid architecture, the α Syn protein can fluctuate between monomeric and polymeric states based on the surrounding environment (Lau et al., 2020). The same theory applies to tauopathy pathology, of which structural deviants of the Tau protein are believed to drive clinical and pathological heterogeneity between patients with Alzheimer's disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and other Tauopathies (Shi et al., 2021).

In the clinic, there is a critical need for methods which distinguish the various synucleinopathies (and tauopathies) from each other, in order to provide accurate prognosis, treatment, and care. In the lab, considering these diseases together, for the purpose of model development, provides a means for determining universal mechanisms driving α Syn-related pathology.

1.2.2. Alpha-synuclein structure, function, distribution, and aggregation

α Syn is a highly soluble 14kDA protein, enriched primarily at presynaptic terminals of neurons in the central and peripheral nervous system (Spillantini et al., 1997). In its native, unfolded state, α Syn exists in a compact monomeric α -helical conformation stabilised mainly by hydrogen bonding (Weinreb et al., 1996). The protein has three major subunits, with an amphipathic N-terminal domain (amino acid 1-60), a central hydrophobic domain (amino acid 61-94), and an acidic C-terminal domain (amino acid 95-140) (Figure 1.2). The amphipathic and central domains mediate interactions between cellular and subcellular targets. In the brain, α Syn displays an intrinsic ability to maintain its structural α -helical integrity by protecting its non-amyloid- β component (NAC) region from undergoing spontaneous aggregation and fibrillisation (Burre et al., 2010; Theillet et al., 2016). Under physiological conditions, α Syn regulates the release of synaptic vesicles in a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated manner (Burre et al., 2010), contributing significantly to synaptic function, mitochondrial metabolism, and synergism between the endoplasmic reticulum and Golgi apparatus (Burre et al., 2010; Wong & Krainc, 2017). α Syn is expressed exclusively in vertebrates and is highly phylogenetically conserved amongst mammalian species, thereby supporting its innate involvement in higher order brain function. α Syn expression in muscle, kidneys, heart, and red blood cells fortifies its role in regulatory signal processing of physiological functions beyond the CNS (Burre, 2015).

N-terminal point mutations in the protein-coding gene SNCA – A53T (alanine to threonine substitution at 53rd amino acid), A30P (alanine to proline substitution at 30th amino acid), or E46K (glutamic acid to lysine substitution at 46th amino acid) for example – can cause the native protein to misfold and structurally accumulate with other misfolded monomers to become higher order β -

sheet-rich oligomeric assemblies. α Syn oligomers can undergo further functional changes as proteinase-resistant oligomers, before eventually transitioning into fibrils (Cremades et al., 2012). Continued protein aggregation leads to the formation of Lewy bodies, proteinaceous neuronal inclusions in the brain, spinal cord, and peripheral nervous system which are rich in α Syn aggregates (Kalia & Lang, 2015) (Figure 1.3). Of the many structural conformers which α Syn can take, fibrillar α Syn has been most closely associated with PD pathology and other neurodegenerative diseases. Unlike monomeric or filamentous α Syn, fibrils can spread from cell-to-cell, and can recruit and promote the hyper-phosphorylation of naïve α Syn, leading to widespread Lewy body accumulation (Lashuel et al., 2013; Mulaj et al., 2014). In addition to being a small protein (140 amino acids), α Syn contains an exposed filament-forming hydrophobic region, which facilitates intermolecular interactions that lead to fibrillisation. In contrast, Tau, one of the amyloidogenic components of intracellular deposits in Alzheimer's disease, is much larger (352 to 441 amino acids), and contains a shielded filament-forming region, preventing conformational changes required for fibrillisation. α Syn fibrillisation reportedly promotes the fibrillisation of tau, through a mechanism specifically dependent on α Syn fibrillisation, since neither non-fibrillogenic α Syn (species not specified) nor fibrillogenic amyloid peptides promote tau fibril formation (Giasson et al., 2003). These observations demonstrate the possibility that sequestration of α Syn into Lewy bodies may reduce the concentration of effective α Syn in nerve terminals, and may contribute to neurodegeneration through their ability to readily fibrillise, seed recruitment of endogenous α Syn, and propagate readily throughout the CNS.

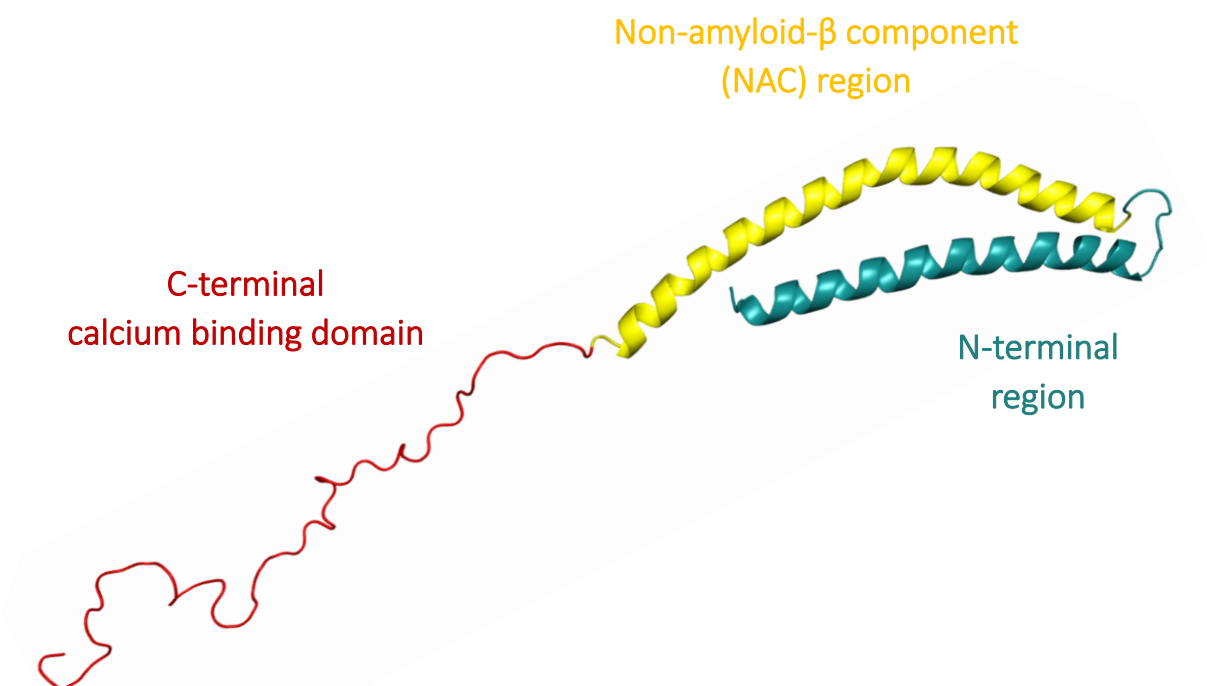


Figure 1.2. The primary structure of α Synuclein. α Syn is composed of 140 amino acids and comprises three distinct domains: the acidic C-terminal region, the highly hydrophobic non-amyloid component (NAC) region, and the N-terminal repeat region. In its native, unfolded state, α Syn exists in a compact monomeric α -helical conformation. Its structure protects it from undergoing spontaneous conformational changes, which can lead to protein misfolding and aggregation. Under pathological conditions, α Syn shows remarkable conformational plasticity, and can exist in different conformational states within a cell.

Unlike monomeric α Syn, fibrils can spread from cell-to-cell causing widespread cellular dysfunction throughout the brain (Lashuel et al., 2013; Mulaj et al., 2014). Braak-staging pathology describes the sequence in which α Syn aggregates are likely to spread throughout the brain of PD-afflicted patients in a topographically predictable sequence, causing distinct patterns of Lewy body formation and associated symptomatology (Braak et al., 2004). Although the mechanism underpinning neuronal vulnerability to Lewy Body formation is not fully understood, cell-to-cell propagation of α Syn fibrils interferes with structural and functional properties of neurons, causing malfunction of key physiological processes for which native α Syn is required (Spillantini et al., 1997).

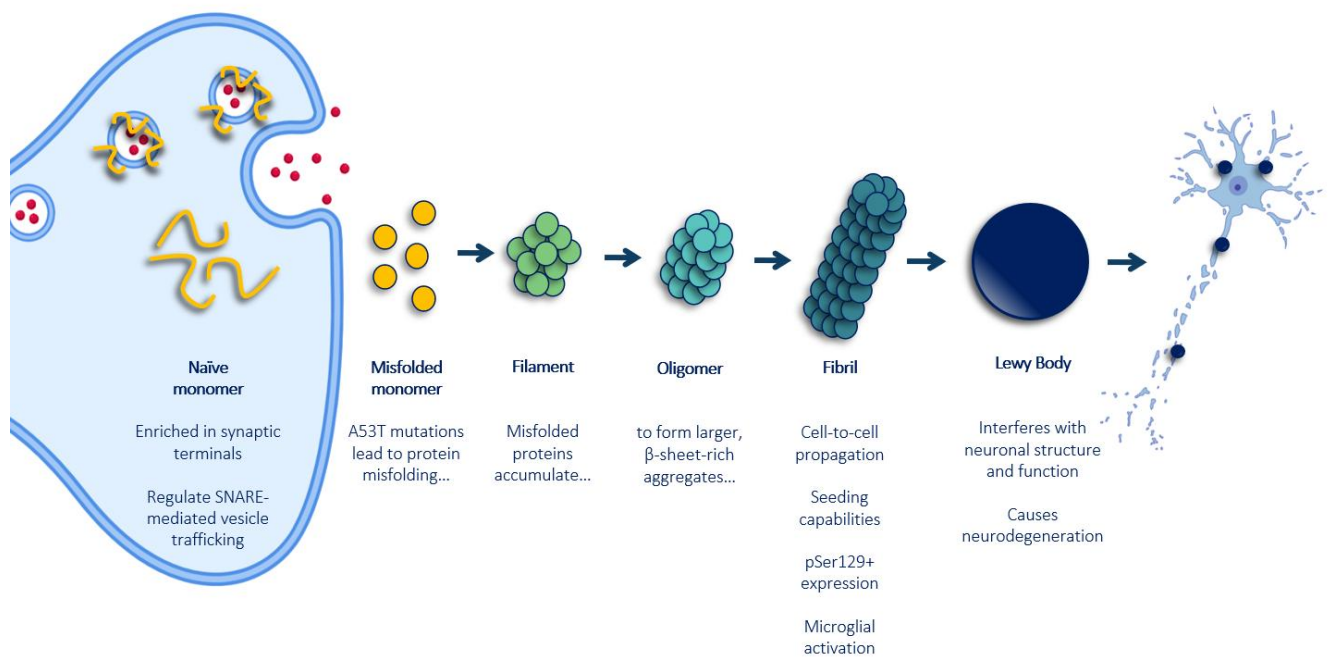


Figure 1.3. α Synuclein fibril formation pathway. α Syn is expressed exclusively in vertebrates and is highly phylogenetically conserved amongst mammalian species, thereby supporting evidence of its innate involvement in higher order brain function. Under physiological conditions, α Syn is enriched at presynaptic neuronal terminals in its natively unfolded and highly disordered monomeric form, and plays a role in regulating the release of synaptic vesicles in a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated manner. Genetic and/or environmental factors can lead to protein misfolding, giving rise to misfolded monomers, which aggregate together to build larger-order intermediate structures, including filaments and oligomers. α Syn fibril formation is thought to be driven by a nucleation reaction, during which soluble monomers aggregate together to form tightly packed insoluble aggregates, rich in β -sheet structures. Intracellular build-up of α Syn aggregates leads to the formation of Lewy bodies, the principal hallmark of PD pathology. While both fibrillar and oligomeric constructs of α Syn are found in high proportions in Lewy bodies, fibrils are thought to be the most neurotoxic form of aggregated α Syn for several reasons. Fibrils can 1) self-propagate between cells through the brain, spinal cord, and peripheral nervous system, spreading their toxicity throughout cellular networks. While doing this, they can 2) act as nucleation templates upon which endogenous α Syn can polymerise to form additional aggregates. 3) α Syn fibrils are also a highly phosphorylated species. Specifically, phosphorylation at Serine residue 129 (pSer129) is the most observed post-translational modification in fibrillar forms of α Syn, and the degree of α Syn phosphorylation in the brain is strongly linked with Parkinson's disease severity. α Syn fibrils have also been shown to 4) drive microglial polarisation towards a pro-inflammatory phenotype, resulting in increased production of inflammatory cytokines, which at unmanaged concentrations, can be cytotoxic to vulnerable neuronal populations. This exacerbated neurotoxicity can in turn further amplify microglial activation, which is known to have severe consequences for neuronal health.

While striatal administration of oligomers induced dopaminergic depletion in the substantia nigra of wild-type (WT) mice, injection of small-sheet fibril fragments produced greater destruction throughout the nigrostriatal tract, resulting in motor dysfunction (Froula et al., 2019). Similar findings were reported previously by Peelaerts and colleagues, who demonstrated more robust motor dysfunction, dopaminergic cell loss, and synaptic impairment in rats following intranigral delivery of fibrils compared to oligomeric assemblies (Peelaerts et al., 2015). Fibrillar fragments may also bind more readily to cell-surface receptors on neurons, impairing physiological function. Shrivastava and colleagues demonstrated obstruction of the sodium and potassium (Na^+/K^+)-activated ATPase (NKA) pump in response to fibrillar clustering, an action not replicated by oligomers or monomers. Fibrillar clustering around this channel was found to reduce NKA-dependent Na^+ extrusion, contributing to cellular physiological dysfunction characteristic of synucleinopathy (Shrivastava et al., 2015). Application of αSyn fibrils, but not oligomers, to neuronal cultures disrupts calcium (Ca^{2+}) homeostasis, which continues to deplete in parallel with progressive accumulation of phosphorylated αSyn (p αSyn) (Gribaudo et al., 2019). In primary mouse neuronal and human induced pluripotent stem cell (iPSC)-derived neuronal cultures, soluble aggregates of αSyn have been shown to reduce neuritic length, whilst αSyn monomers, even at the highest concentration applied, have no impact on dendritic integrity. When applied to mouse hippocampal brain tissue slices, soluble protein aggregates, but not monomers, inhibited theta-burst stimulation-induced long-term potentiation, indicating electrophysiological disturbance associated specifically with αSyn aggregates (Corbett et al., 2020).

As αSyn fibrils self-propagate through the brain, spinal cord, and peripheral nervous system, they act as nucleation templates upon which endogenous αSyn can polymerise to form additional aggregates (Karampetsou et al., 2017; Luk et al., 2012b). αSyn fibrils, not monomers, are a highly phosphorylated species which can recruit and promote the subsequent hyper-phosphorylation of naïve endogenous αSyn , further advancing its fibrillisation and continued propagation (Fujiwara et al., 2002). Serine residue 129 (Ser129) on the C-terminal of the αSyn protein is the site most susceptible to post-translational modification. Specifically, phosphorylation at Serine 129 (pSer129) is the most observed post-translational modification in fibrillar forms of αSyn , mediated by several kinases including G-protein-coupled receptor (GPCR) kinases, casein kinase II, and leucine-rich repeat kinase 2 (Kawahata et al., 2022). In healthy patients, phosphorylated αSyn comprises approximately 4% of total insoluble αSyn in the CNS. In stark contrast, synucleinopathy patients exhibit pSer129+ residues in 90% of total insoluble αSyn (Anderson et al., 2006; Fujiwara et al., 2002; Kawahata et al., 2022).

As such, extensive pSer129 αSyn is a pathological hallmark of αSyn aggregation, which is associated with and contributes to neuronal death (Luk et al., 2012b; Luk et al., 2009). Where fibrils can elongate and recruit endogenous monomers to promote elongation, oligomers have no propensity to do so, rendering their seeding capability deficient (Froula et al., 2019). Striatal delivery of mouse αSyn fibrils to Sprague-Dawley rats demonstrated accumulation of p αSyn inclusions across the striatum, frontal and insular cortices, amygdala, and substantia nigra, six months post-delivery (Paumier et al., 2015). Harms and colleagues observed extensive accumulation of pSer129 αSyn inclusions in the substantia nigra, dopaminergic degeneration, and striatal axon loss three months after administration of mouse fibrils to the rat substantia nigra (Harms et al., 2017). In mice, striatal inoculation of human WT and pSer129+ αSyn fibrils caused seeding of endogenous αSyn , but only

pSer129 fibrils exacerbated Lewy body-like aggregation, nigral dopaminergic neuronal loss, and fine motor impairment at 60 days post-delivery (Karampetsou et al., 2017).

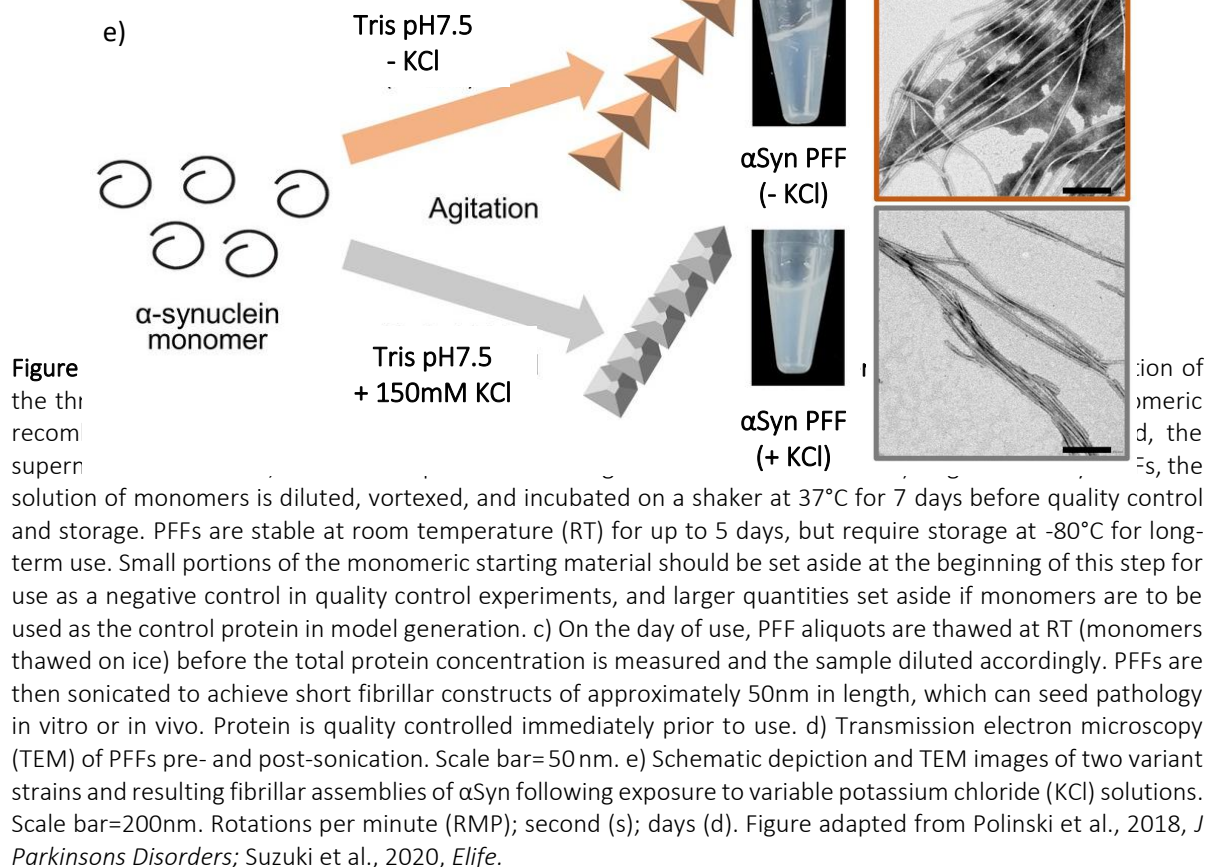
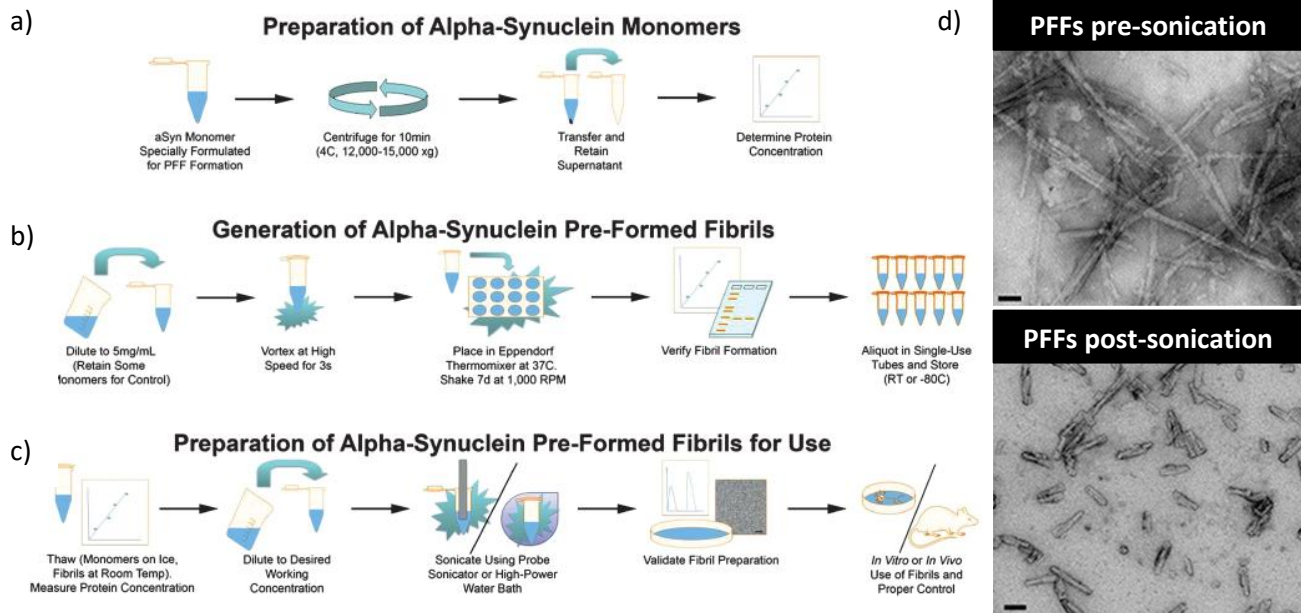
1.2.3. Exploiting the molecular features of fibrillar alpha-synuclein for the development of disease-relevant experimental models of synucleinopathy

The establishment of clinically relevant animal models of PD is critical for understanding disease pathology and developing neuroprotective treatments for the human condition. Development of recombinant preformed fibrils (PFF) has facilitated research towards assessing the relationship between α Syn conformation and neuropathology in models of synucleinopathy. Unlike patient derived samples, a resource of limited availability, recombinant α Syn constructs can be developed in mass quantities, and purified in various conditions to achieve a spectrum of PFFs with distinct structural, biochemical, and pathological properties. The standard protocol for generating PFFs for use in model development involves the incubation of purified α Syn monomers in 100mM sodium chloride buffer at 37°C at physiological pH for 5-7 days, while providing constant agitation (Volpicelli-Daley et al., 2014). Constructs are further agitated by sonication to achieve short fibrillar constructs which can seed pathology *in vitro* or *in vivo*. Fibrillar constructs of larger than 50nm will fail to exhibit pathogenicity upon administration (Abdelmotilib et al., 2017; Froula et al., 2019; Polinski et al., 2018; Tarutani et al., 2016) (Figure 1.4a-d). While unintentional deviations from this strict protocol can give rise to reproducibility issues, deliberate alterations can give rise to unique polymorphs, which have proved useful in explaining heterogeneity in synucleinopathies (Bousset et al., 2013; Henderson et al., 2020a; Lau et al., 2020). Recombinant PFFs can be generated from α Syn containing disease-relevant genetic mutations. Production of A53T-mutant PFFs, for example, differ from WT in their ultrastructural properties, rates of aggregation, resistance to enzymatic digestion, and seeding propensities (Lau et al., 2020). To investigate the relationship between unique conformational states of α Syn and the aggregate fibrils which they form *in vivo*, Lau and colleagues developed two distinct A53T-mutant α Syn constructs by varying the salt buffer conditions during the fibril formation process. Intra-hippocampal delivery of PFF type 1, generated in high-salt conditions, drove early onset of neurological symptoms and limb paralysis in transgenic mice, a phenotype not dissimilar to that seen in common models of MSA. PFF type 2, generated in salt-free conditions, induced delayed cognitive-pathology, and tremor symptoms more characteristic of midbrain Lewy body pathology. Furthermore, the varying strains affected different cell types and brain regions, with one exclusively producing ring-like deposits around neuronal cell bodies, and the other producing large Lewy body-like deposits in neurons and astrocytes (Lau et al., 2020) (Figure 1.4e).

1.2.4. Adeno-associated viral vector tools for increasing alpha-synuclein expression in experimental models of synucleinopathy

Adeno-associated viruses (AAV) comprise a family of small, non-enveloped viruses that contain a genome of single stranded DNA within an icosahedral nucleocapsid. Though remarkably small (4.7kb), AAV's can inherently conduct all functions necessary for its life cycle through its three subsets of naturally occurring genes, *rep* (Replication), *cap* (Capsid), and *assembly* (assembly-activating protein) (Flotte, 2004). These three genes give rise to at least nine gene products through the use of three promoters, alternative translation start sites, and differential splicing. The *rep* genes are required for genome replication and viral life cycle maintenance (Maurer & Weitzman, 2020). The *cap* genes encode the viral capsid proteins, which, with a team of assembly-activating proteins, allow for capsid assembly during recombinant vector production, and cellular recognition and

internalisation (Grosse et al., 2017). Along the AAV's plasmid map, *rep* and *cap* segments are flanked by two inverted terminal repeats (ITR), which contain cis genes required for deoxyribonucleic acid (DNA) replication, packaging, and integration of the virus into the host cell (Bedbrook et al., 2018). AAVs can transduce both replicating



and non-replicating cells, but are non-integrating in nature, and thus can introduce genetic material into a host cell without integrating into the genome of the host cell (Figure 1.5).

AAV vectors have since been developed as gene-delivery vehicles through which select genetic material can be delivered to targeted cellular systems to express a protein of interest over time. Recombinant AAV (rAAV) vectors are generated by replacing the *rep* and *cap* genes with the transgene cassette of interest between the two ITRs. By nature, the removal of the *rep* and *cap* genes, and thus a large portion of viral genetic material, removes viral DNA while also reducing the immunogenicity associated with virus (Maurer & Weitzman, 2020; Muzyczka, 1992; Su et al., 2016). First generation viral vectors were limited in their packaging capacity of approximately 5 kilobases (kb), which restricted their use for transducing larger genes of interest into host cells. Further development has given rise to vectors with larger capsid capacity, some of which can reportedly carry genomes of 14kb, while preserving transduction efficiency (Allocca et al., 2008). Hybrid vectors, which split larger transgenes into smaller components for multiple simultaneous gene delivery, have been developed to enhance strategic expression of larger genes of interest (Duan et al., 2000; Patel et al., 2019).

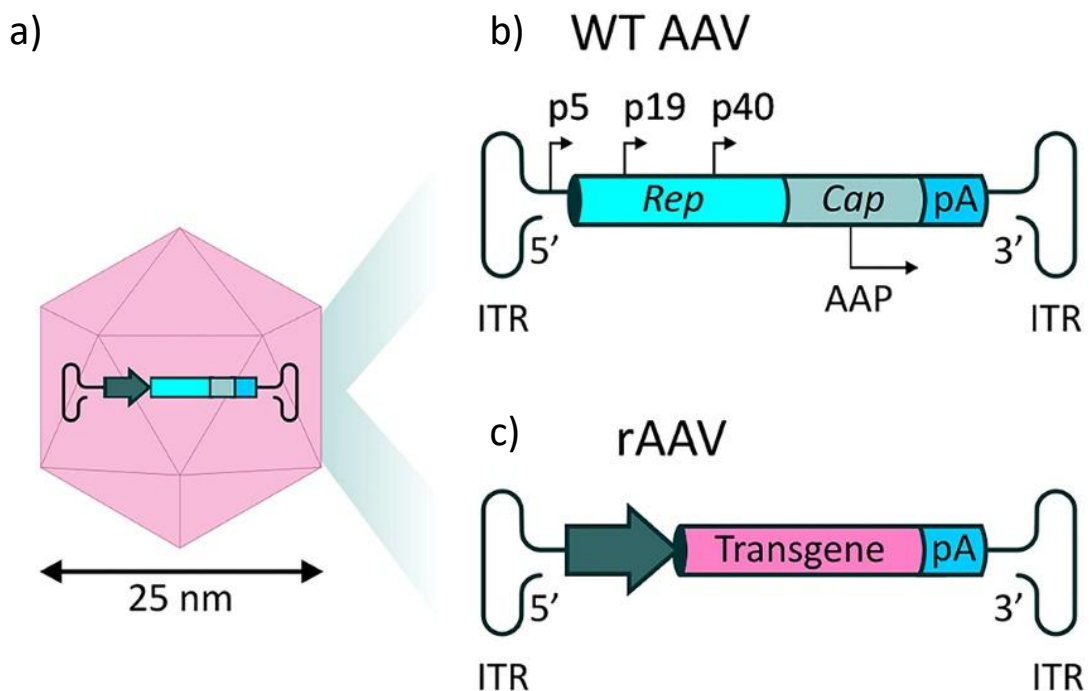


Figure 1.5. Engineering recombinant adeno-associated viral vectors for systemic delivery of a gene of interest. a) Adeno-associated virus (AAV) capsid structure. The AAV capsid is composed of 60 protein monomers arranged in icosahedral symmetry. b) Schematic depiction of the single stranded DNA genome found in naturally occurring wild-type (WT) AAV vectors. Two open reading frames express genes necessary for replication (*rep*) and capsid structure (*cap*). An alternate reading frame overlapping the *cap* gene encodes the assembly-activating protein (AAP), which promotes capsid formation and stability. c) In recombinant AAV-based vectors (rAAV), the viral genes are substituted by a synthetic transgene cassette, the expression of which is driven by a series of promoters (p). Along the genetic sequence, the transgene of interest is followed by a poly-adenosine (pA) tail. Figure adapted from Marino et al., 2022, *Frontiers in Neurology*.

Cellular uptake of rAAV requires the recognition and attachment of virions to host cell surface receptors. This process triggers cellular internalization and trafficking of the vector to the host cell's nucleus, where it will disassemble and deliver its genetic cargo. After uncoating, the virus converts the single-stranded DNA genome into its double-stranded form, which can be translated into a protein of interest. The endocytic mechanisms through which vectors are taken up by cells remain unclear, though initial interactions are thought to involve surface glycan receptor attachment proteins (Huang et al., 2014). Following uptake, either by clathrin-mediated or micropinocytosis-mediated entry (Bartlett et al., 2000; Weinberg et al., 2014), vector cargo is thought to utilize host-cell machinery to access the nucleus for replication (Nicolson & Samulski, 2014). The exact mechanism of genome transduction into a host cell remains to be fully explained, but is likely driven by the ITR sequence of the vector genome, which, upon recruitment of host cell machinery, can act as a promoter to initiate its own transcription, and drive protein expression (Earley et al., 2020) (Figure 1.6). Different cell types show different affinities for recognition and internalisation of different vector serotypes, the appropriate pairing of which is necessary for efficient gene transduction. Serotype subtypes are, in part, determined by the molecular interactions between host cell surface markers and the capsid-protein shell in which the vector genome is encapsulated (Chen et al., 2019). Sequence promoters influence the strength and cell-selectivity of transgene expression, with cytomegalovirus (CMV) and chicken β -actin (CBA) promoters being widely used to transduce genetic expression in cells of the central and peripheral nervous system (Nieuwenhuis et al., 2023; Westhaus et al., 2020). Recently, a toolkit for AAV use was developed to evaluate the transduction capabilities of various AAV serotypes in a range of cell types (Westhaus et al., 2020). This toolkit provides a robust, high-throughput analysis of vector performance *in vitro* and *in vivo* which can be used to identify optimal AAV serotypes for model development, and to investigate novel tropisms and variants of AAV. These advancements to vector development further validate the AAV vector as a viable and effective tool for gene delivery, while exhibiting capacity for future development.

A wide range of vectors encoding human α Syn have been explored in rodents and cell culture models with aims of enhancing model construct validity (Van der Perren et al., 2015b). AAV vectors exhibit tropism for dopaminergic neurons, high transduction efficiency, and low immunogenicity, making them attractive tools for model development of early-stage PD-like pathology. AAV-mediated α Syn expression has been achieved in a variety of model organisms, including mice, rats, and non-human primates (Azeredo da Silveira et al., 2009; Eslamboli et al., 2007; St Martin et al., 2007). In AAV nomenclature, the two numbers of a hybrid vector serotype represent the serotypes of the capsid and the genome. AAV1/2 indicates a virus with an AAV1 capsid and an AAV2 genome. The capsid dictates the tropism (the type of cells the virus will infect), while the genome specifies the genetic information or payload carried by the virus. AAV2/1 indicates a virus with an AAV2 capsid and an AAV1 genome. In this case, the tropism of the virus will be based on the properties of the AAV2 capsid, which often has different tissue targeting characteristics than AAV1 (Chen et al., 2019). Due to their selective tropism, AAV5, 1/2, 2/2, 2/5, and 2/7 are those most commonly utilized to express α Syn, and other pathology-associated proteins in cellular models of neurodegeneration (Srivastava, 2016). AAV-mediated A53T α Syn expression in primary mesencephalic neuronal cultures has proved to be a successful means by which neuronal atrophy, intraneuritic swelling, and cellular inclusions can be modelled *in vitro* (Zach et al., 2007). AAV vectors have also been used to demonstrate gene

silencing, as was shown through downregulation of SNCA expression in SH-SY5Y neuroblastoma cell lines (Han et al., 2011).

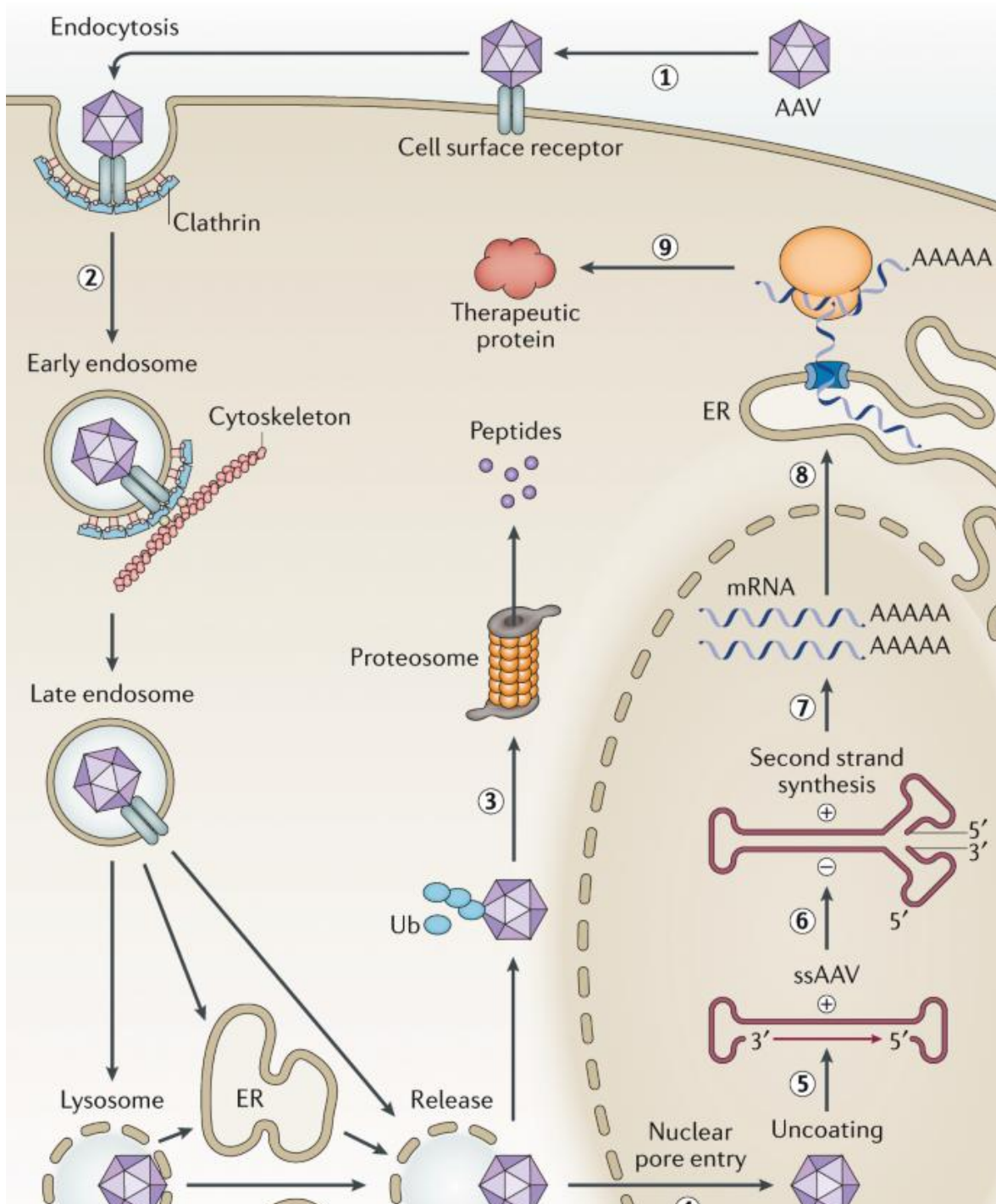


Figure 1.6. Adeno-associated viral vector transduction pathway. 1) Adeno-associated virus (AAV) vector virions bind to receptors on the surface of target cells and are 2) engulfed into endosomes through endocytosis. Once released intracellularly by the endosomes, AAV virions can be 3) identified, ubiquitinated, and targeted for proteasome-mediated degradation, or 4) further trafficked intracellularly to the nucleus. 5) Once inside the nucleus, the AAV capsid is uncoated, and the AAV genome released. The single-stranded DNA genome is 6) converted into double-stranded DNA, 7) transcribed into messenger ribonucleic acid (mRNA), then 8) exported out of the nucleus for 9) translation and expression of the protein of interest. Engineering AAV vectors to affect any step of their transduction pathway impacts their transduction efficiency. Endoplasmic reticulum (ER), single-stranded AAV (ssAAV), ubiquitin (Ub). Figure adapted from Li et al., 2020, *Nature Reviews Genetics*.

AAV-mediated genetic expression in microglia has proved more challenging than in neurons (Maes et al., 2019). Su and colleagues, however, have suggested the AAV2 vector to be less immunogenic than AAV5, 6, 8, or 9, which promote inflammation, and thus a serotype better suited for microglial transduction (Su et al., 2016). In addition to serotypes being cell specific, AAV-mediated genetic delivery is model sensitive. Recent work illustrated a preferential efficiency of gene delivery and expression in cells when applied *in vitro* compared to *in vivo*, despite use of the same vector (Ambrosi et al., 2019).

The consensus from the past decade of AAV research is that delivery of a standard titre of AAV vector expressing human α Syn into the rodent substantia nigra results in key features of the human disease – nigral dopaminergic cell loss, α Syn aggregation, microglial activation, and motor deficits associated with dopamine depletion. However, the magnitude of these outputs, and timelines required for observation of the onset of pathology, vary widely across publications, making reproducibility and transferability of AAV models into other laboratories unclear.

Several rodent models with AAV-mediated α Syn expression have exhibited a reduction of at least 50% of nigral dopaminergic TH+ cells before motor deficits are observed. This finding is consistent with human PD. (Bourdenx et al., 2015; Decressac et al., 2012a; Gombash et al., 2013; Kirik et al., 2002; Klein et al., 2002; Oliveras-Salva et al., 2013; Rodriguez-Perez et al., 2018; Van der Perren et al., 2015a). However, AAV-induced motor deficits have also been widely reported in animal models with less than 40% of TH+ cell loss in the substantia nigra (Campos et al., 2013; Daniel et al., 2015; Decressac et al., 2012a; Ip et al., 2017; Koprach et al., 2011; Thakur et al., 2017; Ulusoy et al., 2010). This variability stems from several sources, including AAV serotype, promoter, viral titre, length of expression timeline, injection protocol, and/or species of rodent. AAV vector-mediated α Syn expression by serotypes 1, 2 and 5 have been recognized for exhibiting enhanced transduction efficiency and expression levels in neurons of the rat substantia nigra, compared to other serotypes (Van der Perren et al., 2015b). As early as 17 days post-intranigral delivery of rAAV2/7 expressing A53T-mutant α Syn (3 μ L; 3.0×10^{11} viral genomes (vg)/mL), Wistar rats exhibited nigral α Syn-containing cytoplasmic inclusions and TH+ cell loss in the substantia nigra, and neuritic stress in the striatum. These α Syn deposits exhibited a fibrillar structure, displayed phosphorylation at serine residue 129, and were colocalised with ubiquitin; three distinct features of human Lewy body pathology (Van der Perren et al., 2015a). Crabbé and colleagues observed limb-use asymmetry in the cylinder test as early as two weeks post intranigral delivery of AAV2/7 expressing human A53T α Syn, but not enhanced green fluorescent protein (eGFP), to female Wistar rats (Crabbe et al., 2019). Similarly, Koprach and colleagues observed limb-use asymmetry, along

with α Syn inclusion in TH+ neurons at three weeks following nigral delivery of 2 μ L of AAV1/2 A53T α Syn at various titres - a low titre [5.1×10^{11} vg/ml], medium titre (1.7×10^{12} vg/ml), and high titre (5.1×10^{12} vg/ml).

Nigral dopaminergic degeneration, striatal dopamine depletion, and behavioural impairment progressed steadily over the course of six weeks in response to medium and high titres. Low titre-treated animals displayed progressive pathology until week 4, after which, neuronal atrophy was self-corrected, with dopamine turnover and striatal TH returning to levels observed at baseline (Koprach et al., 2011; Koprach et al., 2010). This study suggests that some viral titres may drive progressive pathology, against which disease-modifying therapies for late-stage disease can be investigated. In contrast, lower viral titres might prove more useful for investigating mechanisms of

endogenous compensation in early disease states, where pathology is malleable, and disease course modifiable. AAV2/5-mediated nigral overexpression of WT α Syn promoted burst firing within the striatum of female Sprague Dawley rats (Andersen et al., 2019). Female Sprague Dawley rats present with insoluble α Syn inclusions six weeks after intranigral delivery of AAV1/2 A53T α Syn (2 μ L, 5.1×10^{12} vg/ml). Vector delivery also reduced nigral TH+ fibres by about 50% across both the substantia nigra and striatum of the injected hemisphere (Koprach et al., 2010). These findings have since been confounded by work which shows α Syn deposition, but no TH+ cell loss six weeks following intranigral delivery of AAV expressing A53T-mutant α Syn, despite using the same injection volume, titre, serotype, and injection region (Appendix I, Figure A1.1, A1.4). Equivocal results between research laboratories also beg the question as to whether differences in the efficacy of similar constructs may be dependent on source and manufacturer.

1.2.5. Combination of alpha-synuclein preformed fibrils and adeno-associated viral vector-mediated alpha-synuclein expression to recapitulate synucleinopathy in a rodent model

AAV tools which express human α Syn have been used in combination with α Syn PFFs to explore the interaction between α Syn over-expression and α Syn aggregation in rodent models of synucleinopathy.

Injection of human α Syn PFFs into the rat substantia nigra four weeks after an initial injection of AAV vector expressing human α Syn promoted p α Syn, dopaminergic degeneration, striatal dopaminergic depletion, and motor dysfunction. AAV alone was unable to induce pathology. In this model, Lewy body-like pathology was observed through pSer129+ and ubiquitin+ immunohistochemistry as early as 10 days post-PFF injection. These findings indicate the ability of PFFs to accelerate PD-like pathology in the presence of genetic material which increases the expression of monomeric α Syn, further promoting formation of aggregates (Thakur et al., 2017). While animal models are key for the study of PFF-associated behavioural deficits, cellular models are also used for exploring the mechanisms which drive these changes. Treatment of primary mouse hippocampal neurons with human α Syn PFFs induced Lewy body-like inclusions of p α Syn. Immunocytochemical analysis showed no colocalization between exogenous α Syn and p α Syn, indicating an ability for PFFs to recruit endogenous mouse α Syn to form p α Syn inclusions in culture (Earls et al., 2019). A study investigating differential seeding capabilities of α Syn species sheds light on the components of PFF architecture which are associated with formation of p α Syn inclusions. Suzuki and colleagues applied two variants of fibrils with slight architectural differences to mouse primary cortical neurons, and found only one induced marked accumulation of phosphorylated and ubiquitinated protein aggregates (Suzuki et al., 2020). This team attribute varying behaviours of α Syn constructs to structural differences in the C-terminal region, which can contort in response to changing environmental conditions. In the presence of salt, intermolecular repulsion in the acidic and negatively charged C-terminal region of α Syn (residues 96-140) weakens, exposing the C-terminus, rendering a tightly packed fibril. The opposite effect is observed in the absence of salt, where intermolecular repulsion at the C-terminus prevails, exposing and elongating the C-terminus of the aggregate (Suzuki et al., 2020) (Figure 1.4e). The significance of these structural changes relates to the access to phosphorylation and/or ubiquitination sites at which post-translational modifications can be made, thereby influencing toxicity.

1.3. Inflammation at the wheel of disease progression in the alpha-synuclein-burdened brain

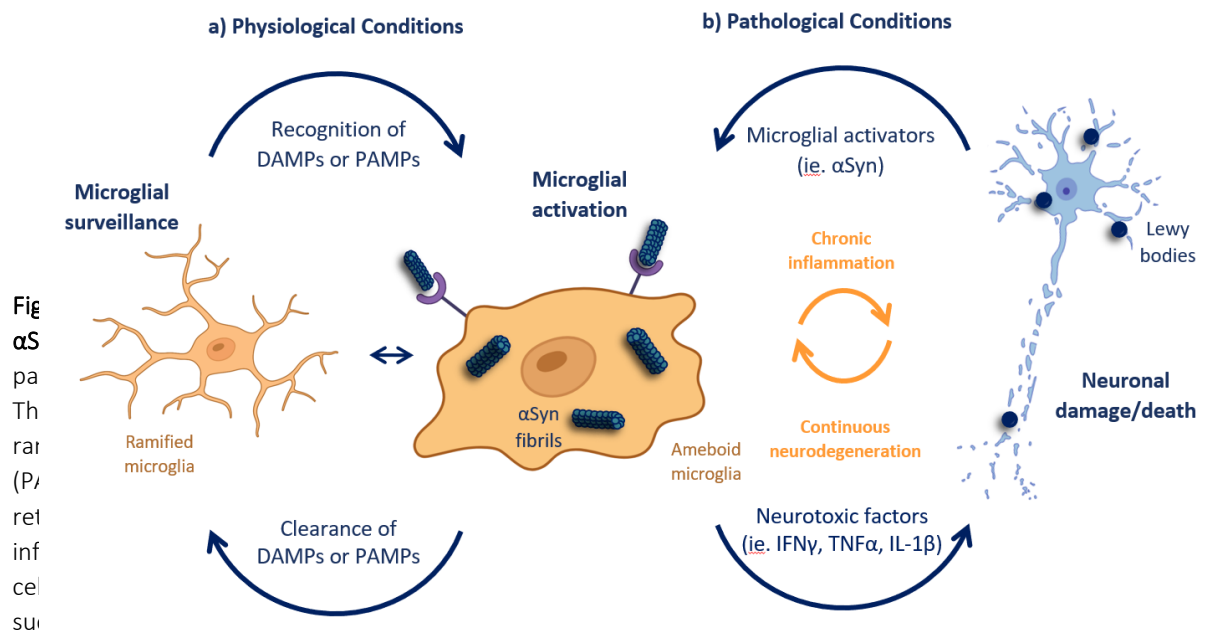
1.3.1. A role for microglia in the healthy brain

Among the many factors known to influence the onset and progression of synucleinopathies, microglial activation has gained great attention in recent years. Microglia are the resident immune cells of the CNS, and constitute about 12% of cells within the human brain (Herculano-Houzel, 2014). Isolated from the peripheral immune system by the blood brain barrier, microglia inhabit a relatively sheltered environment compared to peripheral macrophages, although their functions are not dissimilar. Microglia function primarily as benign surveillants of the CNS microenvironment, ensuring homeostasis of the brain's immunoregulated state. Their oscillating morphologies are distinct. Under physiological conditions, microglia exist in a resting state, displaying extensive ramifications which protrude from a small cell body. They use these extended processes to survey the environment and communicate with surrounding cells. Microglia express a repertoire of pattern-recognition receptors (PRR), which enable them to recognize, react, and respond to different stimuli, including foreign pathogens, cell debris, mutated proteins, and other cellular threats within the CNS. Upon recognition of these non-native elements, which typically exhibit pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP), microglia retract their ramifications and shift into a reactive state, displaying a balloon-like morphology. The phenotypic shift into a pro-inflammatory state is accompanied by upregulation of cell surface proteins which recognize the pathogen and aid in subsequent phagocytosis (Figure 1.7). These include toll-like receptors (TLR), nucleotide-binding oligomerisation domain (NOD)-like receptor proteins (NLRP), and scavenger receptors (SR), which enable microglia to detect and respond accordingly to different stimuli (Gao et al., 2023). Changes in major histocompatibility complex (MHC) protein, complement receptor protein, and cluster of differentiation (CD) protein expression are also evident as microglia shift from resting to reactive state (Cho et al., 2006; Ponomarev et al., 2005). Reactive microglia can sequester non-native cellular factors in a monocyte-like fashion, a process which is physiologically beneficial for localised pathogen clearance (Figure 1.7). Reactive pro-inflammatory microglia release a cache of soluble factors consisting of cytokines, including interleukins (IL), interferons (IFN), tumour necrosis factors (TNF), and chemokines, which collectively signal to neighbouring cells to promote and direct the immune response.

Microglial-derived IL-1 β regulates N-methyl-D-aspartate (NMDA) receptor signalling in post-synaptic neurons, and enhances presynaptic glutamate release (Clark et al., 2015). TNF-alpha (TNF α) enhances signalling at excitatory synapses by influencing surface expression of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Beattie et al., 2002). Additionally, these cytokines lead the regulation of major signalling pathways in the CNS, including nuclear factor- κ B (NF κ B), mitogen-activated protein kinase (MAPK), and caspase-dependent apoptosis pathways (Sabio & Davis, 2014). Certain sub-sets of these secreted proteins also serve as neurotrophic factors which promote dendritic integrity and regulate synaptic plasticity (Streit, 2002; Ueno et al., 2013). Microglia supply neurons with brain-derived neurotrophic factor (BDNF), which contributes to the maturation of inhibitory interneurons in the spinal cord, and plasticity of synaptic connections in the motor cortex, processes required for learning and memory (Parkhurst et al., 2013). Together, microglia conduct a diverse orchestra of processes required for immune regulation and neuronal function, thus maintaining the brain's state of homeostasis.

1.3.2. Overactive microglia impair neuronal function

Sustained microglial activation and chronic production of pro-inflammatory cytokines are features of early stage synucleinopathy, and other age-related degenerative diseases. Positron emission tomography (PET) scanning using radiotracers for activated microglia has shown higher quantities of reactive microglia in the midbrain of early-stage drug-naïve PD patients compared to age-matched healthy controls. Glial activation in PD patients correlates positively with severity of motor dysfunction, as assessed by the Unified Parkinson's Disease Rating Scale, and negatively with DAT detection in the putamen (Ouchi et al., 2005). While chronic microglial activation in the nigrostriatal pathway is apparent in midbrains of healthy subjects undergoing the normal process of aging, microglial activation is increased in disease states where α Syn aggregation prevails (Ouchi et al., 2005). The association of amoeboid-shaped microglia with α Syn pathology in the substantia nigra and hippocampus in autopsied PD brains has been reported (Imamura et al., 2003). Glial-derived TNF α , IL-1 β , and IL-6 are also evident at elevated levels in the nigra, striatum, and putamen of tissue from PD patients, and their concentrations have been directly associated with the severity of symptomatology in patients (Hunot et al., 1996). Elevated levels of IL-1 β , IL-6, IFN γ , and TNF α been recorded in serum and cerebrospinal fluid (CSF) of PD patients with evidence of dopamine depletion (Nagatsu et al., 2000; Schroder et al., 2018). IL-6 and TNF α , found at increased concentrations in PD patient serum, are correlated with the severity of the nonmotor symptoms in PD patients (Lindqvist et al., 2012). Single-cell transcriptome analysis of postmortem midbrain tissue samples from idiopathic PD patients demonstrates increased amoeboid microglial cell counts in the nigra, along with increased levels of IL-1 β (Smajic et al., 2022). Moreover, NLRP3 inflammasome and associated IL-1 β and IL-18 upregulation have been reported in microglial cells in the substantia nigra of PD patients, an inflammatory phenotype which has been linked with neurodegeneration (Gordon et al., 2018; Haque et al., 2020).



damage to neurons, which in turn leads the further release of α Syn and other microglial activators by damaged neurons. This continuous cycle between neuronal release of microglial activators, and microglial release of neurotoxic factors depicts the toxic relationship that microglia and neurons can have in a brain overwhelmed by α Syn.

Chronic microgliosis in PD is also characterised by the over-production of cytotoxic factors including nitric oxide (NO) and superoxide. These factors are deleterious to neuronal cells in the CNS (Colton & Gilbert, 1987; Liu et al., 2002; Moss & Bates, 2001). Similar inflammatory phenotypes have been observed in animal and cellular models associating α Syn aggregation with microglial activation, inflammation, neuronal damage, and neurodegeneration. Tang and colleagues found that lipopolysaccharide (LPS)-stimulated microglial conditioned media containing high levels of IL-1 β , IL-6, TNF α , and inducible nitric oxide synthase (iNOS) was sufficient to induce death of 50% of dopaminergic neurons in mouse ventral midbrain cultures after 24 hour exposure (Tang et al., 2014). A reactive pro-inflammatory microglial production of IL-1 β , TNF α , and iNOS were shown to mediate nigral dopaminergic degradation in both MPTP and LPS mouse models of PD (Du et al., 2018). In a two-hit mouse model comprising both α Syn overexpression and inflammation indicative of PD pathology, Gao et al. (2008) demonstrated, by genetic silencing of α Syn, the dependency of inflammation-mediated dopaminergic degeneration on neuronal α Syn expression. These findings run parallel with *in vitro* work, which demonstrated the relative selectivity of inflammation-mediated neurodegeneration in the presence of α Syn. In primary midbrain neuron-glia co-cultures prepared from transgenic mice expressing human WT α Syn, application of 5 or 10ng/ml LPS resulted in a significant reduction in dendritic length of TH+ neurons, and increased loss of dopaminergic neurons. In contrast, in neuron-glia cultures prepared from transgenic α Syn knockout mice, the same concentrations of LPS failed to induce significant neuronal loss. Moreover, in the neuron-enriched cultures devoid of glia, LPS administration had no effect on neuronal degradation, irrespective of the origin of the cultures. This work indicates a dependency of inflammation-mediated neurotoxicity on the presence of both microglia and α Syn (Gao et al., 2008). While findings from patient samples, rodent models, and cells in culture strongly support the theory that reactive pro-inflammatory microglia are major contributors to neurodegeneration in the α Syn-burdened brain, the mechanisms through which these players interact are still unclear (Figure 7).

Overexpression, aggregation, and propagation of α Syn have been shown to drive microglial polarisation towards a pro-inflammatory phenotype, resulting in increased production of inflammatory cytokines IL-1 β , IL-6, and TNF α , and upregulation of iNOS and free radicals (Calabresi et al., 2023; Du et al., 2018; Sarkar et al., 2020; Tang et al., 2014; Zhang et al., 2005). α Syn aggregates also act as chemo-attractants, directing microglia toward damaged neurons, thus increasing their prevalence in vulnerable brain regions, namely the substantia nigra. Spatial correlations between hypertrophic microglia, infiltrated CD4⁺ and CD8⁺ T lymphocytes, and p α Syn inclusions have been observed post intranigral PFF delivery to the rat brain (Thakur et al., 2017). In early stages of synucleinopathy, microglia play a critical role in clearing extracellular α Syn, a phagocytic process which is thought to be orchestrated directly through glial-expressed transmembrane TLRs. Interestingly, the magnitude of glial pro-inflammatory cytokine production and efficiency of microglial clearance is thought to be dependent on the conformational state of α Syn. For example, full length soluble, fibrillar, oligomeric, and C-terminally truncated α Syn all trigger varied inflammatory responses following interaction with microglia (Lee et al., 2008b; Park et al., 2008b).

A key finding reported by Stefanova and colleagues highlighted TLR4 as a specific mediator through which α Syn can coerce microglia into a pro-inflammatory phenotype, ultimately driving cytotoxic signalling pathways. TLR4 signalling leads to translocation of NF κ B to the nucleus and expression of pro-inflammatory cytokines (Beraud & Maguire-Zeiss, 2012). In a transgenic mouse model overexpressing α Syn, genetic silencing of TLR4 expression increased motor dysfunction, nigrostriatal

dopaminergic degeneration, and α Syn expression within the midbrain, compared to TLR4-expressing controls. The impairment of TLR4-mediated α Syn clearance, and resultant increase in extracellular α Syn, appeared to exacerbate neurodegeneration. Cultured TLR4-deficient BV2 microglial cells also exhibited defective α Syn phagocytosis relative to control BV2 microglia (Stefanova et al., 2011). Subsequent studies by this group identified C-terminally truncated α Syn as a more potent inducer of TLR4-dependent microglial activation, associated IL-6 and TNF α expression, and reactive oxygen species production, compared to monomers or fibrils. These findings suggest that TLR4 plays a key modulatory role on microglial pro-inflammatory responses triggered by α Syn. In contrast to microglia, the uptake of α Syn by cultured astrocytic cells appears not to be dependent on TLR4. Cultured TLR4-deficient astrocytes present with a suppressed pro-inflammatory response and reduced reactive oxygen species production following treatment with α Syn (Fellner et al., 2013).

Quantitative real-time polymerase chain reaction (qRT-PCR) data from mouse microglial cell lines show an ability for PFFs to upregulate messenger ribonucleic acid (mRNA) expression of pro-inflammatory markers including IL-6, TNF α , and IL-1. Subsequent analysis of proteomic and metabolic function further depict mitochondrial damage by PFFs in primary microglia (Sarkar et al., 2020). On the contrary, while Park and colleagues observed no changes to microglial phagocytosis in primary rat cortical microglial cultures treated with either monomeric or fibrillar α Syn on their own, the addition of PFF to monomer-pre-treated cells inhibited phagocytosis (Park et al., 2008b).

Extracellular α Syn is recognized as a DAMP by TLR2, although its ligand activity for α Syn seems to be conformation sensitive. In human peripheral blood mononuclear cells (PBMC) derived from healthy donors, both fibrillar and monomeric α Syn upregulate pro-IL-1 β production through TLR2 receptor engagement. However, only PFFs, and not monomers, subsequently stimulate NLRP3 inflammasome activation, leading to increased secretion of mature IL-1 β , a pro-inflammatory cytokine with neurotoxic properties at high concentrations. Codolo and colleagues suggest that α Syn-associated inflammation to be driven by NLRP3 activation, and that subsequent release of neurotoxic components contributes to neurodegeneration (Codolo et al., 2013). Similar research using monocyte cell lines shows up-regulation of TNF α and IL-1 β following treatment with PFFs, but not monomers or oligomers, in a TLR2 and NLRP3-mediated manner (Gustot et al., 2015). In contrast, results reported by Kim and colleagues have shown in primary mouse microglia devoid of TLR2 expression, a lack of involvement of TLR2 in mediating microglial response to recombinant α Syn monomers, oligomers, or fibrils. Interestingly, a dependency of TLR2 on extracellular α Syn uptake and microglial activation in brains of transgenic mice overexpressing human α Syn has been reported (Kim et al., 2013). While blood and brain tissue samples from PD patients show TLR2 and TLR4 expression to positively correlate with α Syn-associated reactive microgliosis, contradictory evidence from PD mouse models sheds uncertainty on the functional connection between TLR2 and TLR4 and microglia-associated motor deficits (Drouin-Ouellet et al., 2014).

Lyophilized full-length α Syn, as well as α Syn fragments promote the release of pro-inflammatory cytokines IL-6 and TNF α from enriched primary mouse mesencephalic microglia, an effect which was subsequently neutralised by an α Syn capturing antibody (Rabenstein et al., 2019). In addition to upregulation of IL-6 and TNF α , surges in glial secretion of IL-1 α , IL-17 and iNOS, along with increased expression of MHC-II have been reported in primary microglial cultures stimulated with PFFs (Harms et al., 2017).

Regulation of peripheral immune cell activation in the neurodegenerating brain may also involve the microglial expression of MHC-II, given genetic polymorphisms of MHC-II have been linked with sporadic forms of PD (Hamza et al., 2010), and its depletion results in the reduction of glial activation and dopaminergic degeneration in PD mouse models (Harms et al., 2013). MHC-II presentation of α Syn peptides, after microglial phagocytosis, leads to CD4+ T-cell activation and recruitment of the peripheral immune system (Ponomarev et al., 2005; Sanchez-Guajardo et al., 2010). This continuous immune response seems to accelerate α Syn aggregation, thus perpetuating the loop of α Syn-mediated inflammation and associated neurodegeneration.

Exosomal transport of α Syn throughout the CNS has been investigated by Xia and colleagues, who purified α Syn aggregate-containing exosomes from plasma of PD patients. Two weeks following unilateral striatal delivery of PD-patient plasma derived exosomes into eight-month-old mice, this group observed greater exosomal colocalization with amoeboid microglia, microgliosis at the site of injection, and enhanced expression of pro-inflammatory cytokines, compared to mice treated with exosomes derived from healthy controls. Despite unilateral striatal injection, microglial activation, and internalisation of α Syn+ exosomes was observed across the bilateral striatum, substantia nigra, and cortex, further supporting exosomal facilitation of α Syn transport through the CNS. Exosomes preferentially targeted microglia *in vivo*, rather than neurons or astrocytes, as shown by high colocalization with Iba1+ cells, but only partial colocalization with the neuronal marker microtubule associated protein 2 (MAP2) or astrocytic marker glial fibrillary acid protein (GFAP). Nonetheless, delivery of human α Syn+ exosomes significantly reduced TH+ immunoreactivity in striatal dopaminergic neurons (Xia et al., 2019).

In their ability to endocytose α Syn via dynamin-related pathways, astrocytes and macrophages also likely serve as significant conduits for disease transmission (Lee et al., 2008a; Lee et al., 2010). When exposed to α Syn fibrils, human embryonic stem cell-derived astrocytes exhibit increased cell surface expression of costimulatory molecules CD80, CD86 & CD40, which are necessary for T-cell activation. Colocalization of both astrocytic MHC-I and MHC-II with α Syn aggregates following astrocytic uptake, suggests a role for astrocytes as antigen presenting-cells in α Syn-associated pathologies (Rostami et al., 2020).

1.3.3. Inflammatory models of synucleinopathy

Perhaps the most widely used inflammogen to produce neurodegenerative pathology in rodents and in cell models is the endotoxin LPS. LPS is the main component of the outer membrane of Gram-negative bacteria, and mediates a pro-inflammatory response through interaction with TLR4, in association with other receptors. Injection of LPS into various brain regions of rats and mice, including the cerebral cortex, striatum, choroid plexus-cerebral ventricles, substantia nigra, or hippocampus, triggers robust activation of the CNS microglia, driving neuronal degeneration. Dopaminergic neurons within the nigrostriatal bundle have repeatedly been shown to be specifically vulnerable to LPS. As such, the use of LPS in inflammatory models of PD has proven particularly valuable. Injection of LPS (2 μ g in 2 μ L) into the rat substantia nigra or striatum can reduce TH+ neuronal cell bodies and dopamine depletion within three weeks of injection, while serotonergic and GABAergic neurons remain unaffected (Herrera et al., 2000). Unlike toxic agents MPTP or 6-OHDA, which directly damage dopaminergic neurons with the nigra, LPS appears to disrupt neuronal integrity indirectly, through a cytotoxic inflammatory response. Intranigral injection of LPS (5 μ g in 2 μ L) can drive microglial and astrocytic activation, as well as leukocyte infiltration into the rat

substantia nigra within two days of injection. The acute phase of LPS-induced neuroinflammation is characterised by upregulation of pro-inflammatory signalling mediators including TNF α , IL-1 β , and IL-6 at both mRNA and protein levels. Moreover, NO production and lipid peroxidation have been reported within one week of injection, followed not long after by reductions in nigral TH+ cell bodies and striatal dopamine depletion (Flores-Martinez et al., 2018). The short-term physiological consequences of central LPS injection to rodents are apparent following the initial and provisional onset of flu-like sickness behaviours, which are not unlike those exhibited by humans after contracting a bacterial infection. Over time, motoric impairment associated with nigrostriatal dysfunction becomes apparent, the severity of which is quantifiable using behavioural tests including the stepping, cylinder, and amphetamine-induced rotations tests (Garcia-Revilla et al., 2022; Hsieh et al., 2002). As such, the use of LPS to develop animal models has been successful for studies which aim to assess the effects of the inflammatory response system on dopaminergic cells death in experimental inflammatory models of PD. Higher dose injections of 30 μ g (Hsieh et al., 2002) and 50 μ g (Shukuri et al., 2021) into the rat brain have been reported, which undeniably promote robust and reproducible degeneration of the dopaminergic nigrostriatal system, microglial activation, α Syn accumulation, mitochondrial damage, and associated motor impairment. Use of excessive doses of LPS has been criticized for deviating from physiologically relevant concentrations, thus failing to reproduce a disease model more representative of the human disease condition. The reproducibility of PD-like pathologies in LPS rodent models has proven invaluable in trialling new interventions which aim to alleviate neuroinflammation and/or neurodegeneration. Through the use of LPS-associated rodent models, the anti-inflammatory and neuroprotective benefits of a wide range of non-PD drugs have been shown, including dexamethasone, a fluorinated glucocorticoid (Castano et al., 2002), minocycline, a tetracycline antibiotic (Tomas-Camardiel et al., 2004), simvastatin, a lipid-lowering agent (Hernandez-Romero et al., 2008), naloxone, an opioid receptor antagonist (Lu et al., 2000), atomoxetine, a noradrenaline re-uptake inhibitor (Yssel et al., 2018), and formoterol, a noradrenergic beta adrenoceptor (β AR) agonist (O'Neill et al., 2020). The agents may be considered as promising candidates for further evaluation in synucleinopathy-associated models with inflammatory associated pathology.

IFN γ , a pro-inflammatory cytokine produced by neutrophils and type 1 T-helper cells, has also been exploited for development of animal models exhibiting inflammation-associated neurodegeneration (Mamane et al., 1999). Under physiological conditions, IFN γ is a potent activator of microglial cells, and contributes to the management and clearance of invading cerebral insults. Its exaggerated production by T-cell infiltration in the permeable neurodegenerating brain can drive a cytotoxic inflammatory response. IFN γ -mediated microglial activation drives the production of iNOS, IL-6, IL-1 β and TNF α through the Janus kinase (JAK) signal transducer and activator of transcription (STAT) signalling pathway (Mount et al., 2007). IFN γ -triggered increased microglial activation, production of pro-inflammatory signalling molecules, induction of iNOS, and production of NO have been reported both *in vitro* and *in vivo* to be contributing factors in neurodegeneration (Barcia et al., 2012; Mount et al., 2007; Papageorgiou et al., 2016). In male 8-week-old mice, intracerebroventricular delivery of IFN γ (0.5, 1, 2, 4, or 8 μ g in 1 μ L) increased, in a dose-dependent manner, the number of MHC-II⁺Iba1⁺ microglia in the prefrontal cortex and hippocampus 1-, 3-, and 7-days post injection, in observation not seen in vehicle-treated mice. Both vehicle-treated and IFN γ -treated animals resented with increased Iba1⁺ microglia compared to non-injected animals, suggesting evidence of microglial reactivity in response to the injection. IFN γ injection (2 μ g in 1 μ L), and not sham injection,

stably induced the continued upregulation of iNOS, IL-6, IL-1 β , and TNF α mRNA expression in the prefrontal cortex and hippocampus, which was accompanied by reductions in proliferation and survival of immature hippocampal neurons.

In vitro, microglia extracted from IFN γ -treated mice halted proliferation and induced apoptosis of co-cultured primary mouse neural precursor cells. In line with LPS-models, IFN γ -associated models of inflammation have paved the way for the development of disease treatments and interventions. Microglial inhibition with the tetracyclic antibiotic minocycline ameliorated IFN γ -associated pro-inflammatory cytokine production and prevented neuronal damage both in *in vivo* and *in vitro* (Zhang et al., 2020). Similarly, as IFN γ activates microglia through the JAK/STAT pathway, the JAK inhibitor ruxolitinib suppressed upregulation of MHC-II⁺Iba1⁺ microglia and mRNA expression of iNOS, IL-6, IL-1 β and TNF α 1-, 3-, and 7-days post IFN γ intracerebroventricular injection to mice. In cultures, pre-treatment with minocycline or ruxolitinib prior to IFN γ treatment promoted proliferation of neuronal precursor cells and decreased apoptosis of immature neurons (Zhang et al., 2020).

The use of inflammatory models has been, and will continue to drive research efforts forward. However, in lacking consistent α Syn-related pathology, their limitations must be considered. Interestingly, LPS and IFN γ are thought to increase microglial-derived exosome release, a process which has been hypothesized to facilitate the transmission of α Syn to neurons and accelerate neuronal cell death (George et al., 2019; Tsutsumi et al., 2019). There is nonetheless a paucity of literature linking α Syn as an inflammatory stimulus in models of synucleinopathy to neurodegeneration. A rationale therefore exists to further investigate a role for α Syn in mediating microglial activation, inflammation, and inflammation-associated neurodegeneration in experimental models.

1.3.4. Primed microglia exacerbate neurodegeneration in the alpha-synuclein-burdened brain

In synucleinopathic disease, it is proposed that the conversion of α Syn from inherent soluble monomers to aggregated amyloid-like insoluble aggregates drives chronic activation of microglia, to promote a pro-inflammatory and cytotoxic environment within the brain (Fellner et al., 2013; Harms et al., 2017; Stefanova et al., 2011). The presence of additional disease-salient stimuli in the α Syn-burdened brain, possibly resulting from systemic infection, appears to boost the prolonged pro-inflammatory response, contributing significantly to disease progression. Microglia in the amyloidogenic brain are thought to become “primed” following interaction with protein aggregates. In a primed state, glial cells produce an enhanced inflammatory response upon interaction with subsequent disease-salient stimuli, which, prior to protein aggregation, would likely have been surmountable (Perry et al., 2007). The relevance of systemic infection in synucleinopathy has been assessed primarily through intraperitoneal (i.p.) injection of LPS, and other known inflammatory stimuli, in rodent models. Qin and colleagues reported for the first time in mice, the stark reduction in dopaminergic neuronal integrity and upregulation of peripheral TNF α at seven and ten months following a single dose of LPS (5 mg/kg, i.p.) (Qin et al., 2007). Intranasal injection of a non-toxic dose of LPS (0.09 μ g in 2 μ L) increased microglial activation, IL-1 β production, and susceptibility of dopaminergic neurons to 6-OHDA-induced degeneration in Sprague Dawley rats. Continuous subcutaneous (s.c.) infusion of an IL-1 receptor antagonist (3.64 mg/kg/hr) suppressed microglial

TNF α and IFN γ production, and abrogated nigral dopaminergic neuronal loss when assessed 21 days post intranigral 6-OHDA injection (Koprach et al., 2008).

Subsequent studies showed similar results through intranigral injection of the TLR3 immunostimulant polyinosinic:polycytidylic acid (Poly I:C) in rats. Poly I:C-induced glial reactivity within the nigra predisposed rats to exacerbated midbrain dopaminergic neuronal loss and striatal nerve terminal degeneration following subsequent exposure to a low dose of 6-OHDA. An IL-1 receptor antagonist also mitigated this pathology (Deleidi et al., 2010). Systemic LPS-driven IL-1 β production was increased in mice with an intranigral α Syn burden, compared to vehicle-control mice devoid of pre-exposure to α Syn (Couch et al., 2011). In a mouse model of prion disease, systemic injection of LPS (100ug/kg, i.p.) induced microglial activation and central production of IL-1 β , TNF α , and IFN β to an extent far greater than that of LPS-injected healthy controls. An exaggerated dose of LPS (500ug/ml, i.p.) further enhanced the extent of microglial activation, in addition to inducing motor and cognitive dysfunction in prion-infected animals (Cunningham et al., 2009).

In primary cultures, Roodveldt and colleagues demonstrated more robust upregulation of IL-6 and TNF α and suppression of IL-13 in microglial cultures primed with WT α Syn upon subsequent exposure to the TLR7 ligand single stranded ribonucleic acid (ssRNA) and the TLR1/2 ligand Pam3Csk4 (Pam3), compared to non-primed microglial cultures. Interestingly, primed and non-primed microglia responded in comparable ways following subsequent exposure to the TLR4 ligand LPS, which suggests TLR4-mediated downstream signalling to be less involved in the inflammatory pathway driven by primed microglia than non-primed microglia (Roodveldt et al., 2013). It has been suggested that microglia become primed in the brain through concomitant downregulation of neuron-derived mediators which inhibit microglial activation, such as fractalkine, CD47, and CD200 (Wolf et al., 2017).

IFN γ is a potent T-helper cell-derived inflammatory mediator, and as such, has been exploited both *in vitro* and *in vivo* to assess its role in orchestrating enhanced responses of primed microglia in the synucleinopathic brain (Mount et al., 2007). In human PD brains, spatial and temporal distribution α Syn and IFN γ expression seem to be strongly correlated, while healthy brains show no evidence of association (Liscovitch & French, 2014). Plasma and CSF levels of IFN γ are elevated in PD patients, which may reflect increased central IFN γ afforded by lymphocytes and other cells capable of infiltrating the vulnerable CNS (Nakamura et al., 1992; Widner et al., 2002). Further evidence in support of a relationship between IFN γ and microglial activation in PD has been indicated by allelic differences in the IFN γ gene between early- and late- onset PD patients (Hakansson et al., 2005; Mizuta et al., 2001).

In MPTP-induced Parkinsonism in monkeys, microglial and astroglia activation requires expression of IFN γ before reaching full-levels of activation resulting in subsequent dopaminergic cell death (Barcia et al., 2012). Consistent findings from IFN γ -deficient mice show protection against MPTP-induced microglial activation, nigral dopaminergic neuron loss, and striatal fibre depletion. In primary mesencephalic neuron-glia co-cultures, the application of exogenous IFN γ (0.6 and 1ng/ml) caused significant loss of dopaminergic neurons, but this degeneration was not apparent in microglial-depleted cultures. Furthermore, an IFN γ neutralizing antibody attenuated rotenone-induced neuronal loss in neuron-glia co-cultures, indicating a capacity for IFN γ to contribute to dopaminergic cell death through a microglial-mediated process (Mount et al., 2007).

In the α Syn-burdened brain, microglia primed by α Syn likely render the brain ill-equipped to respond appropriately to peripheral stimuli which persist following infection, injury, or stress. On the other hand, the elderly population exhibit greater vulnerability to systemic infection. It is unknown whether normal age-related changes in blood brain barrier integrity and brain permeability predispose the brain to α Syn aggregation and progression of synucleinopathic disease in response to systemic infection. Nonetheless, targeting microglial activation, when primed in response to interaction with α Syn, may provide new opportunities for therapeutic development. In animal and cellular models of neuroinflammation, pharmacological agents which aim to reduce pro-inflammatory glial activation and production of pro-inflammatory mediators and promote expression of neurotrophic factors, also attenuate dopaminergic degeneration and motor dysfunction (Farmer & Pugin, 2000; O'Neill et al., 2020; Qian et al., 2011; Yssel et al., 2018).

Importantly, systemic LPS can be a valuable model for exploring non-motor symptoms associated with prodromal phases of synucleinopathy. Systemic LPS injections have been shown to induce olfactory impairments and anxiety-like behaviours (Deng et al., 2021). Systemic LPS models exhibit early-stage α Syn aggregation and associated symptoms of gastrointestinal disturbance (Kelly et al., 2014). Furthermore, systemic LPS has been shown to promote a Braak-like pattern of sequential degeneration, beginning in the LC, progressing to the substantia nigra, and finally reaching the cortex and hippocampus (Song et al., 2019).

1.4. Noradrenaline: an innate anti-inflammatory neurotransmitter

1.4.1. Noradrenergic vulnerability in the synucleinopathic brain

Noradrenaline is a catecholaminergic neurotransmitter which is synthesized from dopamine by the dopamine- β -hydroxylase enzyme within synaptic vesicles of noradrenergic neurons in the LC. The LC, which lies within the dorsolateral pontine tegmentum, is the primary hub of noradrenergic cell bodies, and the principal source from which noradrenaline is supplied to the neocortex, hippocampus, amygdala, thalamus, cerebellum, and spinal cord. The noradrenergic system facilitates synaptic plasticity and long-term potentiation across all regions of the brain to which its projections extend (Benarroch, 2018). Its role in cognitive processes including attention, memory, alertness, sleep, and mood, is well established (Holland et al., 2021). Extra-synaptic noradrenaline can activate astrocytic glycogen metabolism and Ca^{2+} signalling, contributing to the control of blood flow throughout the circadian cycle (O'Donnell et al., 2015). The noradrenergic system also plays a major role in maintenance of the inflammatory system in the CNS, regulating glial production of pro-inflammatory cytokines and NO (Yao et al., 2015). The physiological effects of noradrenaline are mediated through three families of adrenergic GPCRs - α_1 , α_2 and β - each of which consists of several subtypes. α_1 , β_1 , and β_2 receptors are present primarily at postsynaptic sites, while α_2 receptors are expressed on somato-dendrites and presynaptic terminals of LC neurons, and act predominantly as inhibitory autoreceptors. Adrenergic α_1 receptors mediate mainly excitatory effects through the phospholipase C, inositol triphosphate, and protein kinase C pathways. While β_1 receptors are mainly expressed in the heart, β_2 ARs are detectable in the vascular, bronchial, gastrointestinal, and genitourinary smooth muscles, and in nerve terminals involved in the release of noradrenaline. Given their regional expression, β_2 ARs are likely the primary receptors through which noradrenaline controls vasodilation, bronchodilation, and relaxation of uterine and gastrointestinal muscles. β_2 ARs are positively coupled to adenylyl cyclase, which, through production of cyclic adenosine monophosphate (cAMP), promote synaptic excitability and plasticity via protein kinase A (PKA)-

triggered cascades (Benarroch, 2018; Tank & Lee Wong, 2015). The specific expression of β_2 ARs on microglial and astrocytic cell membranes also contribute to their regulation within the CNS (Yao et al., 2015).

Historically, compounds which drive noradrenergic modulation have been prevalent players in disease treatment and modification. Thousands of years of traditional Chinese medicinal records have accounted for the effective use of the *ephedra sinica* plant for treatment of asthma, bronchitis, hay fever, and cough (Lee, 2011). The plant's active ingredient, ephedrine, and its synthetic derivative pseudoephedrine, act as indirect α/β agonists, and have become gold-standard over-the-counter remedies for respiratory-related disorders. The myorelaxant properties of β_2 ARs have also been employed as tocolytics (Caughey & Parer, 2001). Noradrenaline transporter (NAT) blockers, which enhance noradrenergic transmission by inhibiting its reuptake, are widely used in the clinic for treatment of attention deficit hyperactivity disorder (ADHD) and symptoms of adolescent depression (Zhou, 2004).

Although PD and related synucleinopathies are characterized primarily by nigrostriatal dopaminergic loss, it has been well established that degeneration of noradrenergic neurons in the LC occur during prodromal stages of disease (Bernheimer et al., 1973; Braak et al., 2004). Degeneration of the LC neuronal bundle (and regions to which it projects) reportedly precedes that of nigral dopaminergic degeneration (Del Tredici & Braak, 2013). PD-patient autopsies show greater reduction of neuronal cell bodies in the LC than in the substantia nigra (Zarow et al., 2003). Given noradrenaline's role in muscle relaxation, cognitive processing, attention and mood, sleep-wake cycles, and immune regulation, constraints on its availability likely explain many of the prodromal symptoms of PD. Studies by Rommelfanger and colleagues have shown, through a battery of coordinated movement tests, the consequences of noradrenergic loss on motor function. Lesioning of the mouse LC system with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), a noradrenergic neurotoxin, induced more severe motoric dysfunction and spontaneous dyskinesia, despite intact striatal dopamine content, compared to MPTP-lesioned animals, whose striatal dopaminergic nerve terminals were reduced by 80%. Noradrenergic ablation by DSP-4 treatment (50 mg/kg i.p. injection every two weeks) has also been shown to accelerate LPS (5 mg/kg i.p.)-induced dopaminergic neuron loss in the mouse substantia nigra (Jiang et al., 2015). Dopamine β -hydroxylase knockout mice, which lack noradrenaline, also exhibited motor dysfunction greater than that of MPTP-injected animals, a phenotype which was restored by acute pharmacological restoration of central noradrenaline content. Furthermore, this group has reported attenuation of MPTP-induced nigrostriatal dopaminergic depletion by enhancing the noradrenergic tone, through both genetic silencing and pharmacological blockade of the NAT protein (Rommelfanger et al., 2007). Noradrenaline deficiency exacerbated the degenerative phenotype of experimental autoimmune encephalomyelitis (EAE) mice, a rodent model of multiple sclerosis, while treatment with the noradrenaline reuptake inhibitor (NRI) atomoxetine, in combination with droxidopa, a synthetic amino acid prodrug of noradrenaline, ameliorated limb paresis (Simonini et al., 2010). Atomoxetine, alongside other NAT inhibitors, has recently gained traction following discovery of its anti-inflammatory and neuroprotective properties in inflammatory models of neurodegeneration (Levey et al., 2022; O'Neill et al., 2020; Yssel et al., 2018).

1.4.2. Reconciling alpha-synuclein aggregation, inflammation, and dopaminergic degeneration by enhancing the noradrenergic tone

LC degeneration and ensuing cortical noradrenaline depletion are well documented to negatively influence microglial function, and further drive the progression of neurodegenerative disease pathology *in vivo* (Jiang et al., 2015). Such documented datasets illustrate the reliance of the CNS on balanced noradrenergic tone for neuroprotection and motor function. While evidence from classic inflammatory models of neurodegenerative disease highlights robust neuroprotective effects of glial β_2 AR modulation (O'Neill & Harkin, 2018; O'Neill et al., 2020; Yssel et al., 2018), the ability of noradrenaline to alleviate α Syn-induced inflammation via β_2 AR activation has largely been unexplored. In recent years, the putative neuroprotective properties of noradrenaline mediated via β_2 AR activation have gained traction following discovery of the ability of noradrenaline, and β_2 AR agonists to 1) reduce microglial activation and pro-inflammatory gene expression in the degenerating substantia nigra, 2) regulate α Syn expression at both the gene and protein levels, and 3) promote astrocytic production of neurotrophic factors. A seemingly all-encompassing target, the β_2 AR's regulation of three contributing elements to synucleinopathic neurodegeneration - α Syn accumulation, inflammation, and loss of neurotrophic support - holds promise for the repurposing of β_2 AR agonists for treatment of α Syn-associated and -related neurodegenerative diseases.

1.4.2.1. Beta2 adrenergic receptor activation regulates alpha-synuclein gene expression and translation to protein

One report has provided evidence to support a novel theory, which suggests that activation of glial β_2 ARs is linked with downregulation of SNCA mRNA transcription and α Syn protein expression. Modulation of α Syn expression via β_2 AR is hypothesized by Mittal and colleagues to occur through transcriptional regulation by acetylation or deacetylation of histone 3 lysine 27 (H3K27) along the SNCA promoter and enhancer sites. As such, β_2 AR agonism or antagonism, drive a decrease or increase, respectively, in the permissive acetylated H3K27, thus impacting transcription of SNCA. High-throughput screening and enzyme-linked immunosorbent assays (ELISA) linked β_2 AR agonists salbutamol, clenbuterol, and metaproterenol with downregulation of SNCA transcription and reduced α Syn translation in rat primary cortical neuronal cultures. Clenbuterol treatment has been shown to reduce SNCA mRNA and α Syn protein expression in the substantia nigra of WT mice, and protect against MPTP-associated dopaminergic nigral degeneration (Mittal et al., 2017). Clenbuterol treatment also ameliorated mitochondria-associated superoxide production and TH+ cell death in PD patient-derived iPSC-derived precursor neurons carrying SNCA triplication to a degree similar to that of SNCA knockdown (Flierl et al., 2014; Mittal et al., 2017). Accordingly, the SNCA mRNA and α Syn protein downregulation by clenbuterol were abrogated by genetic deletion of β_2 AR and pharmacological antagonism by propranolol, further supporting the theory of β_2 AR-mediated α Syn translation. A population analysis of Norwegian asthma patients demonstrated reduced risk rate of PD development in salbutamol-treated asthmatics compared to corticosteroid-treated asthmatics. In the same vein, use of β_2 AR antagonists, namely the hypertension medication propranolol, correlated with increased risk rate (Mittal et al., 2017). Similar findings from an Israeli population study associated both short-acting and long-acting β_2 AR agonists with reduced risk of PD, and nonselective β AR antagonists with increased risk of PD (Gronich et al., 2018), although these findings have been challenged by French and American studies which show these associations to be confounded by other behavioural, health, and/or environmental factors (de Germaey et al., 2020; Searles Nielsen et al., 2018). Patterson and colleagues report only minimal effects of repeated

clenbuterol administration on α Syn mRNA expression in rats, mice, and neuronal cultures, and suggest that the limited changes are perhaps due to β_2 AR desensitization and/or increased drug metabolism. Lower levels of clenbuterol were observed in both brain and plasma of rats and mice following a treatment regime involving repeated drug administration, compared a regime involving single injection (Patterson et al., 2022). These data, albeit preliminary, certainly provide rationale for the continued study of β_2 AR modulation of SNCA translation with respect to PD pathology progression.

1.4.2.2. Beta2 adrenergic receptor activation suppresses microglial activation and cytokine expression

Prolonged microglial activation is a self-perpetuating and neurotoxic feature of pathological inflammatory states in neurodegenerative disease (Kettenmann et al., 2011; Qin et al., 2004). Chronic microglial activation and microgliosis are characterised by over production of neurotoxic factors including NO, superoxide, and pro-inflammatory cytokines TNF α , IL-1 β , and IL-6, which have been shown to directly impair nigrostriatal integrity in cellular and animal models. The innate ability of noradrenaline, and noradrenergic agonists, to combat these proponents in inflammation-associated neurodegeneration has been well documented. Primary rat microglia express α_1 , α_2 , β_1 - and β_2 ARs in culture, and reportedly express higher amounts of β_2 ARs than any other cell type in the brain (Mori et al., 2002). In primary rat neuron-glia cultures devoid of noradrenergic neurons, pre-treatment with exogenous noradrenaline for 30 minutes protected against LPS-induced microglial activation, production of superoxide, NO and TNF α , and subsequent dopaminergic cell death. Pre-treatment with noradrenaline conferred no protection against dopaminergic depletion in microglia-depleted co-cultures, indicating a glial-mediated mechanism through which noradrenaline provides neuroprotection. Through the use of β_2 AR-deficient glial cultures, this group theorize the protective effects of noradrenaline (when available at high concentrations) to be predominantly mediated through interaction with glial-expressed β_2 AR (Jiang et al., 2015). Mesencephalic neuron-glia cultures pretreated with either the short-acting β_2 AR agonist salbutamol, or long-acting β_2 AR agonists bambuterol, clenbuterol, formoterol, and salmeterol at a wide range of concentrations (10^{-7} – 10^{-12} M) for 30 minutes prior to the addition of LPS, also conferred protection against LPS-induced neuronal toxicity. Salmeterol reduced pro-inflammatory cytokine production, protecting neuronal cells when applied 2 hours after LPS treatment. Follow-up *in vitro* studies show salmeterol-mediated neuroprotection to be mediated through the inhibition of LPS-induced microglial activation, and production of pro-inflammatory neurotoxic mediators TNF α , superoxide and NO. Other long-acting β_2 AR agonists including formoterol and clenbuterol inhibit microglial production of the proinflammatory mediators TNF α and NO (Qian et al., 2011).

The anti-inflammatory and neuroprotective effects of salmeterol are thought not to be mediated through the conventional GPCR/cAMP pathway, as salmeterol failed to induce microglial cAMP production. Instead, salmeterol likely ameliorates microglial activation through a β_2 AR/ β -arrestin2-dependent (but cAMP-independent) pathway, thereby conferring neuroprotection. Reports from this investigation demonstrate potent protection afforded by salbutamol against both LPS and MPTP-induced superoxide, inducible reactive oxygen species, TNF α , NO production, and dopaminergic neurotoxicity in mice (Qian et al., 2011).

1.4.2.3. Beta2 adrenergic receptor activation promotes astrocytic production of neurotrophic factors

In PD and other synucleinopathic degenerative diseases, LC degeneration and noradrenaline depletion occur alongside reductions in endogenous growth factor production. In addition, astrocytic sparsity further depletes the source of neurotrophic support, leaving dopaminergic neurons in the dying substantia nigra (and other vulnerable populations) at an increased risk of degeneration. In primary astrocytic cultures, 4-hour exposure to 1, 10, and 100 μ M of β_2 AR agonist clenbuterol led to upregulation of nerve growth factor (NGF) mRNA and protein expression. In neuron-astrocyte co-cultures, clenbuterol-driven increases in astrocytic NGF secretion proved to be protective against glutamate-induced excitotoxicity (Culmsee et al., 1999). In human NT2-derived neuronal and primary rat hippocampal cultures modelling Alzheimer's disease, β_2 AR activation by noradrenaline has been shown to boost NGF and BDNF production. NGF and BDNF in turn activate neuronal tyrosine protein kinase (Trk)A and TrkB receptors, respectively, to provide protection against amyloid β -associated degeneration (Counts & Mufson, 2010; Liu et al., 2015).

1.4.2.4. Beta2 adrenergic receptor activation confers neuroprotection in animal models of neurodegeneration

The neuroprotective effects of β_2 AR activation have been investigated in animal and cell models of disease states exhibiting inflammation and neurodegeneration. In a mouse model of amyotrophic lateral sclerosis (ALS), i.p. injection of clenbuterol exhibited protection against loss of lumbar motoneurons, resulting in improved motor performance on the rotarod test, compared to vehicle treatment (Teng et al., 2006). In a mouse stroke model of permanent focal cerebral ischemia, clenbuterol in combination with NMDA receptor antagonist memantine, proved effective in increasing production of neurotrophic factors and ameliorating glutamate excitotoxicity (Culmsee et al., 2004). Selective serotonin and noradrenaline reuptake inhibitors (SNRI), including duloxetine or venlafaxine, have shown neuroprotection by suppressing cytotoxic glial production of IL-6 and IFN γ in neuron-glia co-cultures (Vollmar et al., 2008). The NAT inhibitor venlafaxine has also been shown to boost BDNF expression, while simultaneously suppressing transcription of IL-12, TNF α , IFN γ , CD3, CD8, and other pro-inflammatory mediators in the inflamed spinal cord of a mouse EAE model (Vollmar et al., 2009). Taken together, these findings fit in line with the theory that noradrenaline exerts a neuroprotective role in the brain via the inhibition of pro-inflammatory mediator release and stimulation of growth factor production from glial cells.

Three exploratory clinical studies have been conducted to exploit β -adrenergic stimulation in PD patients, the earliest of which dates back to 1992. The first open label study investigated in nine PD patients the effects of co-administration of β_2 AR agonist salbutamol on mediating symptoms of "wearing off", a phenomenon associated with long-term use of L-DOPA treatment. The results suggest that the addition of salbutamol to L-DOPA treatment regime to be safe (Hishida et al., 1992). Similar observations were reported in a second study, in which eight PD patients showed persistent improvements in parkinsonian symptoms after salbutamol was added as an adjunct to L-DOPA. Consequently, these patients reported symptoms of mild tachycardia, nervousness, and/or worsening of dyskinesia (Alexander et al., 1994b). Although results were encouraging, other therapeutic strategies were favoured until this theory was re-appraised in 2003. A third open label study included eight L-DOPA-treated PD patients with motor fluctuations, but no major dyskinesia. Salbutamol co-administration with L-DOPA showed consequent motor improvement in seven out of

the seven patients who completed the study. One participant withdrew due to symptoms of headache, jitteriness, and anxiety (Uc et al., 2003). Short-term salbutamol treatment exhibited promising immunomodulatory effects in primary progressive multiple sclerosis patients, by reducing plasma concentrations of IL-12, and IL-12-producing monocytes and dendritic cells (Makhoul et al., 2001). Nonetheless, with no large randomized controlled trials performed, the clinical utility of this approach remains an open question.

1.5. Evaluating the potential of formoterol in the treatment of alpha-synuclein-associated inflammation in preclinical models of neurodegenerative disease

Formoterol is a β_2 AR agonist used primarily for the treatment of asthma and symptoms of chronic obstructive pulmonary disease (COPD). In the periphery, formoterol rapidly induces bronchodilation, vasodilation, and smooth muscle relaxation, with minimal risk of unwanted side effects (Rosenhall et al., 2002). A blood–brain barrier permeant, formoterol can enter the CNS parenchyma to exert pharmacological effects on glial cells. As a long acting selective β_2 AR agonist, formoterol displays a higher lipophilicity and higher affinity, selectivity, and potency at glial-expressed β_2 ARs than most short-acting or non-selective β_2 AR agonists (Linden et al., 1996).

The use of formoterol as a treatment for curtailing inflammation and neurodegeneration in the diseased brain remains only loosely explored. In an inflammatory rat model of PD, formoterol attenuated the loss of nigral TH+ dopaminergic neurons and striatal TH+ dopaminergic nerve terminals, prevented the depletion of midbrain and striatal dopamine content, reduced microglial activation, and ameliorated unilateral motor deficits induced by intranigral injection of LPS. Clenbuterol also produced protective effects, but was unable to fully attenuate LPS-induced TH+ striatal denervation, dopamine depletion, or microgliosis (O'Neill et al., 2020). The superior neuroprotective efficacy of formoterol relative to clenbuterol in this study may be dose dependent, but could also likely be accounted for by formoterol's brain penetrance to target microglia (Tanaka et al., 2002) and greater binding affinity for β_2 ARs over β_1 ARs (Anderson, 1993). In the same LPS rat model, atomoxetine treatment exhibited efficacy in suppressing LPS-associated nigral dopaminergic neuronal degeneration, striatal dopaminergic nerve fibre degeneration, nigrostriatal dopamine depletion, and microglial activation, and provided partial protection against unilateral motor impairments (Yssel et al., 2018). Co-treatment of atomoxetine with the β_2 -adrenoceptor antagonist ICI 118551 blocked the protective effects of atomoxetine, in support of the proposed β_2 AR-dependent pathway through which anti-inflammatory and neuroprotective actions are afforded (O'Neill et al., 2020). Pre-treatment of mesencephalic neuron-glia co-cultures with formoterol over a range of concentrations (10^{-7} – 10^{-12} M) for 30 minutes prior to stimulation with LPS imparted protection against LPS-induced toxicity *in vitro* (Qian et al., 2011). Noradrenaline and β AR agonists increase the level of cAMP in microglia by activating receptors and inhibiting proinflammatory cytokines IL-6, TNF- α , and free radicals, thereby controlling the microglial response to cellular damage. In Wistar rats, formoterol treatment ameliorated nigral and striatal dopamine depletion and nigral TH+ cell loss, repressing NF κ B and subsequent production of TNF α , and alleviating motor dysfunction, as measured through open field, grip strength-meter, and rotarod tests, in a rotenone-induced toxin model of PD. Rats received rotenone (1.5 mg/kg; s.c.) every other day for three weeks with or without formoterol treatment (25 μ g/kg/day; i.p.) one hour after rotenone administration, starting from day 11. In parallel, Western blot analysis of striatal homogenate showed a tight inverse correlation between downregulation of β_2 AR expression and upregulation of striatal α Syn protein ($r = -0.99$, $p = 0.0003$). In this model, β_2 AR agonism by formoterol is suggested to mitigate α Syn

accumulation through activation of mitophagy, the molecular process of selective degradation and clearance of defective mitochondria resulting from treatment with the complex I inhibitor, rotenone. This study demonstrates a neuron survival cascade afforded by β_2 AR activation through enhancing mitophagy and mitochondrial biogenesis to remove α Syn aggregates. Moreover, β_2 AR activation by formoterol boosts dopaminergic neuronal integrity through PI3k/Akt/CREB signalling-mediated BDNF production and expression of TH (Khidr et al., 2023).

Taken together, exploratory research reported to date supports a hypothesis that β_2 AR agonists, such as formoterol, exert neuroprotective action in animal models of PD via the inhibition of pro-inflammatory mediator release and promotion of an anti-inflammatory and neurotrophic phenotype.

Although currently marketed treatments for PD are efficacious in combatting motoric dysfunction in many patients, continuous use and gradual dose increases leads to an array of undesirable adverse effects which reduce the patient's quality of life. As β_2 AR agonists are not currently FDA-approved for PD treatment, evaluation in additional experimental models and in clinical trials will be required to determine whether the insights gained on their anti-inflammatory and neuroprotective properties can be translated to patients with PD.

Drug repurposing is a readily exploitable opportunity to discover new indications for existing drugs, particularly with respect to treating neurodegenerative diseases. Specifically, there is rationale for utilising drugs which target glial-expressed β_2 ARs to drive an anti-inflammatory phenotype, promote production of neurotrophic factors, and regulate SNCA transcription, to protect against degeneration in the α Syn-burdened brain. Development of clinically relevant animal models will advance research efforts towards the development of disease modifying treatments and their translation into the clinic.

1.6. Aims and objectives of this thesis

In pursuit of the development of a unilateral rat model of synucleinopathy, the primary aims of this thesis were to investigate the immunogenic, neurodegenerative, and behavioural implications of stereotactic delivery of human recombinant α Syn PFFs into the rat substantia nigra, and explore α Syn-mediated glial and neuronal interactions *in vivo*. Clinically, modulatory agents which target microglial activation may prove useful for the treatment of neurodegenerative conditions associated with inflammation and neuronal atrophy. With a better understanding of the disease-relevant phenotypes driven by α Syn fibrils in the rat, subsequent *in vivo* investigations aimed to assess the potential for formoterol, a pharmacological β_2 AR agonist, to alleviate α Syn-associated glial and neuronal pathology in the newly established rat model of synucleinopathy.

The first objective of this thesis was to establish and characterise a unilateral rat model of early stage synucleinopathy by delivering α Syn PFFs alone, or in combination with AAV vectors expressing α Syn, into the substantia nigra. The effects of exogenous α Syn fibril deposition and α Syn expression on 1) motor function, 2) phosphorylation of endogenous α Syn, 3) microglial cell counts and 4) dopaminergic neuronal cell counts in the substantia nigra were assessed, as was the capacity for treatment with formoterol to influence these measures. Skilled motor function and limb-use asymmetry were assessed through application of the stepping and cylinder tests, respectively. Rotational behaviour in the amphetamine-induced rotations test was assessed to functionally evaluate the presence of a unilateral nigral lesion. pSer129 immunofluorescence, Iba1

immunohistochemistry, and TH immunohistochemistry were conducted on cryopreserved midbrain tissue sections to assess phosphorylated α Syn expression, microglial cell activation, and dopaminergic neuronal integrity, respectively, in the substantia nigra six weeks after α Syn delivery.

Complementary *in vitro* experiments aimed to assess the glial and neuronal interactions associated with α Syn burden in primary rat cultured mixed glial, enriched microglial, and neuronal cultures. Human recombinant α Syn filaments, oligomers, WT PFFs or A53T-mutant PFFs were applied to primary cortical mixed glial cell cultures to assess microglial activation state in response to variant forms of the α Syn protein. Next, primary cortical mixed glial cultures were pre-treated with α Syn A53T PFFs, and subsequently stimulated with IFN γ , a potent microglial activator, to further explore the potential response of α Syn-primed microglia to additional inflammatory stimuli. Microglial activation state was assessed through Iba1+ immunocytochemistry and cell morphology analysis. qRT-PCR was conducted on glial cell lysate to assess changes in mRNA transcription of target cytokines IL-1 β , IL-1 α , IL-6, and TNF α in response to α Syn treatment.

Conditioned media from treated glial cultures was transferred to primary neuronal cultures to assess changes in neuronal atrophy and integrity afforded by α Syn-associated glial factors. Conditioned media was transferred from glial cultures treated with variant forms of human recombinant α Syn, including filaments, oligomers, and PFFs, either with or without IFN γ , to mature primary neurons in culture. Neuronal complexity was assessed with MAP2 immunocytochemistry and Sholl analysis. Lactate dehydrogenase (LDH) assay was used to assess the viability of glial cultures in response to treatment with α Syn species, and neuronal cultures following the transfer of conditioned media from treated glia.

Chapter 2: Materials and Methods

2.1. Materials

Animals

Male and female Wistar rats

Trinity College Comparative Medicines Unit

Laboratory equipment

Autoclave

TOMY SX-700E, Mason Technology

Benchtop large centrifuge

Legend RT+, Sorvall

Benchtop mini centrifuge

Heraeus Pico 17, Thermo Fisher Scientific

Biosafety cabinet

Chemflow, Chemical Systems Control

Cryostat

CM1850, Leica

Epifluorescence microscope

Axio Imager.Z1, Carl Zeiss

Epifluorescence microscope camera

Axiocam 305 mono, Carl Zeiss

Gyratory rocker

SSL3, Stuart

Haemocytometer

Thermo Fisher Scientific

Isoflurane vaporizer

Vet Tech 3 VT1499, Vet Tech

Laminar flow hood

Optimale 18, ADS Laminaire

Light microscope

BX51, Olympus

Light microscope digital camera

DP23, Olympus

Micro-drill

3000 series, DREMEL

Nanodrop spectrophotometer

ND1000, Thermo Fisher Scientific

Oxygen concentrator

Bitmos Medizintechnik

Peristaltic fluid pump

GP1100, Fisher Scientific

pH meter

Mettler Toledo

Polycarbonate cylinder

N/A

Probe sonicator

XL-2000, Misonix

Real-time PCR system

QuantStudio 5, Thermo Fisher Scientific

Stereotactic rat surgical frame

Stoelting

SteriCycle CO² incubator

371, Thermo Fisher Scientific

Thermal cycler

PTC-200, Bio Rad

Video Camera

Cannon, Logitech

Vortex

Vortex Genie 2, Scientific Industries

96-well microplate reader

Biotek

Laboratory consumables

Cell culture flasks (T75cm ²)	Sarstedt
Cell culture plates (24-well, 96-well)	Sarstedt
Cell strainers (40µm)	Fisher
Conical tubes (15ml, 50ml)	Falcon
Glass round coverslips (13mm)	Sigma-Aldrich
Glass slide coverslips (76mm x 26mm)	Sigma-Aldrich
Micropipette tips, filtered (10, 20, 100, 200, 1000µl)	Starlab
Micropipette tips, unfiltered (10, 20, 100, 200, 1000µl)	Sarstedt
Micropipettes (p1000, p200, p100, p20, p10)	Eppendorf
Microscope slides (76mm x 26mm)	Eppendorf
Microtome blades (MX35 Ultra™, 35°, 80 mm)	Eppendorf
Microtubes (0.5ml, 1.5ml, 2ml)	Eppendorf
Neuros surgical needles	NRS75 5.0µl (33/20/3), Hamilton
Neuros surgical syringe	7002 KH SYR, Hamilton
Pasteur pipettes (10ml, 3.5ml)	Sarstedt
Petri dishes (90mm)	Fisher
Sterile scalpels	Sarstedt
Syringe needles (26G x 13mm) (BD Ireland)	BD
Syringe-filters (0.22µm)	Sigma-Aldrich
Syringes (0.05ml, 1ml, 10ml, 20 ml)	Sarstedt

Laboratory reagents

Biocidal ZF™	WAK-Chemie Medical GmbH
Chromium (III) potassium sulfate (KCr(SO ₄) ₂)	Fischer scientific
Dimethyl sulfoxide	Sigma-Aldrich
DPX mounting medium	Sigma-Aldrich
Dulbecco's phosphate buffered saline	Sigma-Aldrich
Gelatin	Sigma-Aldrich
Hydrochloric acid	Sigma-Aldrich
Hydrogen peroxide	Sigma-Aldrich
Isopentane (2-methylbutane)	Sigma-Aldrich
Molecular grade ethanol	Sigma-Aldrich
Molecular grade methanol	Sigma-Aldrich
Optimal cutting temperature (OCT) compound	Sakura Finetek
Paraformaldehyde	Sigma-Aldrich
RNase-free water	Thermo Fisher Scientific
RNaseZap	Thermo Fisher Scientific
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium dihydrogen phosphate (NaH ₂ PO ₄)	Sigma-Aldrich

Sodium phosphate dibasic (Na ₂ HPO ₄)	Sigma-Aldrich
Sucrose solution	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Virkon	Sigma-Aldrich
Xylene	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich
3,3' -diaminobenzidine (DAB)	Sigma-Aldrich

***In vivo* experimental materials**

Buprenorphine	Chanelle Veterinary
d-Amphetamine	Sigma-Aldrich
Formoterol hemifumarate	Tocris Biosciences
Isoflurane	Chanelle Veterinary
LACRI-LUBE eye ointment	Conefrey's Pharmacy
Lidocaine topical cream, 5%	Conefrey's Pharmacy
Lidocaine topical spray, 5%	Conefrey's Pharmacy
Lipopolysaccharide L2630-10MG (Escherichia coli O111:B4 serotype)	Sigma-Aldrich
Povidone iodine, 10%	Duggan Veterinary
Sodium pentobarbital	Duggan Veterinary
Surgibond tissue adhesive	Duggan Veterinary

***In Vitro* experimental materials**

B-27 Supplement	Sigma-Aldrich
Bovine serum albumin	Sigma-Aldrich
Donkey serum	Sigma-Aldrich
Dulbecco's modified Eagle medium: F12	Sigma-Aldrich
Foetal Bovine Serum- heat inactivated	Sigma-Aldrich
Fungizone	Sigma-Aldrich
Glutamax	Sigma-Aldrich
Neurobasal medium	Sigma-Aldrich
Normal goat serum	Sigma-Aldrich
Normal horse serum	Sigma-Aldrich
Penicillin-streptomycin	Sigma-Aldrich
Poly-D-lysine hydrobromide	Sigma-Aldrich
Poly-L-lysine hydrobromide	Sigma-Aldrich
Recombinant human αSyn dopamine-stabilised oligomers (SPR-466)	Stressmarq Biosciences
Recombinant human αSyn	Stressmarq Biosciences
A53T-mutant preformed fibrils (SPR-326)	
Recombinant human αSyn epigallocatechin	Stressmarq Biosciences

gallate-stabilised oligomers (SPR-469)	
Recombinant human α Syn filaments (SPR-450)	Stressmarq Biosciences
Recombinant human α Syn preformed fibrils (SPR-322)	Stressmarq Biosciences
Recombinant rat IFN γ	Peptotech
Trypan Blue (0.4%)	Sigma-Aldrich
Trypsin-EDTA solution (0.25%)	Sigma-Aldrich
Vectashield mounting medium with DAPI	Thermo Fisher Scientific

Primers

GAPDH: Rn01775763_g1 (VIC-MGB)	Thermo Fisher Scientific
IL-6: Rn01410330_m1 (FAM-MGB)	Thermo Fisher Scientific
IL-10: Rn01483988_g1 (FAM-MGB)	Thermo Fisher Scientific
IL-1 α : Rn00566700_m1 (FAM-MGB)	Thermo Fisher Scientific
IL-1 β : Rn00580432_m1 (FAM-MGB)	Thermo Fisher Scientific
TNF- α : Rn00562055_m1 (FAM-MGB)	Thermo Fisher Scientific

Antibodies

Alexa Fluor 488 goat anti-rabbit (A11008)	Thermo Fisher Scientific
Alexa Fluor 546 goat anti-mouse (A11003)	Thermo Fisher Scientific
Mouse anti-MAP2 (M1406)	Sigma-Aldrich
Mouse anti-tyrosine hydroxylase (MAB318)	Millipore
Rabbit anti-Iba1 (019-19741)	WAKO, FUJIFILM
Rabbit anti-pSer129+ α Syn (SMC-600)	Stressmarq Biosciences

Assay Kits

Elite Vectastain ABC Kit (mouse IgG, avidin and biotin conjugates, and normal horse serum)	Vector Laboratories
Elite Vectastain ABC Kit (Rabbit IgG, avidin and biotin conjugates, and normal goat serum)	Vector Laboratories
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific
Lactate Dehydrogenase Assay	Thermo Fisher Scientific
NucleoSpin RNA, Mini kit	Macherney-Nagel

Viral Vectors

AAV1/2-CMV-human A53T α Syn ($>5 \times 10^{12}$ vg/mL)	Vigene Biosciences
AAV5-CBA-human WT α Syn (1×10^{13} vg/mL)	Viral Vector Core, University of Iowa, United States

Gas

Laboratory carbon dioxide (CO₂)

BOC gas

Medical oxygen (O₂)

BOC gas

Software

ANYmaze

Stoelting

CellSens

Olympus

ImageJ

National Institute of Health

MATLAB

MathWorks

Prism

Graphpad

Zeiss Imager

Carl Zeiss

2.2. *In vivo* methods

2.2.1. Animals

All animal experiments were conducted in compliance with the European directive 2010/63/EU on the protection of animals used for scientific purposes, approved by the Animal Research Ethics Committee in Trinity College Dublin, and performed under a licence granted by the Health Products Regulatory Authority. Animal studies are conducted in fulfilment of the 3R's (Replacement, Reduction and Refinement), and reported in compliance with the Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines. 7–8-month-old male and female Wistar rats obtained from the Comparative Medicine Unit in Trinity College Dublin were housed two per cage in hard-bottomed, individually-ventilated polypropylene cages with stainless steel wire tops and wood shavings for bedding. Animals were provided with a polycarbonate tunnel and kraft nesting paper for environmental enrichment. Animals were maintained on a 12:12-hr light–dark cycle in a climate-controlled room at 21°C ($\pm 2^\circ\text{C}$) with 50% humidity ($\pm 10\%$), and were allowed food and water *ad libitum*.

2.2.2. Preformed fibril preparation for use *in vivo*

Human recombinant WT αSyn PFFs (SPR-322) and A53T-mutant αSyn PFFs (SPR-326) were acquired from Stressmarq Biosciences at a stock concentration of 2mg/ml in phosphate buffered saline (PBS) buffer of pH 7.4. For use *in vivo*, no further dilution was necessary. PFFs were stored at -80°C until use. PFFs were thawed at room temperature and gently agitated before use to ensure homogeneity of sample. PFFs were sonicated with a micro probe sonicator (Misonix Ultrasonic Liquid Processor XL-2000) for 6 sets of 10 x 0.5 second pulses, with a 10 second pause in between each set to account for heat production (Volpicelli-Daley et al., 2014). PFFs were administered into the rodent brain via stereotactic surgery immediately after sonication at a concentration of 2mg/ml. Sterile PBS was delivered to control animals.

2.2.3. Adeno-associated viral vector preparation for use *in vivo*

AAV5-CBA-human WT αSyn (product code N/A) was acquired from the Viral Vector Core of the University of Iowa, United States at a viral titre of 1×10^{13} vg/ml. AAV1/2-CMV-human A53T αSyn (GD1001-RV) was acquired from Vigene Biosciences at a viral titre of $> 5 \times 10^{12}$ vg/mL in PBS buffer with 1 mM MgCl_2 and 0.001% Lutrol F68. AAVs were stored at -80°C until use. For use *in vivo*, no further dilution was necessary. Vectors were thawed on ice, and the required injection volume was administered *in vivo* immediately after thawing.

2.2.4. Stereotactic surgery

Surgical procedures were performed using aseptic technique under isoflurane anaesthesia. Animals were anaesthetised with an initial induction of 5% isoflurane, which was reduced and maintained to 2% at a flow rate of 1.5L/min throughout the surgery. After induction, the rats head was shaved, topical anaesthesia was applied to the ears, and the animal was fit into the stereotactic frame using non-rupture ear bars. The incision area was cleansed with betadine anti-septic, and topically anaesthetised with lidocaine spray. Eyes were lubricated with Lacrilube to minimise dryness procedure. Upon confirmation of sufficient plane of anaesthesia, a midline incision was made with a sterile scalpel, and the connective tissue removed to reveal bregma and lambda. A burr hole was drilled in appropriate locations using bregma and lambda as coordinate references. For single injection paradigms, the coordinates for infusion in males were as follows; AP: -5.3 from bregma,

ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for infusion in females were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. For double injection paradigms, the coordinates for the first infusion in males were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for the first infusion in females were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. The coordinates for the second infusion in males were as follows; AP: -4.9 from bregma, ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for the second infusion in females were as follows; AP: -4.9 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. The loaded Hamilton® Neuros syringe (7002 KH SYR; Hamilton, Switzerland) mounted with a 33-gauge needle was gradually lowered to the injection site over the course of 2 minutes. The injection solution was delivered at a rate of 0.5µl/min. After injection, the needle was left in place for an additional 8 minutes to allow diffusion of treatment into the targeted brain region. The needle was then slowly retracted over the course of 2 minutes to limit efflux along the needle tract. The incision was closed with surgical adhesive, and the animal was removed from the stereotactic frame and allowed recover from anaesthesia. Buprenorphine (0.05mg/kg, s.c.) was administered immediately after surgery and every 12-hours for 72 hours after surgery. Buprenorphine is the most commonly used opioid analgesic to combat postoperative pain in laboratory animals, primarily because of its long duration of action and low adverse-effect profile. The typical recommended dose range of buprenorphine in rats is 0.05 mg/kg s.c., administered twice a day for three days after surgery (Reifenrath et al., 2022). A major implication of prolonged exposure to isoflurane anaesthesia during surgery combined with repeated administration of post-operative opioid analgesics is pica, a possibly lethal behaviour which describes the compulsive eating of objects which are non-digestible or not intended to be eaten. Without intervention, severe pica can result in fatal choking, or immobility, lethargy, balance/gait disturbances, and laboured breathing at levels indicative of humane endpoints. The onset and severity of pica is associated with the administration of opioid analgesics, secondary to the isoflurane. Lower doses of buprenorphine help to minimize post-operative pica, as does reducing the length of exposure to isoflurane during surgeries. These tangible refinements were implemented to minimise animal stress and avoid any unnecessary adverse post-surgical effects.

2.2.5. Formoterol preparation

Formoterol hemifumarate (Tocris Biosciences, 1448) was acquired from Tocris biosciences. 10mg formoterol hemifumarate was diluted in 476ul of 100% dimethyl sulfoxide (DMSO) to achieve a stock solution of 21mg/ml. Stock was further diluted to a working concentration of 0.1mg/ml in sterile saline (NaCl). Two weeks post-surgery, rats were treated with 0.1mg/kg of formoterol in an injection volume of 1ml/kg, i.p., once daily for seven days. Sterile NaCl was prepared in DMSO as described above, and administered to control animals in an injection volume of 1ml/kg, i.p., once daily for seven days.

2.2.6. Behavioural Assessments

2.2.6.1. Cylinder test of limb-use asymmetry

The cylinder test was used as a measure of forelimb-use asymmetry (Schallert et al., 2000). The rat was placed in a clear glass cylinder (18.5cm diameter x 50cm high) and its explorative behaviours were recorded for 5 minutes. During this session, the numbers of wall placements and floor placements made with each forepaw were recorded. A wall placement was recorded when the rat contacted the cylinder walls with its forepaw after rearing. A floor placement was recorded when the

rat contacted the cylinder floor before landing on all paws. Wall or floor placements made with both paws at the same time were counted, but excluded from analyses. Combined scores for contralateral and ipsilateral wall and floor placements represent the total number of placements made during the 5-minute testing session. Rearing scores represent the number of wall placements only. Contralateral limb-use was further represented by placing total score for the contralateral limb over total score for the ipsilateral limb expressed as a percentage of limb-use. Rats underwent the cylinder test once before surgery to obtain a baseline score, and once four weeks after surgery for comparison with baseline and between experimental treatment groups. Scores post-surgery were also expressed as a percentage of scores at baseline. The cylinder was sufficiently high to prevent the rats from reaching the top and wide enough to allow an approximate 2cm gap from the base of the tail and the cylinder wall when the rat was on all fours. The test was carried out under dim lighting conditions. The cylinder was cleaned with 70% ethanol (EtOH) between individual animals being exposed to the cylinder test to remove olfactory cues which influence behaviour in the test (Figure 2.1a).

2.2.6.2. Stepping test of forelimb akinesia

The stepping test was used as a measurement of forelimb akinesia (Lundblad et al., 2002; Olsson et al., 1995). In the stepping test, the rat was held in the experimenter's hand, with both of its back limbs and one of its forelimbs restrained. The rat was positioned so that its frontal plane was parallel with the tabletop, and the free forelimb was placed on the table. The rat was guided forward along the edge of a 90cm table in a span of 5 seconds, and the numbers of steps taken with the free limb were recorded. The rat was then guided backwards along the same edge for 5 seconds, while backward steps were recorded. 1 trial comprised of one forward walk and one backward walk along the table on each limb. Each session consisted of 3 trials with 30 minutes rest in between each trial. Rats underwent 1 session of training and habituation each day for 3 consecutive days prior to the baseline test sessions. At baseline, 1 session was recorded each day for 3 consecutive days, and the average score of all sessions was used for analysis. After surgery, each rat was given one practice trial in both directions for each forelimb before post-surgery scores were recorded. At week 5, 1 session was recorded each day for 3 consecutive days, and the average score of all sessions was used for analysis. Overall score was calculated by combining the number of forward steps and backward steps made in each trial and taking the average of three consecutive trials. Forward scores were calculated by adding the number of forward steps made in each trial and taking the average of three trials. Contralateral stepping was further presented by expressing total score for the contralateral limb over total score for the ipsilateral limb and expressing as a percentage of stepping. Scores post-surgery were also expressed as a percentage of scores at baseline. The test was carried out under dim lighting conditions. The table was cleaned with 70% EtOH in between testing sessions to remove olfactory cues which influence behaviour in the test (Figure 2.1b).

2.2.6.3. Amphetamine-induced rotations test

The amphetamine-induced rotation test is a behavioural paradigm conducted to indirectly assess the behavioural impact of a lesion in the nigrostriatal bundle (Bjorklund & Dunnett, 2019; Torres et al., 2011). Rats were placed in a circular arena (female arena: 19cm diameter x 50cm height; male arena: 29cm diameter x 44cm height) and allowed to acclimatize for 20 minutes. Rats were then injected with d-amphetamine (5mg/kg), in an injection volume of 1ml/kg i.p., and their rotational behaviour was recorded for 90 minutes, commencing five minutes after injection. An amphetamine-

induced rotation is scored when the animal moves in a wide circle around the arena and completes a turn of 360° in either direction. The number of rotations in the directions ipsilateral and contralateral to the lesion were recorded using ANYmaze behavioural tracking software (Stoelting Co. version 7.1). Total rotations and net ipsilateral rotations were calculated from the raw data. A period of at least 4 days was scheduled between amphetamine-injection and euthanasia to allow for the acute effects of amphetamine administration on the dopaminergic system to wash out before tissue collection. A dose of 5mg/kg was selected following publication by Yssel and colleagues (Yssel et al., 2018), but future experiments should utilize lower a dose ranging between 1-3mg/kg (Bjorklund & Dunnett, 2019) (Figure 2.1c).

a) Cylinder test of limb-use asymmetry

- **Task:** Free exploration in a clear glasses cylinder for 5 minutes
- **Conducted:** Baseline, 4 weeks post-surgery
- **Measured output:** numbers of wall placements & number of floor placements made with each forepaw
- **Data presentation:** lateral wall placements, lateral wall and floor placements combined, % contralateral limb / ipsilateral limb, % 4-weeks post-surgery / baseline



b) Stepping test of forelimb akinesia

- **Task:** Guided stepping across 90cm table with isolated forepaw
- **Conducted:** Training, baseline, 5 weeks post-surgery
- **Measured output:** numbers of forward steps & number backward steps made with each forepaw
- **Data presentation:** lateral forward steps, lateral forward and backward steps combined, % contralateral limb / ipsilateral limb, % 4-weeks post-surgery / baseline



c) Amphetamine-induced rotations test

- **Task:** Systemic amphetamine injection-induced rotating in a circular arena for 90 minutes
- **Conducted:** 6 weeks post-surgery
- **Measured output:** number of rotations made in the directions ipsilateral and contralateral to the lesion

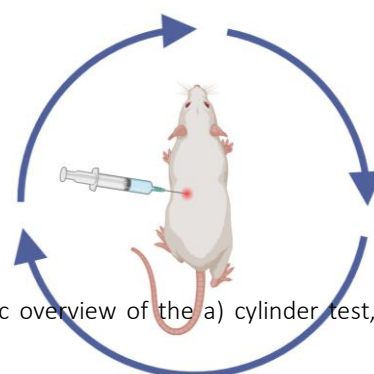


Figure 2.1: Schematic representation of behavioural tests. Schematic overview of the a) cylinder test, b) stepping test, and c) amphetamine-induced rotations test.

2.2.7. Transcardial perfusion and tissue cryopreservation

Awake rats were administered a terminal dose of sodium pentobarbital (100mg/kg). A thoracotomy was performed to expose the beating heart, the left ventricle was pierced with a 23-gauge injection needle, and an incision was made in the right ventricle to allow efflux during the perfusion. Ice-cold PBS perfusate was pumped at a rate of 15ml/min with a perfusion pump (Gilson) until liver was cleared of blood and runoff perfusate expelling from right atrium ran clear. PBS perfusate was then replaced with 4% paraformaldehyde and pumped at a rate of 15ml/min until sufficient tissue fixation. Animals were then decapitated, and the brains were removed and post-fixed overnight in 4% paraformaldehyde at 4°C. Brains were transferred to 30% sucrose solution for 24-72 hours for cryoprotection, and snap frozen in isopentane before storage at -80°C.

2.2.8. Tissue processing

Prior to cryosectioning, brains were embedded in cryotome freezing medium. Coronal sections of 35µm thickness were collected in serial sequence on gelatine-coated glass slides and stored in light-protected slide boxes at -80°C until further use.

2.2.9. pSer129 immunofluorescence (with TH co-stain)

The phosphorylation of α Syn at serine residue 129 is widely accepted as the post-translational modification most closely associated with pathology in synucleinopathies (Luk et al., 2012b; Luk et al., 2009; Waxman & Giasson, 2008). For this reason, pSer129 immunofluorescence was used in this study to identify pathologic inclusions in the rat substantia nigra through optimised adaptation of methods previously described (Awa et al., 2022; Fujiwara et al., 2002). Dual immunofluorescence with TH was utilized to confirm localization of pSer129+ inclusions in the substantia nigra. Slide mounted tissue sections were thawed at room temperature. Sections were outlined with a hydrophobic pen to allow for even dispersal of reagents throughout the staining process. Sections were rehydrated by washing 3 times in PBS with 0.1% Triton-X (PBS-T) for 10 minutes per wash with gentle agitation. Non-specific binding sites were blocked with 10% normal goat serum (NGS) in 0.1% PBS-T for 2 hours at room temperature, and primary antibody cocktail was applied overnight in a light protected incubation chamber. Primary antibody cocktail consisted of rabbit anti-pSer129 α Syn primary antibody (Stressmarq Biosciences SMC-600, 1:1000) and mouse anti-TH primary antibody (Merck Millipore MAB318, 1:2000) in 3% NGS in 0.1% PBS-T. The next day, sections were washed 3 times in 0.1% PBS-T for 10 minutes per wash with gentle agitation, and secondary antibody cocktail was applied for 2 hours in a light protected incubation chamber. Secondary antibody cocktail consisted of Alexa Fluor 488 goat anti-rabbit (Invitrogen A11008, 1:1000) and Alexa Fluor 546 goat anti-mouse (Invitrogen A11003, 1:1000) in PBS. Sections were washed twice in PBS for 10 minutes per wash with gentle agitation. Nuclei were counter stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, 1:20,000) in PBS for 10 minutes. Sections were washed in a final set of 3 x 10-minute washes in PBS before mounting under glass coverslips with anti-fade mounting media. Sections were stored at 4°C in a light protected environment.

2.2.10. Imaging and analysis of pSer129+ alpha-synuclein immunofluorescence

Images were taken at 20x magnification using a Zeiss AxioCam camera mounted on an AxioImager Z1 epifluorescence microscope. Three images were taken from each hemisphere to collate the full substantia nigra in each section. ImageJ software (version 1.54d) was used to convert images to 8-bit and apply a consistent threshold to delineate positive staining from background fluorescence.

The average grey value of each thresholded image was measured. The following ImageJ macro was developed internally to semi-automate the process: `SetOption("ScaleConversions", true); run("8-bit"); run("Auto Threshold", "method=Triangle white"); run("Measure");`. The mean grey values of each image were averaged to obtain an average fluorescence value for the injected and non-injected hemisphere of each section. A minimum of 6 sections were analysed per animal. For each animal, the mean fluorescence of the injected hemisphere and non-injected hemisphere was calculated by finding the mean grey value of all sections per hemisphere. Mean fluorescence expressed as a % of the non-injected side was calculated first per section, then used to determine the % value for each animal.

2.2.11. Iba1 immunohistochemistry

Iba1, a calcium-binding adapter molecule coded for by the AIF1 gene, is a widely used marker for the immunohistochemical analysis of microglia. It is expressed ubiquitously throughout all functional microglial phenotypes (Lier et al., 2021; Walker & Lue, 2015). Slide mounted tissue sections were thawed at room temperature and rehydrated in PBS-T for three 10-minute washes with gentle agitation. Endogenous peroxidase activity was quenched in 3% H₂O₂ in 20% methanol (MeOH) in dH₂O for 20 minutes at room temperature. Sections were washed 3 times in PBS-T for 10 minutes per wash with gentle agitation. Sections were outlined with a hydrophobic pen to allow for even dispersal of reagents throughout the staining process. Non-specific bindings sites were blocked with 10% NGS in PBS-T for 2 hours at room temperature, and rabbit anti-Iba1 primary antibody (Wako 019-19741, 1:1000) in 3% NGS in PBS-T was applied overnight at room temperature in a light protected incubation chamber. The next day, sections were washed 3 times in PBS-T for 10 minutes per wash with gentle agitation, and incubated with horse radish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody for 2 hours at room temperature in the incubation chamber. Sections were again washed 3 times in PBS for 10 minutes per wash with gentle agitation, and incubated with avidin biotin complex (ABC) for 2 hours at room temperature. The ABC method was used to visualise staining using peroxidase as enzyme, hydrogen peroxide as substrate, and 3,3-diaminobenzidine (DAB) as chromogen to visualise the antibody-bound peroxidase complex. Sections were washed in a final set 3 x 10-minute washes in PBS before 5-minute incubation in DAB (or until sufficient reaction occurred). Sections were submerged in dH₂O to stop the reaction, and excess chromogen was removed by washing in a series of dH₂O baths. Finally, sections were dehydrated in a series of ascending EtOH concentrations (70% EtOH, 90% EtOH, 95% EtOH, 100% EtOH) for 2 minutes each, and cleared with 2 x 2-minute rounds of in xylene incubation. DPX mounting medium was used to apply glass coverslips and sections are left to dry overnight.

2.2.12. Imaging and analysis of Iba1+ microglia

Images were taken at 20x magnification on an Olympus BX51 microscope with DP23 camera. 3 images were taken from each hemisphere to collate the full substantia nigra in each section. ImageJ software (version 1.54d) was used to convert images to 8-bit for cell counting. The number of Iba1+ microglia within the substantia nigra of each hemisphere was counted per section. A minimum of 6 sections were analysed per animal. The average cell count was first calculated per section, then calculated per animal. Cell counts were also expressed as a % of the uninjected side, which was first calculated per section, then per animal.

2.2.13. TH immunohistochemistry

TH is the rate limiting enzyme in the dopamine biosynthesis pathway, and a phenotypic marker of dopaminergic neurons in the substantia nigra (Albert et al., 2019). Slide mounted tissue sections were thawed at room temperature and rehydrated in PBS-T for 3 x 10-minute washes with gentle agitation. Endogenous peroxidase activity was quenched in 3% H₂O₂ in 20% MeOH in dH₂O for 20 minutes at room temperature. Sections were washed 3 times in PBS-T for 10 minutes per wash with gentle agitation. Sections were outlined with a hydrophobic pen to allow for even dispersal of reagents throughout the staining process. Non-specific bindings sites were blocked with 10% normal horse serum (NHS) in PBS-T for 2 hours at room temperature, and mouse anti-TH primary antibody (Millipore MAB318, 1:2000) in 3% NHS in PBS-T was applied overnight at room temperature in a light protected incubation chamber. The next day, sections were washed 3 times in PBS-T for 10 minutes per wash with gentle agitation, and incubated with HRP conjugated horse anti-mouse secondary antibody for 2 hours at room temperature in the incubation chamber. Sections were again washed 3 times in PBS for 10 minutes per wash with gentle agitation, and incubated with ABC for 2 hours at room temperature. Sections were washed in a final set of 3 x 10-minute PBS washed before a 5-minute incubation in DAB (or until sufficient reaction occurred). Sections were submerged in dH₂O to stop the reaction, and excess chromogen was removed by washing in a series of dH₂O baths. Finally, sections were dehydrated in a series of ascending EtOH concentrations (70% EtOH, 90% EtOH, 95% EtOH, 100% EtOH) for 2 minutes each, and cleared with 2 x 2-minute rounds of in xylene incubation. DPX mounting medium was used to apply glass coverslips and sections were left to dry overnight.

2.2.14. Image and analysis of TH+ neurons

Images were taken at 20x magnification on an Olympus BX51 microscope with DP23 camera. 3 images were taken from each hemisphere to collate the full substantia nigra in each section. ImageJ software (version 1.54d) was used to convert images to 8-bit for cell counting. The number of TH+ cell bodies within the substantia nigra of each hemisphere was recorded per section. A minimum of 6 sections were analysed per animal. The average cell count was first calculated per section, then calculated per animal. Cell counts were also expressed as a % of the uninjected side, which was first calculated per section, then per animal.

2.3. *In vitro* methods

2.3.1. Aseptic technique

All *in vitro* work was conducted using aseptic technique under a sterile laminar flow hood. When not in the laminar flow hood, cell cultures were maintained at 37°C in a 5% carbon dioxide humidified incubation chamber.

2.3.2. Preparation of primary cortical neuronal cultures

Neurobasal media (NBM) was supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin, 1% (v/v) Glutamax, and 0.1% (v/v) fungizone to achieve plating NBM (pNBM). NBM was supplemented with 1% (v/v) penicillin-streptomycin, 1% (v/v) Glutamax, 0.1% (v/v) fungizone, and 1% (v/v) B27 neuronal supplement to achieve feeding NBM (fNBM). Medias were pre-warmed at 37°C before culture preparation. Rat cortices from 1- or 2-day old (P1, P2) Wistar rat pups were obtained from the Comparative Medicine Unit in Trinity College Dublin. Pups were decapitated and cortical tissue was isolated, homogenized by chopping with a

sterile scalpel, and dissociated in Trypsin-Ethylenediaminetetraacetic acid (EDTA) for 2 minutes at 37°C. pNBM was added to deactivate the Trypsin-EDTA. Tissue was triturated twice and centrifuged at 2,000 rotations per minute (rpm) for 4 minutes at 21°C. The homogenate was re-suspended in fresh pNBM, filtered through a 40µm cell strainer, and centrifuged again. The supernatant was discarded, and the cell pellet re-suspended in fresh pNBM. Cells were plated at a seeding density of 5×10^5 cells/mL on poly-D-lysine coated glass coverslips in 24 well plates. Poly-D-lysine is the preferred substrate for promoting adhesion of neuronal cells in long-term cultures, which may release proteases capable of degrading poly-L-lysine. After 15-24 hours, media is replaced with 300uL of fresh pNBM per well. After 96 hours, and once per week after that, 50% of media was removed and replaced with fresh pNBM, with an additional 20% volume to account for evaporation. Mature neurons were treated with compounds between 14-21 days *in vitro* (DIV) and fixed for immunocytochemistry subsequently as described below.

2.3.3. Preparation of primary cortical mixed glial cultures

Dulbecco's modified Eagle's medium (DMEM) F12 was supplemented with 10% (v/v) heat inactivated FBS, 1% (v/v) penicillin-streptomycin, and 0.1% (v/v) fungizone to achieve complete mixed glial DMEM (mxgDMEM). Medias were pre-warmed at 37°C before culture preparation. Cortical tissue was isolated from brains of P2-3 neonate Wistar rat pups as described above. Homogenate was dissociated in mxgDMEM for 20 minutes at 37°C. Cell suspension was triturated, filtered through a 40µm cell strainer, and centrifuged at 2,000 rpm for 4 minutes at 21°C. Supernatant was discarded, and the cell pellet re-suspended in fresh mxgDMEM. Cells were seeded at 5×10^6 cells/mL on poly-L-lysine coated glass coverslips in 24-well plates. Poly-L-lysine is a synthetic polymer which adequately promotes glial cell adhesion in culture, cell proliferation, and cell spreading, which is ideal for glial morphology investigations. Every 4-5 days, 50% of the media was removed and replaced with fresh mxgDMEM, with an additional 20% volume to account for evaporation. Mature mixed glial cultures were treated with compounds between DIV14-21. The resulting conditioned media was removed, filter-sterilised, and saved for cytokine analysis or treatment of neuronal cultures. Cells were then fixed for immunocytochemistry subsequently as described below.

2.3.4. Preparation of primary cortical enriched microglial cultures

mxgDMEM was prepared as described above with additional supplement of 10ng/ml granulocyte-macrophage colony-stimulating factor (gmCSF) to achieve enriched glial DMEM (engDMEM). Medias were pre-warmed at 37°C before culture preparation. Mixed glial cultures were prepared and maintained as described above in poly-L-coated T75 flasks. On DIV13, flasks were shaken for 3 hours at 200rpm at 37°C to lift microglia from the flask surface. Astrocytes remained adherent. Homogenate was transferred to a 50mL sterile tube, and centrifuged at 2000rpm for 5 minutes at 21°C. Supernatant was discarded, and the cell pellet re-suspended in fresh engDMEM. Cells were seeded at 5×10^6 cells/mL on poly-L-coated glass coverslips in 24-well plates. After 24 hours, 50% of the media was removed and replaced with fresh engDMEM, with an additional 20% volume to account for evaporation. Mature enriched glial cultures were treated with compounds between DIV14-21. The resulting conditioned media was removed, filter-sterilised, and saved for cytokine analysis or treatment of neuronal cultures. Cells were then fixed for immunocytochemistry subsequently as described below.

2.3.5. Preparation of alpha-synuclein species for treatment of primary neuronal and glial cultures

All α Syn species (provided by Stressmarq Biosciences) were thawed at room temperature under the laminar flow hood. Preparations utilised in the present study included WT PFFs, A53T-mutant PFFs, dopamine-stabilised (DS) oligomers (DSO), epigallocatechin gallate-stabilised (EGCG) oligomers (EGCGO), or soluble filaments (FILL). Fibrils and filaments (not oligomers) were sonicated with a probe sonicator (Misonix Ultrasonic Liquid Processor XL-2000) at 10% power for 6 sets of 10 pulses, 0.5 seconds per pulse, with a 10 second break in between each set to prevent protein denaturation. Preparations were diluted to a desired working concentration of 4 μ g/ml in cell culture media.

2.3.6. Collection of mixed glial and enriched microglial conditioned media

Upon completion of treatment protocol, mixed glial or enriched microglial conditioned media was removed, filter sterilized through a 0.22 μ m syringe filter, and frozen at -20°C until required for cytotoxicity assay or neuronal treatments.

2.3.7. Lactate dehydrogenase cytotoxicity assay

LDH is a cytosolic enzyme present in many different cell types, and is released extracellularly upon damage to the plasma membrane. Damage of the plasma membranes of cells *in vitro* results in a release of LDH into the cell culture medium. Extracellular LDH can be quantified by a coupled enzymatic reaction in which LDH catalyses the conversion of lactate to pyruvate via nicotinamide adenine dinucleotide (NAD⁺) reduction to NAD and hydrogen (NADH). Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that can be measured at 490nm. The quantity of formazan is directly proportional to the amount of LDH released into culture medium. LDH is a well-established and reliable indicator of neuronal and glial cellular toxicity, and as such, can be measured to assess levels of cytotoxicity and cell death *in vitro* in response to cell culture treatments (Chan et al., 2013; Sun et al., 2018). LDH assays (Invitrogen, C20300) were conducted on cell culture supernatant to assess cytotoxicity. 50 μ L of glial or neuronal cell supernatant samples were loaded into individual wells of an optically clear 96 well plate and incubated with 50 μ L of reaction mixture in a light-protected environment at room temperature for 30 minutes. 50 μ L of stop solution was then added to each well before the absorbance was read at 490 nm and 680 nm on a microplate reader (Synergy HT, BioTek Instruments). Background absorbance value at 680 nm was subtracted from 490 nm to extrapolate LDH activity and cell cytotoxicity levels. LDH activity is presented as absorbance expressed as a percentage of controls, where 100% represents no LDH, and 1000% represents LDH from cells treated with 0.01% hydrogen peroxide (and LDH levels from positive controls supplied within the assay kit).

2.3.8. Iba1 immunocytochemistry

Mixed glial cultures prepared for the analysis of microglial morphology were fixed with 4% paraformaldehyde for 30 minutes. Cells were washed with PBS and blocked for 30 minutes with 5% NGS in PBS-T. Rabbit anti-Iba1 primary antibody (Wako 019-19741, 1:1000) in 5% NGS in PBS-T was applied overnight at 4°C. Cells were washed and Alexa Fluor 488 goat anti-rabbit (Invitrogen A11008, 1:500) in PBS was applied for 2 hours at room temperature in a light-protected environment. Coverslips were washed and mounted onto glass slides with Vectashield fluorescent mounting media containing a counterstain for DAPI. Slides were stored in a light-protected environment at 4°C.

2.3.9. Imaging and analysis of Iba1+ microglia

Stained cells on coverslips were visualised at 20X magnification using an AxioImager Z1 epifluorescent microscope with a Zeiss AxioCam HR camera and AxioVision 4.8.2 software. A constant exposure was maintained throughout capturing all images within a coverslip. The outline of each cell was manually traced using the freehand selection tool on ImageJ (1.53e), and the area, perimeter, circularity, Feret's diameter, and solidity of each cell is quantified (Figure 2.2). Area and perimeter are parameters which describe the extent to which cells are elongated and ramified, while Feret's diameter, is representative of cell ellipticity (Nogradi et al., 2020; Zanier et al., 2015). Circularity and solidity are parameters which range from 0-1, where a value of 0 describes a linear polygon, and a value of 1 describes a perfect circle. Together, these parameters indicate a cells proximity to that of a perfect circle or irregular ameboid figure (Zanier et al., 2015).

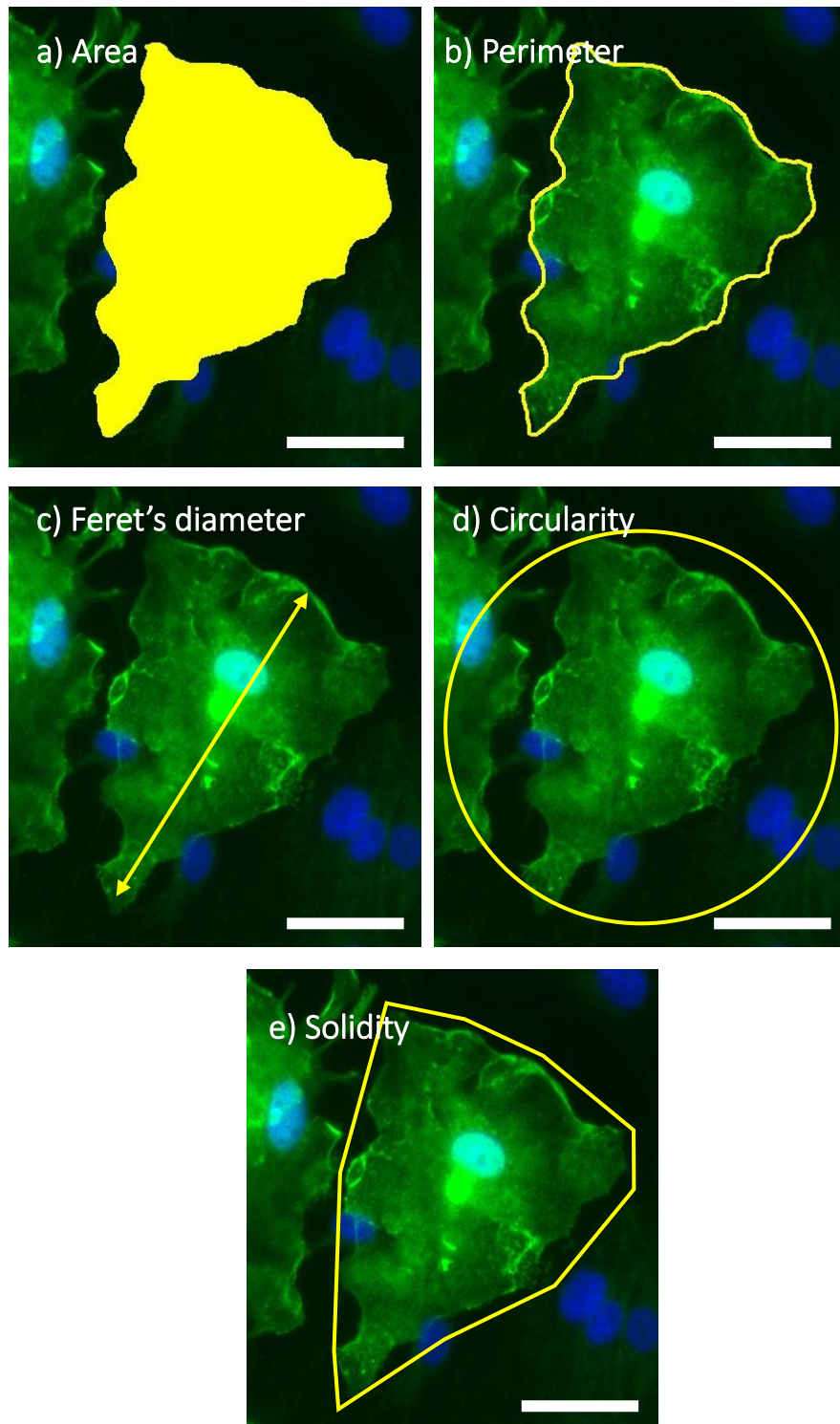


Figure 2.2: Imaging and analysis of fluorescently stained Iba1+ microglia using ImageJ. Representative 20x images of mature primary cortical microglia fluorescently labelled with Iba1 in green and DAPI in blue. The outline of each Iba1+ cell was manually traced using the freehand selection tool on ImageJ (1.53e), and the a) area, b) perimeter, c) feret's diameter, d) circularity, and e) solidity of each cell was quantified. Yellow annotations depict the cellular regions which these measures represent. Scale bar = 25 μm .

2.3.10. MAP2 immunocytochemistry

MAP2 is a cytoskeletal protein expressed abundantly throughout mature neurons in culture and can be fluorescently labelled to visualize and quantify neuronal complexity (Sanchez et al., 2000). Mature neuronal cultures prepared for the analysis of neuronal complexity were fixed with 4% paraformaldehyde in PBS for 20 minutes. Cells were washed and blocked for 2 hours with 2% NGS and 2% bovine serum albumin (BSA) in PBS-T. Mouse anti-MAP2 primary antibody (Sigma-Aldrich M1406, 1:1000) in 2% NGS and 2% BSA in PBS-T was applied overnight at 4°C. Cells were washed and stained with Alexa Fluor 546 goat anti-mouse (Invitrogen A11003, 1:500) PBS-T for 2 hours at room temperature in a light-protected environment. Coverslips were washed and mounted onto glass slides with Vectashield fluorescent mounting media containing a counterstain for DAPI. Slides were stored in a light-protected environment at 4°C.

2.3.11. Imaging and analysis of MAP2+ mature neurons

Sholl analysis is a validated method for quantifying and graphically representing neurite morphology and neuronal complexity (Gutierrez & Davies, 2007; Sholl, 1953). For each cell, the cell soma, number of primary neurites, ends of each neurite, and bifurcations along those neurites are identified manually using the freehand selection tool on MATLAB R2023a. A set of 25 concentric rings with 10µm radial increments between each ring is overlayed on each cell, centred round the soma identified. The number of neurites intersecting each ring is quantified using the following equation: $x_i = x_{i-1} + b_i - t_i$, where “ x_i ” is the number of primary neurites in the “ i th” segment, “ b_i ” is the number of branches occurring in the “ i th” segment, and “ t_i ” the number of neurite terminations in the “ i th” segment. This equation is programmed into Matlab to allow for a semi-automated procedure. With this procedure, the number of primary neurites, relative length of primary neurites, number of branches, and relative Sholl profile (number of intersections at each radial distance from the soma) was measured for each cell. Primary neurites were defined as neurites extending directly from the cell soma. Neuritic branches were defined as extensions from primary neurites of at least 5µm in length. Relative length of primary neurites refers to a value extrapolated from the total number of estimated intersections per neuron, a value which is linearly proportional to the metric length of the neuritic arbores (Figure 2.3).

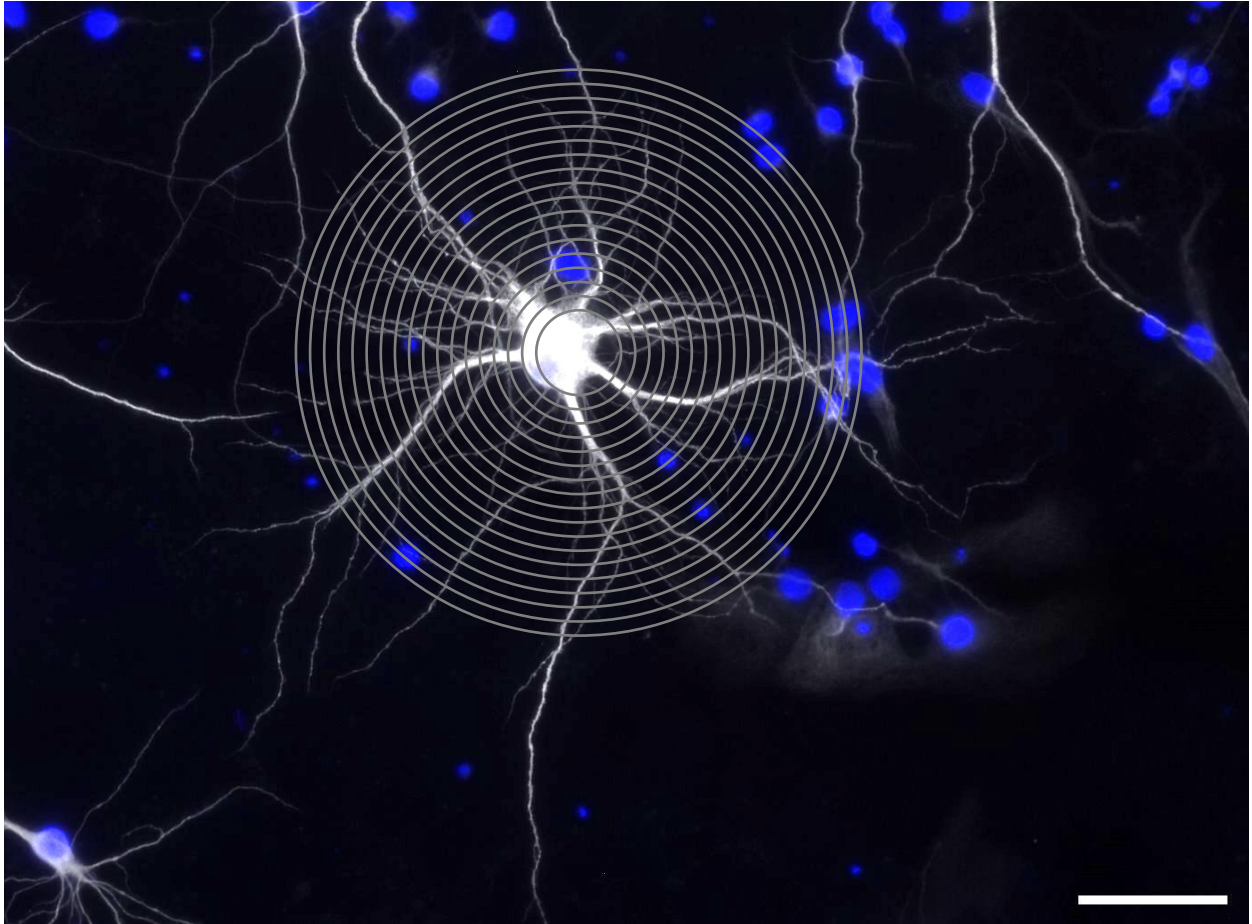


Figure 2.3: Imaging and analysis of fluorescently stained MAP2+ primary neurons using Sholl analysis. Representative 20x image of mature primary cortical neurons fluorescently labelled with MAP2 in white and DAPI in blue. Sholl analysis employs a set of 25 concentric rings centred round the soma, with 10µm radial increments between each ring, to assess neuronal complexity. Scale bar = 50µm.

2.3.12. Quantitative real time polymerase chain reaction (qRT-PCR)

2.3.12.1. Cell lysis

Cell lysis and sample preparation was conducted using the Macherey-Nagel™ NucleoSpin™ ribonucleic acid (RNA) isolation kit (740955.50, Macherey Nagel). Cells were lysed with buffer comprising β -mercaptoethanol in RA1 lysis buffer (1:100) and scraped into RNase-free 1mL Eppendorf tubes. Lysate was stored at -80°C until use.

2.3.12.2. RNA isolation

RNA isolation was conducted with the Macherey-Nagel™ NucleoSpin™ RNA isolation kit (740955.50, Macherey Nagel) in accordance with the manufacturer's protocol. Samples were thawed on ice and triturated with a micropipette to redistribute lysate following freeze-thaw. Cell lysate was passed through a violet ring filter and centrifuged at 11,000g for 1 minute to clear the lysate. Samples were diluted 1:1 with 70% culture-grade RNase-free EtOH to adjust RNA binding conditions, and triturated gently until a homogenous solution was achieved. EtOH-lysate solution was passed through a blue ring filter and centrifuged at 11,000g for 30 seconds to bind RNA to the filter. Sample was washed with membrane desalting buffer and centrifuged at 11,000g for 1 minute. rDNase reaction mixture was gently pipetted into the base of the blue ring filter and incubated for 15 minutes at room temperature to digest DNA. rDNase was deactivated with Macherey-Nagel™ RAW2 buffer, and centrifuged at 11,000g for 30 seconds. RA3 buffer was passed through the filter and centrifuged first for 30 seconds at 11,000g, then passed through again and centrifuged for 2 minutes at 11,000g. The blue ring filter was placed into an RNase-free Eppendorf tube, and RNA is eluted in RNase-free dH₂O by centrifugation at 11,000g for 1 minute. RNA was then stored at -80°C until use.

2.3.12.3. cDNA manufacture

Prior to complementary DNA (cDNA) manufacture, RNA quantification was conducted with a nanodrop spectrophotometer (Nanodrop 2000 spectrophotometer, ThermoFisher Scientific). RNA samples were equalised to the lowest concentration by dilution in RNase-free dH₂O. cDNA was manufactured from equalised RNA sampled using high-capacity cDNA reverse transcription kit following the manufacturer's protocol (Applied Biosystems, ThermoFisher Scientific). Equalised RNA samples were diluted 1:1 with reverse transcription master mix and were centrifuged at 1,200xg for 30 seconds. cDNA was then amplified in a thermocycler (PTC-200 Peltier Thermal Cycler, MJ Research) through the following protocol: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, 4°C ∞ . Once cDNA thermal cycling was complete, samples were stored at -20°C.

2.3.12.4. Polymerase chain reaction

For each cDNA sample, 6 μ L of qRT-PCR master mix was prepared by combining 5 μ L of Taqman master mix with 0.5 μ L of primer for endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 0.5 μ L of desired target primer. 6 μ L of qRT-PCR mix was added to 4 μ L of equalised cDNA sample in independent wells of a 0.1mL MicroAmp® Fast Optical 96-well reaction plate to total 10 μ L per well. Once loaded, the plate was sealed and centrifuged at 1,200g for 30 seconds. Samples were then run using QuantStudio™ 5 System through the following programme: Cover: 105°C; Hold Stage 1: 50°C for 2 minutes; Hold Stage 2: 95°C for 20 seconds; Cycling Stage: 40 cycles of 95°C for 1 second + 60°C for 20 seconds. Data were analysed with the QuantStudio™ Design and Analysis software using the Comparative CT method ($\Delta\Delta$ CT).

2.4. Data analysis and graphical presentation

All datasets were analysed using GraphPad Prism 10. Outliers were detected using the outlier function of GraphPad Prism. Datasets were tested for normality using the Shapiro-Wilk test before applying parametric analyses. Data are presented as mean with standard error of the mean (SEM) between biological replicates, where each dot plotted represents an individual biological replicate. In *in vivo* investigations, a biological replicate refers to one animal. In *in vitro* investigations, a biological replicate refers to one experiment comprising 3-4 technical replicates per treatment group. A minimum of 3 experiments were conducted and included in statistical analyses. A range of 7-10 cells were included in the analysis of each technical replicate. Student's t-tests, one-way analyses of variance (ANOVA), or two-way ANOVAs were performed where appropriate. Nonparametric Mann-Whitney U-test and Kruskal Wallis test by ranks were performed for analysis of not-normally distributed data. Where statistically significant effects were shown following a one-way ANOVA, Tukey's post hoc comparisons test was performed to identify group differences compared to controls. Where statistically significant effects were shown following a two-way ANOVA, Fisher's or Tukey's post hoc comparisons tests were performed to identify group differences between multiple groups, while Dunnett's post hoc comparisons test was used to identify group differences compared to controls. Results were deemed significant when $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), or $P < 0.0001$ (****).

Chapter 3: An assessment of PD-related pathologies six weeks following unilateral delivery of wild-type or A53T-mutant human alpha-synuclein preformed fibrils to the rat substantia nigra

3.1. Introduction

The α Syn propagation theory of PD progression has been proposed as one of the major components underlying PD pathogenesis. Fibrillar α Syn can propagate through neuronal networks of the central and peripheral nervous systems, and template the phosphorylation and fibrillisation of naïve endogenous α Syn (Karampetsou et al., 2017; Lashuel et al., 2013; Luk et al., 2012b). As they spread, large α Syn aggregates provoke a cytotoxic pro-inflammatory response from glial cells, while concomitantly inducing damage to neurons directly through intracellular accumulation into Lewy body plaques. α Syn fibrils can further promote the fibrillisation of other amyloidogenic proteins (Tau, for example), while non-fibrillar α Syn has no propensity to do so. Sedimentation analyses of *in vitro* fibrillisation assays have reported the intrinsic ability of α Syn to fibrillise independently, while other amyloidogenic proteins, including Tau, require co-factors to acquire aggregate conformation. Likely abetted by its small size (140 amino acids), α Syn's intrinsic nucleation capacity is potentiated by the exposed filament-forming hydrophobic region, which facilitates intermolecular interactions that lead to fibrillisation. Both qualities are kinetically favourable for fibril-formation. Tau, in contrast, is much larger (352 to 441 amino acids), and contains a shielded filament-forming region, thereby disabling spontaneous conformational changes and fibrillisation (Giasson et al., 2003).

The development of clinically relevant animal models of PD is crucial for understanding this aspect of disease pathology and developing neuroprotective treatments for the human condition. In accordance with findings from human brain samples, α Syn in its fibrillar form can promote the hyperphosphorylation of α Syn, microglial activation and related inflammation, and dopamine neuronal cell loss, to a greater degree than non-fibrillar α Syn in rodent and cellular models of synucleinopathy (Harms et al., 2017; Luk et al., 2012a; Luk et al., 2012b; Paumier et al., 2015). The structural, biochemical, and pathological homology between fibrillar aggregates in the human PD brain and synthetic recombinant PFFs utilized in rodent models gives credence to their construct validity in model development.

The role of the α Syn protein in presynaptic function has been intensely studied over the past 35 years (Burre, 2015). Under physiological conditions, α Syn functions as a chaperone to the assembly of the SNARE-complex in presynaptic terminals. Through interaction with presynaptic proteins, including synapsin III (Zaltieri et al., 2015), vesicular monoamine transporter (VMAT) 2 (Guo et al., 2008), and the reinit of SNARE proteins (Burre, 2015; Burre et al., 2014; Burre et al., 2010), α Syn aids in regulating vesicular transport and signal transmission. The loss of effective α Syn function has been linked with exacerbated vesicle release at dopaminergic terminals (Abeliovich et al., 2000; Nemani et al., 2010). Conversely, the overexpression of α Syn has been linked with reduced synaptic vesicle density and vesicle recycling, which implies its normal function relates to regulating vesicle release through a positive feedback mechanism (Nemani et al., 2010). Mutations in the gene encoding the α Syn protein can further disrupt physiological synaptic function.

Of specific interest within this line of research is the A53T mutation, an autosomal dominant point mutation in the gene encoding α Syn, which is linked to rare early-onset forms of familial PD (Kruger et al., 1998; Polymeropoulos et al., 1997). Both WT and A53T-mutant α Syn exist as natively unfolded

monomers at physiological concentrations (Conway et al., 1998; Giasson et al., 1999). Both strains can form amyloidogenic fibrils of similar morphology at high concentrations (Conway, 2000; Conway et al., 2000; Narhi et al., 1999), by what appears to be a nucleation-dependent mechanism (Wood et al., 1999). Both strains also have capacity to materialize into nonfibrillar intermediates, including oligomers, which are thought to be analogous to the amyloid- β protofibril, distinctive to proteinaceous plaques in Alzheimer's disease. Other than the prevalence of the A53T-mutant α Syn, familial PD is nearly indistinguishable from idiopathic PD. The role and relevance of the A53T-mutation in disease pathology is therefore unclear, however compelling evidence supports a theory that the A53T mutation influences the fibrillisation of α Syn. Cellular and animal models have been developed to decipher differences between WT and A53T-mutant α Syn with respect to their fibrillisation and propagation properties.

The fibrillisation capacity of A53T-mutant α Syn has been well reported *in vitro* (Conway et al., 1998; Giasson et al., 2003; Giasson et al., 1999; Kotzbauer et al., 2004; Narhi et al., 1999), but less so *in vivo*. AAV-mediated A53T overexpression and A53T-mutant PFFs induce synucleinopathic pathology in animal models. Koprach and colleagues showed that following a single 2 μ l injection into the rat substantia nigra, AAV-A53T α Syn drove aggregation of insoluble α Syn in substantia nigra dopaminergic neurons. Neuronal viability was reduced by approximately 50% as a result of A53T α Syn expression, compared to animals that had received empty vectors. AAV1/2-A53T α Syn produced dystrophic neurites in the striatum and a significant reduction in TH+ immunoreactivity within 3-6 weeks of injection (Koprach et al., 2010). Giasson and colleagues reported severe motor impairment, intracytoplasmic neuronal α Syn inclusions, and neurodegeneration in transgenic mice expressing A53T α Syn, compared to WT, albeit they suggest that with longer exposure, evidence of pathology in WT transgenic mice may have been observed (Giasson et al., 2002). Thioflavin T-staining has demonstrated more rapid fibrillisation of A53T-mutant C-terminal-truncated α Syn aggregates compared to naïve α Syn. Moreover, C-terminal-truncated A53T mutants promote aggregation of full-length A53T α Syn more rapidly (shorter lag phase) than parallel combinations of α Syn aggregates without the A53T mutation. The significance of α Syn truncation in synucleinopathies stems from an immunoblot analysis of human brain tissue, which showed truncated α Syn in the substantia nigra and cingulate cortex of PD and Lewy body dementia patients, but not aged-matched healthy controls (Liu et al., 2005).

When co-assembled with Tau, recombinant A53T α Syn, but not WT, accelerates the polymerisation of Tau (Kotzbauer et al., 2004). In fact, some studies have reported neuroprotective properties of WT α Syn, albeit the aims of these studies were directed towards addressing the physiological requirement for α Syn in neuronal function, rather than distinguishing between pathological features of various aggregate strains (Chandra et al., 2005; Machida et al., 2005; Tofaris et al., 2006). Multiple groups have found that A53T and A30P mutations (and others which are major genetic pre-determinants in the development of PD), accelerated α Syn oligomerisation, but not fibrillisation (Conway et al., 2000). In addition to enhanced fibrillisation capacity, A53T- and A30P-mutant α Syn have been found to bind more readily to the lysosome-associated membrane protein (LAMP) 2, than WT α Syn, preventing chaperone-mediated autophagy and lysosomal cargo loading (Cuervo et al., 2004). In 14-month-old transgenic mice expressing A30P-mutant α Syn, i.p. administration of an α Syn protofibril-selective monoclonal antibody over a 14 week treatment period, reduced levels of both soluble and membrane-associated fibrillar α Syn in the spinal cord, and alleviated motor dysfunction (Lindstrom et al., 2014). Indeed, contradictory reports from transgenic mice expressing either the

A30P or the A53T mutated form of α Syn failed to identify nigral neuropathology associated with the delivery of these mutated forms of α Syn (Matsuoka et al., 2001; Rathke-Hartlieb et al., 2001). Differing pathological profiles generated from *in vivo* studies utilising PFFs likely result from differences in ultrastructure between PFFs, the slightest of which can result in pronounced biochemical and behavioural changes (Lau et al., 2020).

In other environments, mutant variants of the α Syn protein have shown no greater propensity to induce pathology when compared to WT α Syn. In Sprague Dawley rats, AAV2/DJ-mediated overexpression of WT α Syn promoted aggregation of cytoplasmic inclusions in dopaminergic neurons in the substantia nigra and ventral tegmental area at comparable levels to α Syn with the E46K mutation. WT α Syn induced more pronounced motor dysfunction, as measured in the cylinder test, and greater nigral degeneration, compared to E46K α Syn (von Hovel et al., 2019). This study suggests that enhanced expression of some α Syn mutations (in this case, E46K), may mimic presymptomatic stages of disease, while the overexpression of α Syn in its WT form may be more indicative of peri symptomatic PD-like pathology in rats. In contrast, AAV-mediated WT and A53T α Syn overexpression reportedly produced similar degrees of cell death, and comparable levels of soluble and insoluble α Syn expression over time when equal titres were delivered to the adult mouse substantia nigra (Oliveras-Salv , 2013). WT and A53T α Syn-expressing transgenic mice developed consistent levels of soluble full-length α Syn in the cortex and brainstem at 1, 2, 4, 6 and 8 months of age (Liu et al., 2005). In primary midbrain and hippocampal neuronal cultures, the overexpression of WT and A53T α Syn interfered equally with presynaptic transmission by suppressing vesicular recycling and depleting pools of readily releasable synaptic vesicles (Nemani et al., 2010). Furthermore, AAV-mediated expression of WT and Ser129-mutant α Syn (2 μ l; 1.4×10^{13} vg/ml) resulted in equal dopaminergic cell loss and α Syn aggregate distribution in the rat substantia nigra six weeks post-intranigral delivery (McFarland et al., 2009a).

Together, this research substantiates an association between α Syn in its fibrillar form (irrespective of mutation), or α Syn expressing the A53T mutation (irrespective of conformation) and PD-like disease progression. It remains unclear, however, what role the mutant forms of α Syn play, or to what extent their overexpression drives Parkinson-like pathology and neurodegeneration in nigrostriatal neurons, in the context of the rodent.

Furthermore, although classic inflammatory agents like LPS have shown strong propensity to induce PD-related pathology in the rat within six weeks of intranigral delivery (O'Neill et al., 2020; Yssel et al., 2018), it remains underexplored whether α Syn PFFs can replicate these pathologies within this timeline.

The current status of research provides rationale for exploring the role of A53T-mutant fibrillar α Syn in driving pathology within six weeks in a rodent model of synucleinopathy. As chronic inflammation is thought to further drive disease progression, assessing the microglial response to WT and A53T-mutant α Syn fibrils in the rat brain will further elucidate their role in driving early-stage pathology and/or motor behavioural deficits.

3.2. Aims and objectives

The primary aim of the current investigation was to deliver human recombinant α Syn PFFs unilaterally to the rat substantia nigra, to establish a model of early stage synucleinopathic disease exhibiting motoric dysfunction, α Syn aggregation, microglial activation, and nigral dopaminergic neuronal degeneration within six weeks of delivery. Although the classic inflammatory agent, LPS, has shown strong propensity to induce PD-related pathology in the rat within six weeks of intranigral delivery, it remains underexplored whether α Syn PFFs can replicate these pathologies within this timeline. Both WT and A53T-mutant α Syn PFFs were utilised to further assess mutant forms of α Syn in driving synucleinopathic pathology in the rat.

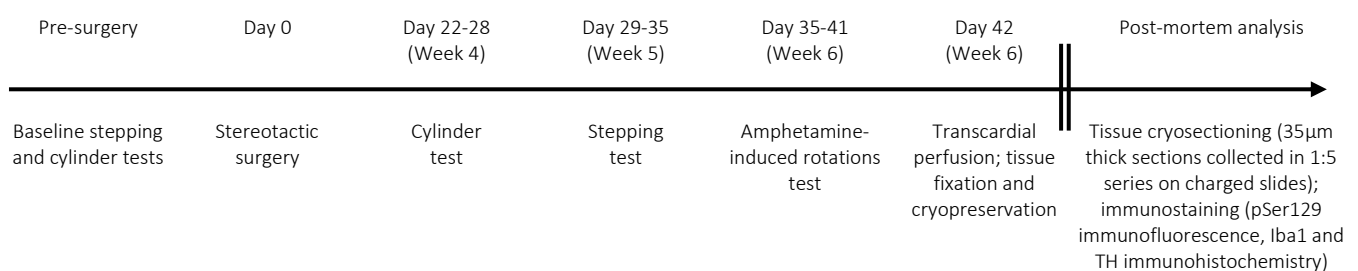
The objective of this study was to deliver WT or A53T-mutant α Syn PFFs into the rat substantia nigra by stereotactic delivery, and assess the efficacy of these constructs to affect skilled motor function, microglial activation, and dopaminergic neuronal degeneration within a period of six weeks. Skilled motor function and limb-use asymmetry were assessed through application of the stepping and cylinder tests, respectively. Rotational behaviour in the amphetamine-induced rotations test was assessed to functionally evaluate the presence of a unilateral nigral lesion. pSer129 immunofluorescence, Iba1 immunohistochemistry, and TH immunohistochemistry were conducted on cryopreserved midbrain tissue sections to assess phosphorylated α Syn expression, microglial cell activation, and dopaminergic neuronal integrity, respectively, in the substantia nigra six weeks after PFF delivery. Comparisons could then be made between WT PFF- and A53T PFF-treated animals to distinguish between mutant and non-mutant constructs, and validate their utility for driving behavioural and cellular changes associated with α Syn fibrillisation in the substantia nigra.

3.3. Experimental design

29 adult (7–8-month-old) Wistar rats (13 female, 16 male) underwent behavioural training and baseline testing before being randomly assigned to a treatment group. Animals received a single intranigral stereotactic injection of 2μL containing PBS, human recombinant WT αSyn PFFs, or A53T-mutant αSyn PFFs. The coordinates for delivery into the substantia nigra in males were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for delivery into the substantia nigra in females were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. In weeks four, five, and six following intranigral delivery, rats were subjected to the cylinder test, stepping test, and amphetamine-induced rotations test, respectively. Six weeks post-surgery, animals were euthanised by sodium pentobarbital overdose and transcardial perfusion/fixation, in preparation for immunofluorescence and histochemical analysis of pSer129+ αSyn, Iba1+ microglia, and TH+ neuronal cell bodies in the substantia nigra.

Treatment groups (n = 9-10):

1. Sterile PBS Vehicle (single unilateral injection of 2μl)
 - n = 9 (4 females, 5 males)
2. Human WT αSyn PFF (single unilateral injection of 2μl)
 - n = 10 (5 females, 5 males)
 - PFF concentration: 2mg/ml (4μg in 2μL)
3. Human A53T αSyn PFF (single unilateral injection of 2μl)
 - n = 10 (4 females, 6 males)
 - PFF concentration: 2mg/ml (4μg in 2μL)



3.4. Methods

All methods were conducted as described in Chapter 2.

3.5. Results

3.5.1. Cylinder test

A two-way repeated measures ANOVA of combined ipsilateral placements shows no effect of treatment [$F(2,26)=0.07220$, $p=0.9305$], time [$F(1,26)=4.009$, $p=0.0558$], or interaction between treatment and time [$F(2,26)=0.08349$, $p=0.9201$] (Figure 3.1a). A two-way repeated measures ANOVA of combined contralateral placements shows no effect of treatment [$F(2,26)=0.1142$, $p=0.8925$], an effect of time [$F(1,26)=19.49$, $p=0.0002$], and no interaction between treatment and time [$F(2,26)=2.656$, $p=0.0892$]. Post hoc comparisons reveal a reduction in combined contralateral placements four weeks post-delivery of WT PFFs ($p=0.0003$) and A53T PFFs ($p=0.0107$), compared to baseline (Figure 3.1b). No change in PBS-treated animals is observed overtime ($p=0.4291$). A two-way repeated measures ANOVA of % contralateral placements shows no effect of treatment [$F(2,26)=0.07866$, $p=0.9246$], an effect of time [$F(1,26)=7.593$, $p=0.0106$], and an interaction between treatment and time [$F(2,26)=7.231$, $p=0.0032$]. Post hoc comparisons reveal reduced % contralateral limb placements four weeks following intranigral delivery of WT PFF ($p=0.0004$) and A53T PFF ($p=0.0409$), compared to baseline (Figure 3.1c).

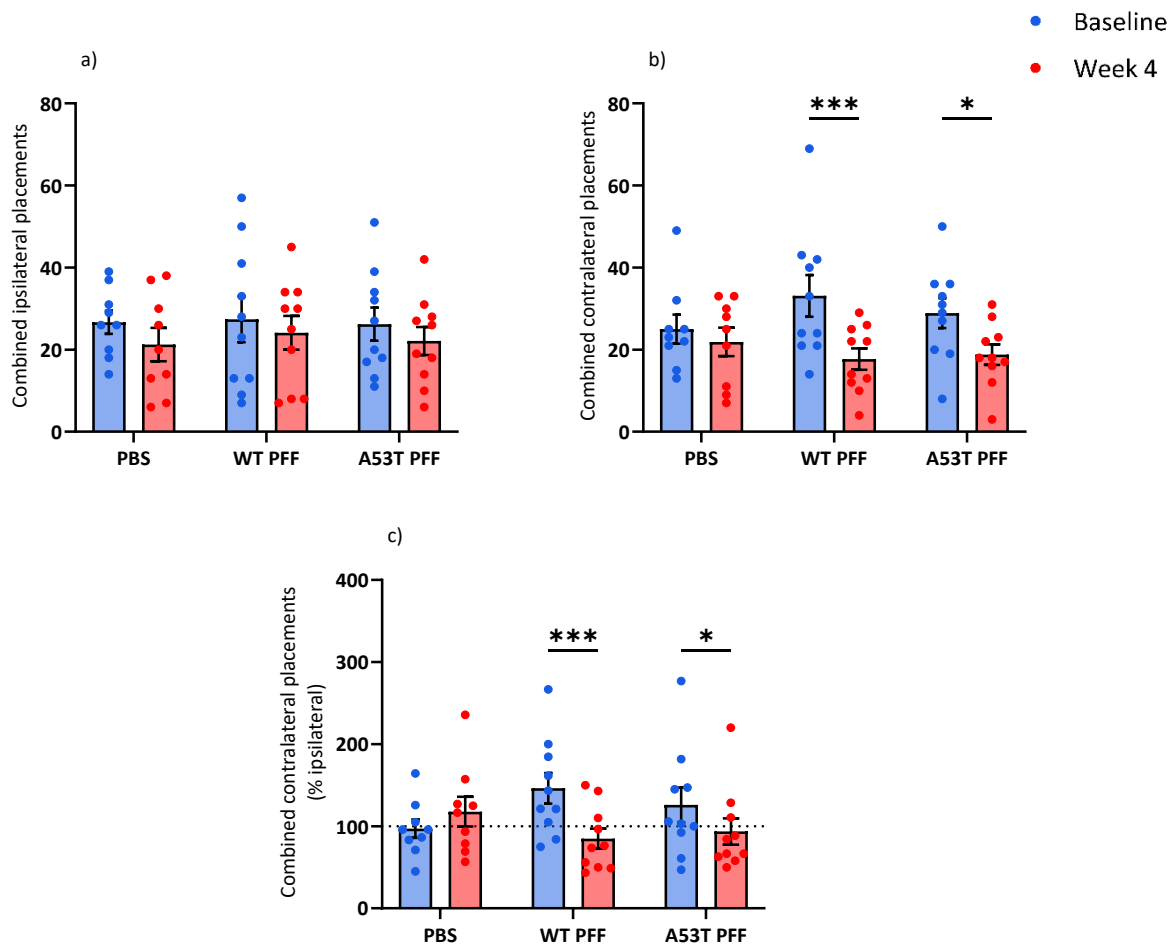


Figure 3.1. WT PFF and A53T PFF reduce placements made with the forelimb contralateral to the injection four weeks after intranigral delivery. a) Combined placements made with the limb ipsilateral to the injection, b) combined placements made with the limb contralateral to the injection, c) combined contralateral placements expressed as a percentage of combined ipsilateral placements. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=9-10$. *= $p\leq 0.05$, ***= $p\leq 0.001$.

A two-way repeated measures ANOVA of ipsilateral wall placements shows no effect of treatment [$F(2,26)=0.1218$, $p=0.8858$], time [$F(1,26)=2.479$, $p=0.1275$], or interaction between treatment and time [$F(2,26)=0.4717$, $p=0.6292$] (Figure 3.2a). A two-way repeated measures ANOVA of contralateral wall placements shows no effect of treatment [$F(2,26)=0.6412$, $p=0.5348$], an effect of time [$F(1,26)=8.898$, $p=0.0061$] and interaction between treatment and time [$F(2,26)=3.567$, $p=0.0428$]. Post hoc comparisons reveal reductions in contralateral wall placements in the WT PFF ($p=0.0020$) and A53T PFF ($p=0.0352$) groups four weeks post-delivery compared to baseline (Figure 3.2b). A two-way repeated measures ANOVA of % contralateral placements shows no effect treatment [$F(2,26)=0.009057$, $p=0.9910$], time [$F(1,26)=0.02079$, $p=0.8865$], or interaction between treatment and time [$F(2,26)=2.317$, $p=0.1185$] (Figure 3.2c).

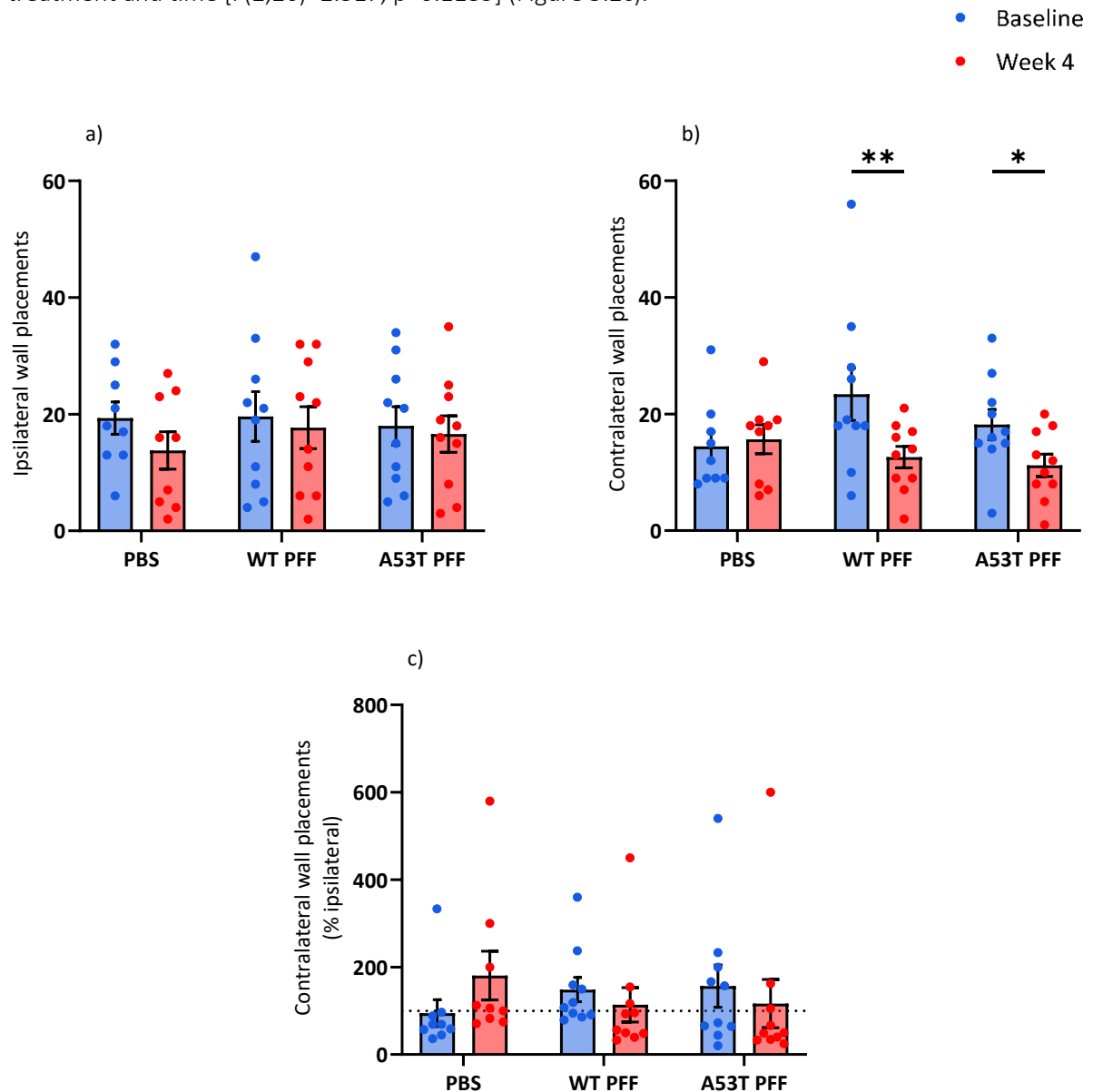


Figure 3.2. WT PFF and A53T PFF reduce wall placements made with the forelimb contralateral to the injection four weeks after intranigral delivery. a) Wall placements made with the limb ipsilateral to the injection, b) wall placements made with the limb contralateral to the injection, c) contralateral wall placements expressed as a percentage of ipsilateral wall placements. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=9-10$. *= $p\leq 0.05$, **= $p\leq 0.01$.

Kruskal Wallance ANOVA of combined ipsilateral placements expressed as % of baseline shows no effect of treatment [$H(3)=1.784$, $p=0.4098$] (Figure 3.3a). Kruskal Wallance ANOVA of combined contralateral placements expressed as % of baseline shows no effect of treatment [$H(3)=2.904$, $p=0.2341$], although parametric ANOVA shows an effect of treatment [$F(2,26)=3.463$, $p=0.0464$]. Post hoc comparisons reveal reduced combined contralateral placements expressed as % baseline in WT PFF-treated animals compared to PBS-treated controls ($p=0.0333$) (Figure 3.3b). Kruskal Wallance ANOVA of ipsilateral wall placements expressed as % of baseline shows no effect of treatment [$H(3)=1.457$, $p=0.4827$] (Figure 3.3c). Kruskal Wallance ANOVA of contralateral wall placements expressed as % of baseline shows no effect of treatment [$H(3)=5.750$, $p=0.0564$], although a trend indicates reduced contralateral wall placements expressed as % baseline in WT PFF-treated animals compared to PBS-treated controls ($p=0.0535$) (Figure 3.3d).

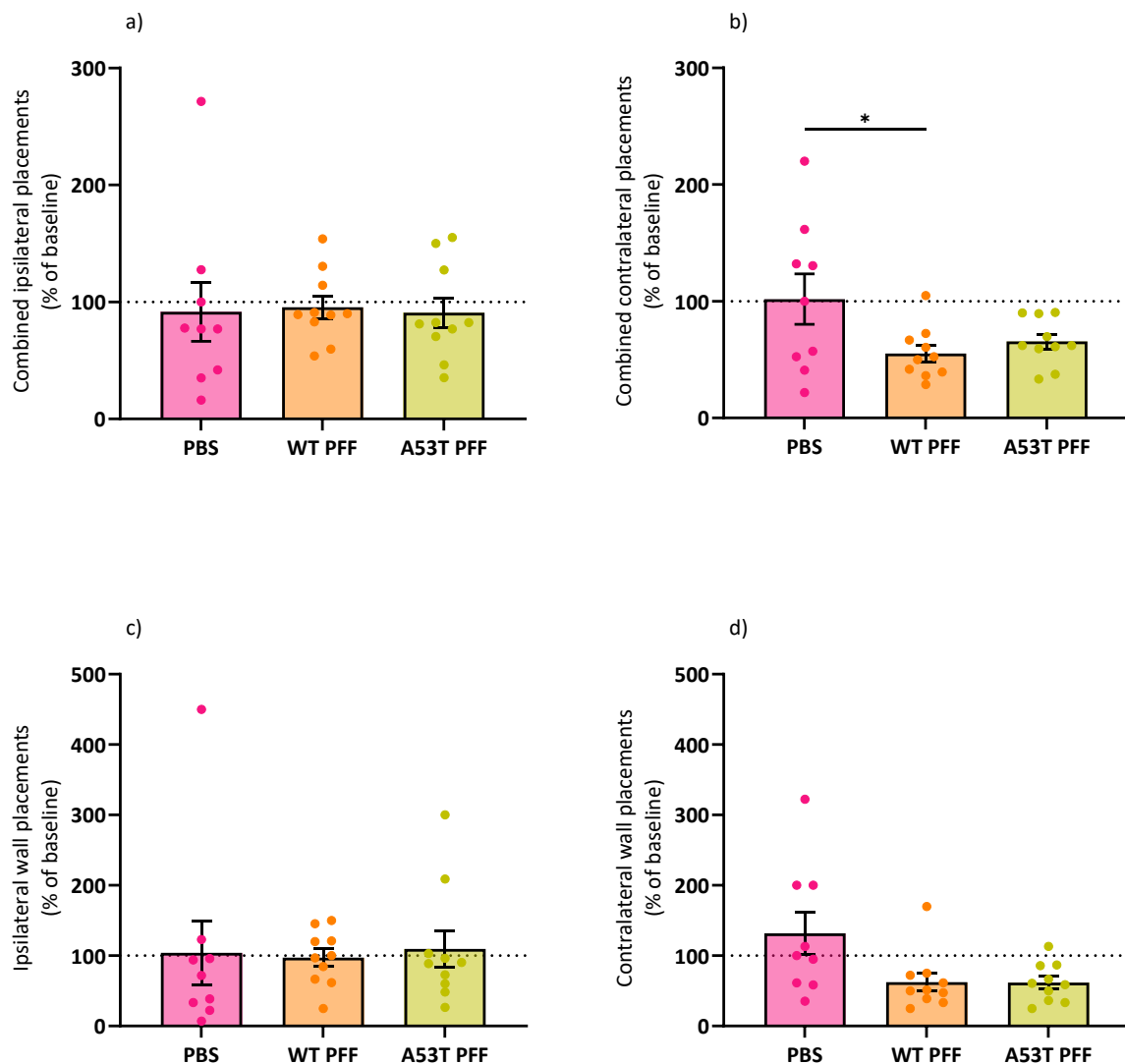


Figure 3.3. Intranigral delivery of WT PFF reduces contralateral forelimb-use in the cylinder test four weeks after intranigral delivery. Scores of a) combined ipsilateral placements, b) combined contralateral placements, c) ipsilateral wall placements, and d) contralateral wall placements, expressed as % of baseline. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Kruskal Wallis ANOVA for non-normally distributed datasets (a,c,d) or one-way ANOVA for normally distributed datasets (b) followed by Dunnett's test for multiple comparisons. $n=9-10$. $*=p\leq 0.05$.

A two-way ANOVA of combined placements expressed as % of baseline shows no effect of treatment [$F(2,26)=0.7978$, $p=0.4610$], laterality [$F(1,52)=2.289$, $p=0.1364$], or interaction between treatment and laterality [$F(2,26)=2.818$, $p=0.0780$] (Figure 3.4a). A two-way ANOVA of wall placements expressed as % of baseline shows no effects of treatment [$F(2,52)=1.307$, $p=0.2795$], laterality [$F(1,52)=0.8053$, $p=0.3737$], or interaction between treatment and laterality [$F(2,52)=1.268$, $p=0.2901$] (Figure 3.4b).

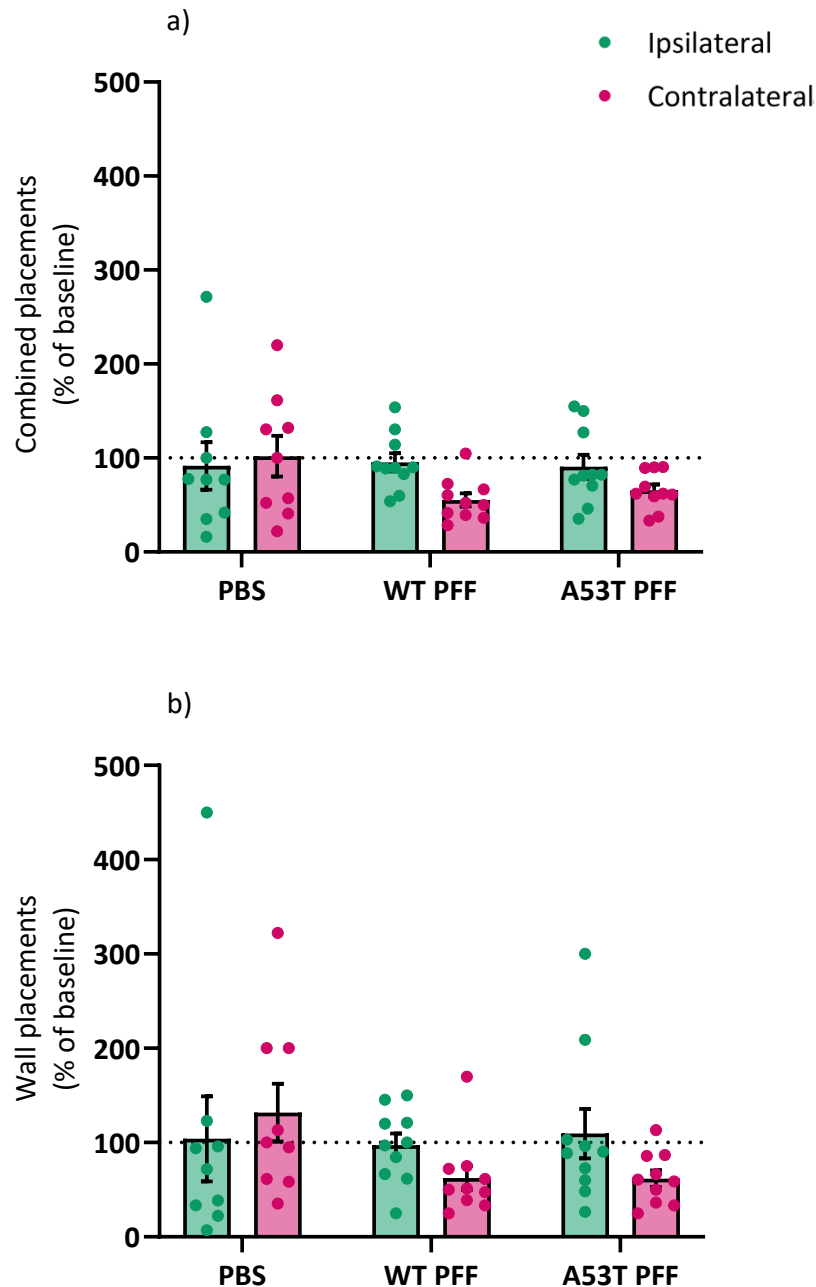


Figure 3.4. WT and A53T PFF appear to reduce contralateral forelimb-use in the cylinder test four weeks after intranigral delivery when number of placements are expressed as % of baseline. a) Combined placements expressed as a percentage of baseline b) wall placements expressed as percentage of baseline. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Two-way ANOVA. $n = 9-10$.

3.5.2. Stepping test

A two-way repeated measures ANOVA of combined (forward + backward) steps made with the limb ipsilateral to the injection shows no effect of treatment [$F(2,26)=1.645$, $p=0.2125$], time [$F(1,26)=0.9790$, $p=0.3316$], or interaction between treatment and time [$F(2,26)=2.150$, $p=0.1367$] (Figure 3.5a). Two-way repeated measures ANOVA of combined steps made with the limb contralateral to the injection shows no effect of treatment [$F(2,26)=0.7503$, $p=0.4822$], an effect of time [$F(1,26)=17.16$, $p=0.0003$], and no interaction between treatment and time [$F(2,26)=0.2474$, $p=0.7826$]. Post hoc comparisons reveal reductions in combined contralateral steps made five weeks after intranigral delivery of PBS ($p=0.0437$), WT PFF ($p=0.0488$) and A53T PFF ($p=0.0058$), compared to baseline (Figure 3.5b). Two-way repeated measures ANOVA of % contralateral steps shows no effect of treatment [$F(2,26)=0.7364$, $p=0.4886$], time [$F(1,26)=3.909$, $p=0.0587$], or interaction between treatment and time [$F(2,26)=0.9020$, $p=0.4181$] (Figure 3.5c).

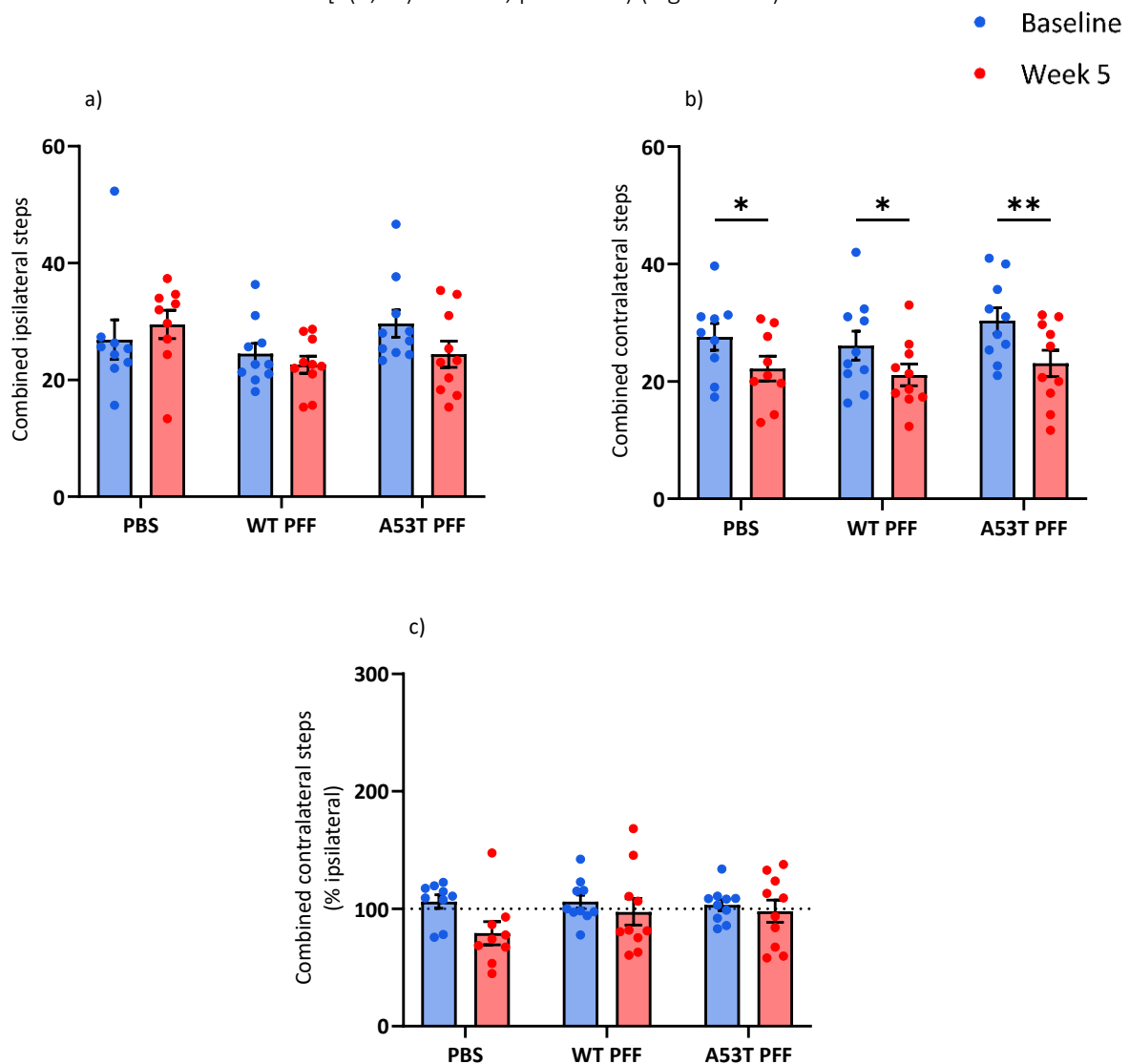


Figure 3.5. Reduced stepping with the limb contralateral to the injected hemisphere in all treatment groups. a) Combined steps made with the limb ipsilateral to the injection, b) combined steps made with the limb contralateral to the injection, c) combined contralateral steps expressed as a percentage of combined ipsilateral steps. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=9-10$. *= $p \leq 0.05$, **= $p \leq 0.01$.

A two-way repeated measures ANOVA of forward ipsilateral steps shows no effect of treatment [$F(2,26)=2.89$, $p=0.0736$], an effect of time [$F(1,26)=8.91$, $p=0.0061$], and no interaction between treatment and time [$F(2,26)=0.22$, $p=0.8043$]. Post hoc comparisons reveal a reduction in ipsilateral steps five weeks following A53T PFF delivery compared to baseline ($p=0.0317$) (Figure 3.6a). A two-way repeated measures ANOVA of forward contralateral steps shows no effect of treatment [$F(2, 26)=0.97$, $p=0.3932$], an effect of time [$F(1, 26)=39.60$, $p<0.0001$], and no interaction between treatment and time [$F(2, 26)=0.011$, $p=0.9894$]. Post hoc comparisons reveal reduced contralateral forward steps five weeks post intranigral delivery of PBS ($p=0.0017$), WT PFF ($p=0.0013$), and A53T PFF ($p=0.0008$) when compared to baseline (Figure 3.6b). A two-way repeated measures ANOVA of % contralateral steps shows no effect of treatment [$F(2, 26)=1.215$, $p=0.3131$], time [$F(1, 26)=1.503$, $p=0.2311$], or interaction between treatment and time [$F(2, 26)=0.01764$, $p=0.9825$] (Figure 3.6c).

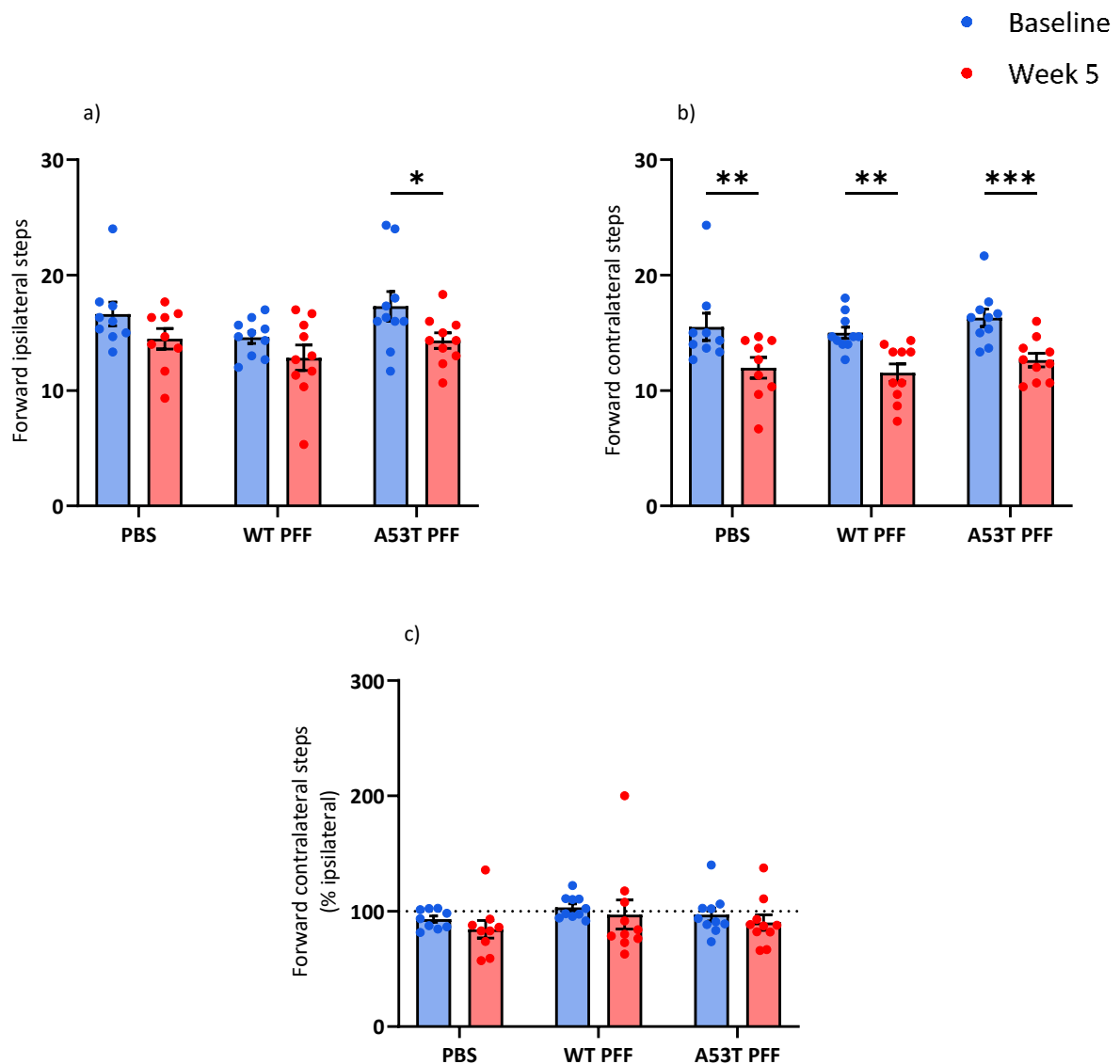


Figure 3.6. Reduced forward stepping with the limb contralateral to the injected hemisphere. a) Forward steps made with the limb ipsilateral to the injection, b) forward steps made with the limb contralateral to the injection, c) forward contralateral steps expressed as a percentage of forward ipsilateral steps. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=9-10$. *= $p\leq 0.05$, **= $p\leq 0.01$, ***= $p\leq 0.001$.

Independent one-way ANOVAs show no effects of treatment on combined ipsilateral steps [$F(2,26)=3.126$, $p=0.0607$] (Figure 3.7a), combined contralateral steps [$F(2,26)=0.1846$, $p=0.8325$] (Figure 3.7b), forward ipsilateral steps [$F(2,26)=0.02339$, $p=0.9769$] (Figure 3.7c), or forward contralateral steps [$F(2,26)=0.01350$, $p=0.9866$] when expressed as % of baseline (Figure 3.7d).

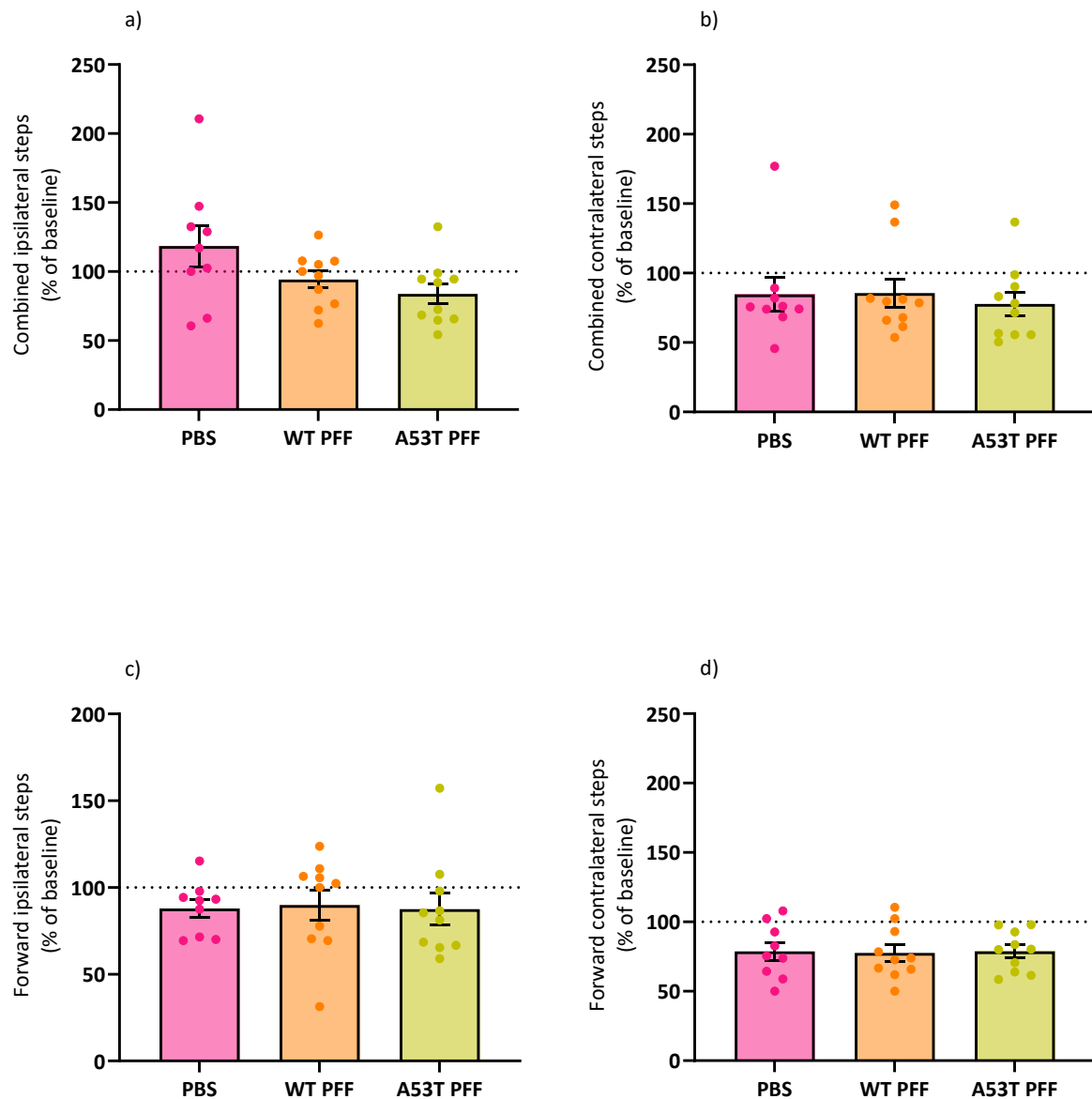


Figure 3.7. Intranigral delivery of WT or A53T PFF do not effect stepping behaviour five weeks after delivery. a) Combined ipsilateral steps, b) combined contralateral steps, c) ipsilateral steps in the forward direction alone, and d) contralateral steps in the forward direction alone, all expressed as % of baseline. Data is expressed as mean $\pm$ SEM. One-way ANOVA. $n=9-10$.

A two-way ANOVA of combined steps expressed as % of baseline shows no effect of treatment [$F(2,52)=2.107$, $p=0.1319$], laterality [$F(1,52)=3.881$, $p=0.0542$], or interaction between treatment and laterality [$F(2,52)=1.116$, $p=0.3353$] (Figure 3.8a). A two-way ANOVA of forward steps expressed as a % of baseline shows no effect of treatment [$F(2,52)=0.003408$, $p=0.9966$], laterality [$F(1,52)=3.136$, $p=0.0824$], or interaction between treatment and laterality [$F(2,52)=0.03667$, $p=0.9640$] (Figure 3.8b).

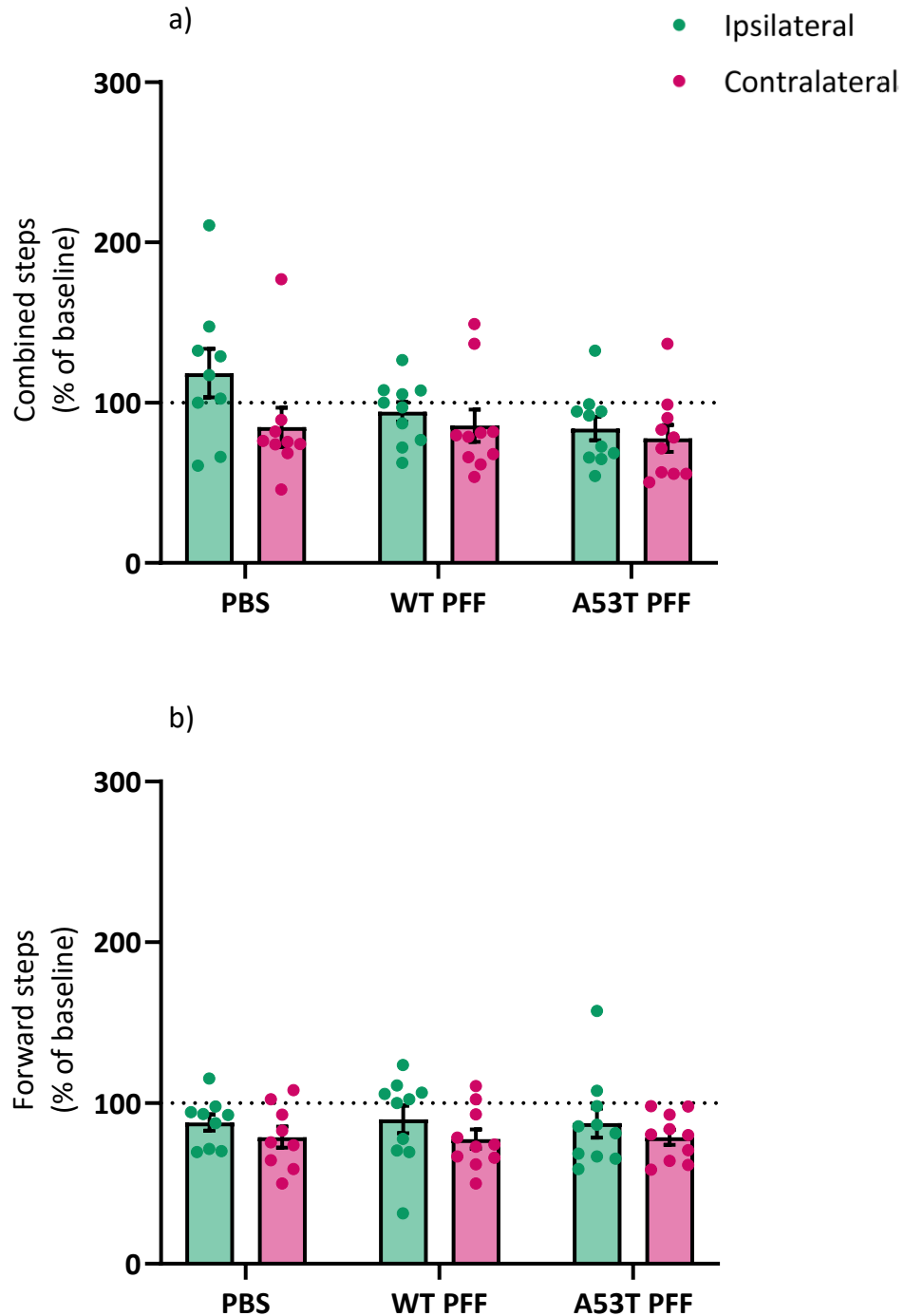


Figure 3.8. Forelimb-use asymmetry is not observed in the stepping test five weeks after intranigral delivery of WT or A53T PFFs. a) Combined steps expressed as a percentage of baseline b) forward steps expressed as a percentage of baseline. Data is expressed as mean $\pm$ SEM. Two-way ANOVA. $n=9-10$.

3.5.3. Amphetamine-induced rotations test

Independent one-way ANOVAs show no effects of treatment on ipsilateral rotations [$F(2,26)=0.1235$, $p=0.8843$] (Figure 3.9a), contralateral rotations [$F(2,26)=0.2979$, $p=0.7449$] (Figure 3.9b), total rotations [$F(2,26)=0.5807$, $p=0.5666$] (Figure 3.9c), or net ipsilateral rotations [$F(2,26)=0.01959$, $p=0.9806$] (Figure 3.9d).

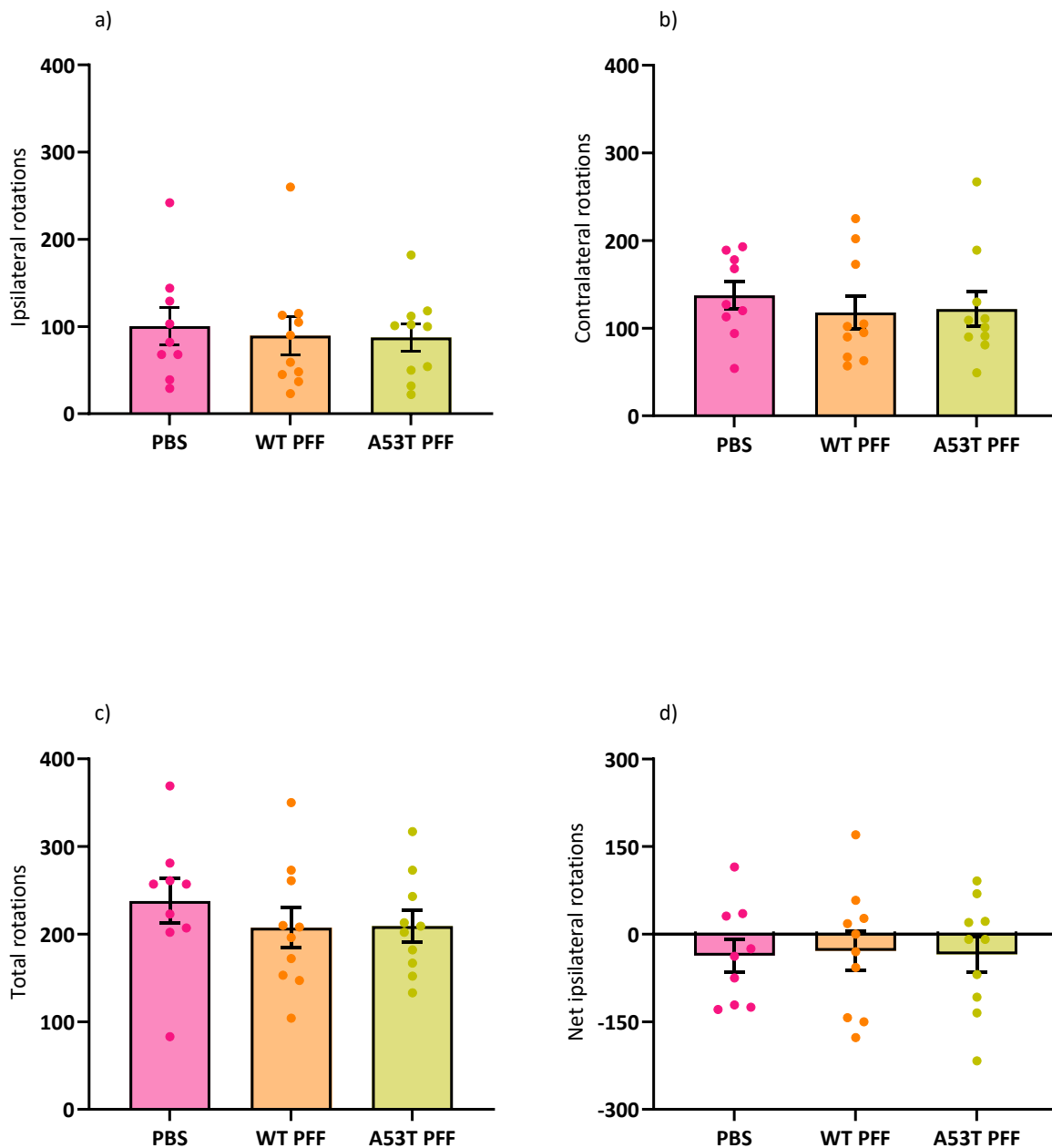


Figure 3.9. WT or A53T PFFs do not affect amphetamine-induced rotations six weeks after intranigral injection. a) Number of ipsilateral rotations, b) number of contralateral rotations, c) number of total rotations, and d) net ipsilateral rotations. Test duration of 90 minutes. Data is expressed as mean $\pm$ SEM. One-way ANOVA. $n=9-10$.

3.5.4. pSer129 immunofluorescence

A two-way ANOVA of pSer129 immunofluorescence shows an effect of treatment [$F(2,46)=8.314$, $p=0.0008$], laterality [$F(1,46)=15.12$, $p=0.0003$], and interaction between treatment and laterality [$F(2,46)=13.87$, $p<0.0001$]. Post hoc comparisons reveal increased pSer129 immunofluorescence in the ipsilateral hemisphere of WT PFF-treated animals compared to the contralateral hemisphere ($p<0.0001$), and compared to the ipsilateral hemisphere of PBS-treated controls ($p<0.0001$) (Figure 3.10a). A one-way ANOVA shows an effect of treatment on pSer129 immunofluorescence expressed as % of the contralateral hemisphere [$F(2,22)=9.499$, $p=0.0011$]. Post hoc comparisons reveal increased pSer129 immunofluorescence in WT PFF-treated animals compared to PBS-treated controls ($p=0.0010$) (Figure 3.10b).

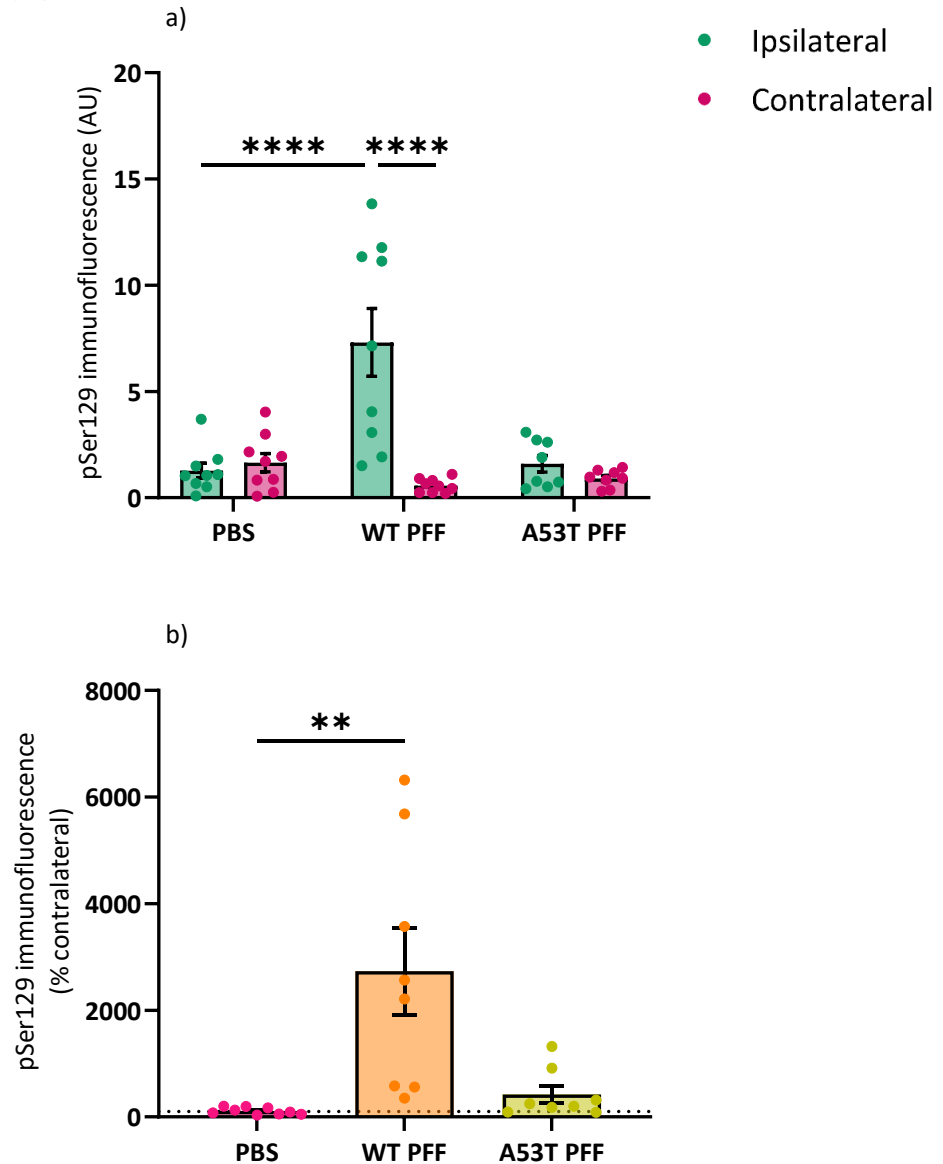


Figure 3.10. WT PFF increases pSer129+ α Syn immunoreactivity in the ipsilateral hemisphere six weeks after intranigral delivery. a) Mean grey value of pSer129 immunofluorescence in the ipsilateral and contralateral substantia nigra, b) mean grey value of pSer129 immunofluorescence in the ipsilateral hemisphere expressed as a percentage of the contralateral hemisphere. Data is expressed as mean $\pm$ SEM. a) Two-way ANOVA followed by Fisher's test for multiple comparisons, b) One-way ANOVA followed by Dunnett's test for multiple comparisons. $n=8-9$. **= $p<0.01$, ****= $p<0.0001$.

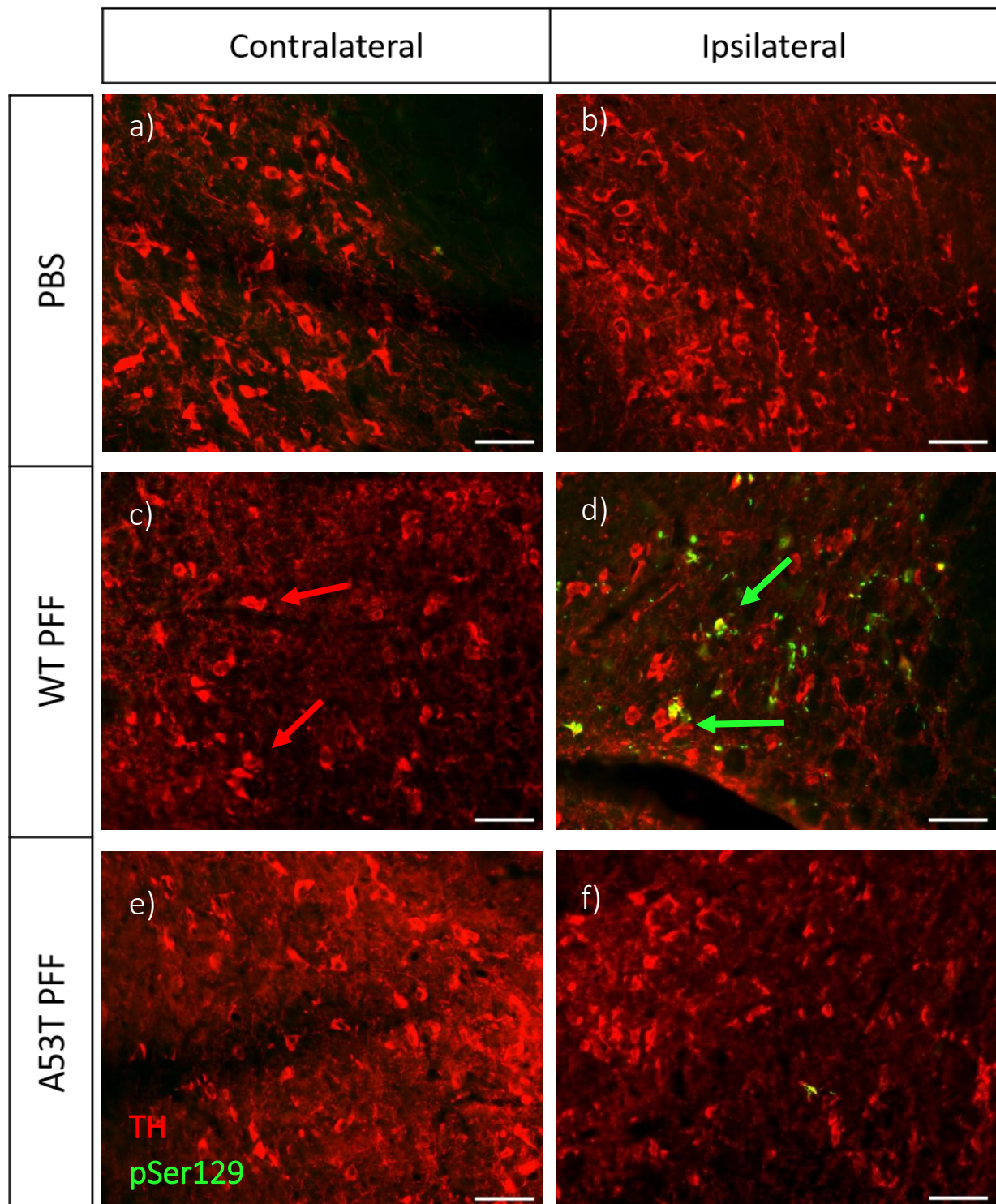


Figure 3.11. pSer129+ α Syn immunofluorescence is increased in the ipsilateral substantia nigra six weeks after intranigral delivery of WT PFF. Six weeks following intranigral delivery, brain tissue was cryosectioned and fluorescently stained for pSer129+ α Syn and TH to confirm location within the substantia nigra. Representative 20x images show pSer129+ staining in green and TH+ staining in red in the a) contralateral hemisphere of PBS group, b) ipsilateral hemisphere of PBS group, c) contralateral hemisphere of WT PFF group, d) ipsilateral hemisphere of WT PFF group, e) contralateral hemisphere of A53T PFF group, and f) ipsilateral hemisphere of A53T PFF group. Scale bar=50 μ m.

3.5.5. Iba1 immunohistochemistry

A two-way ANOVA of Iba1+ cell bodies shows an effect treatment [$F(2,40)=10.53$, $p=0.0002$], no effect of laterality [$F(1,40)=0.3756$, $p=0.5434$], or interaction between treatment and laterality [$F(2,40)=0.5598$, $p=0.5758$]. Post hoc comparisons reveal an increase in Iba1+ cells in the ipsilateral hemisphere of WT PFF-treated animals compared to the ipsilateral hemisphere of PBS-treated controls ($p=0.0011$), and an increase in Iba1+ cells in the contralateral hemisphere of WT PFF-treated animals compared to the contralateral hemisphere of PBS-treated controls ($p=0.0060$) (Figure 3.12a). A one-way ANOVA shows no effect of treatment on the number of Iba1+ cells expressed as a % of the contralateral hemisphere [$F(2,20)=1.356$, $p=0.2804$] (Figure 3.12b).

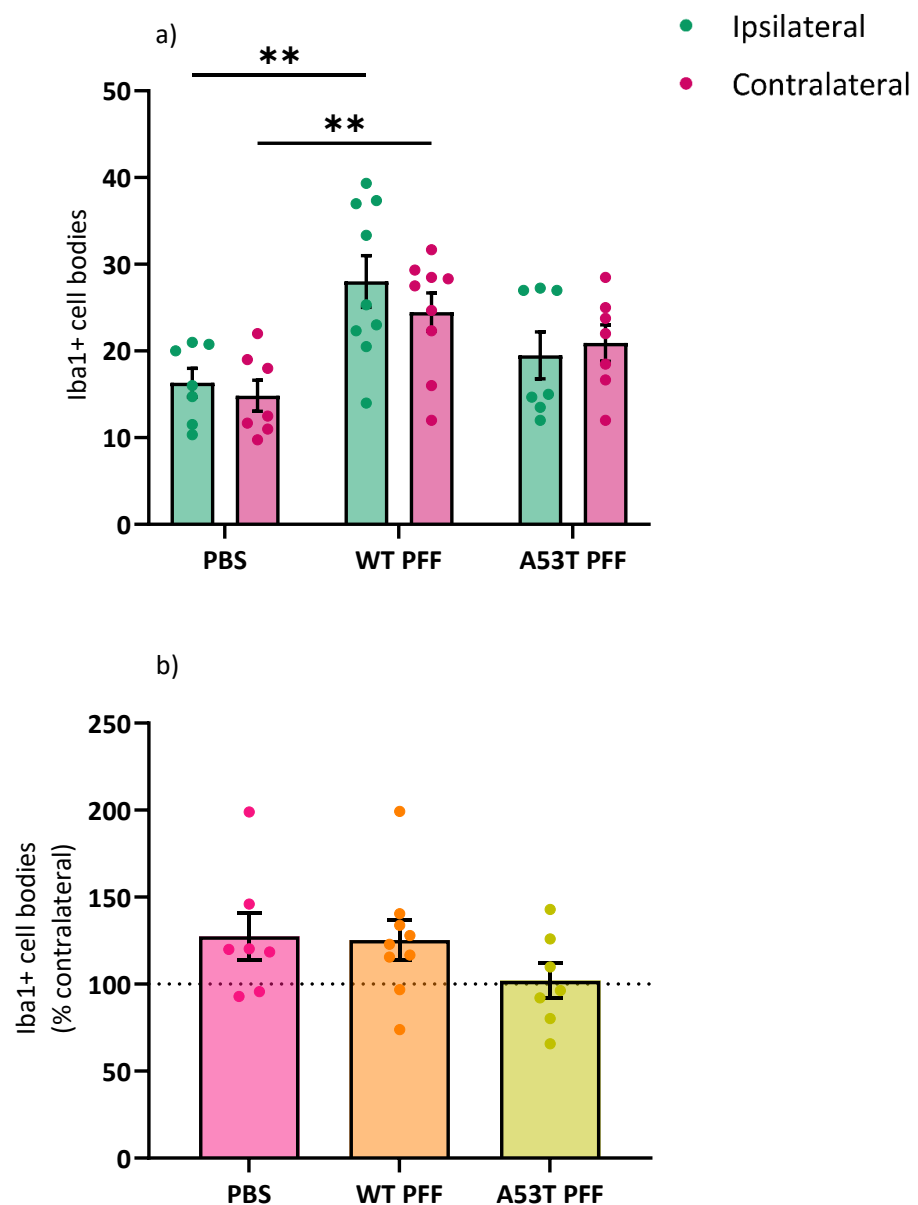


Figure 3.12. WT PFFs increase the number of Iba1+ microglia in the ipsilateral and contralateral hemispheres six weeks after intranigral delivery. a) Number of Iba1+ cells in the ipsilateral and contralateral substantia nigra, b) number of Iba1+ cells in the ipsilateral hemisphere expressed as a percentage the contralateral hemisphere. a) Two-way ANOVA followed by Fisher's post hoc test, b) One-way ANOVA. $n=7-9$. $*$ = $p\leq 0.05$, $**$ = $p\leq 0.01$.

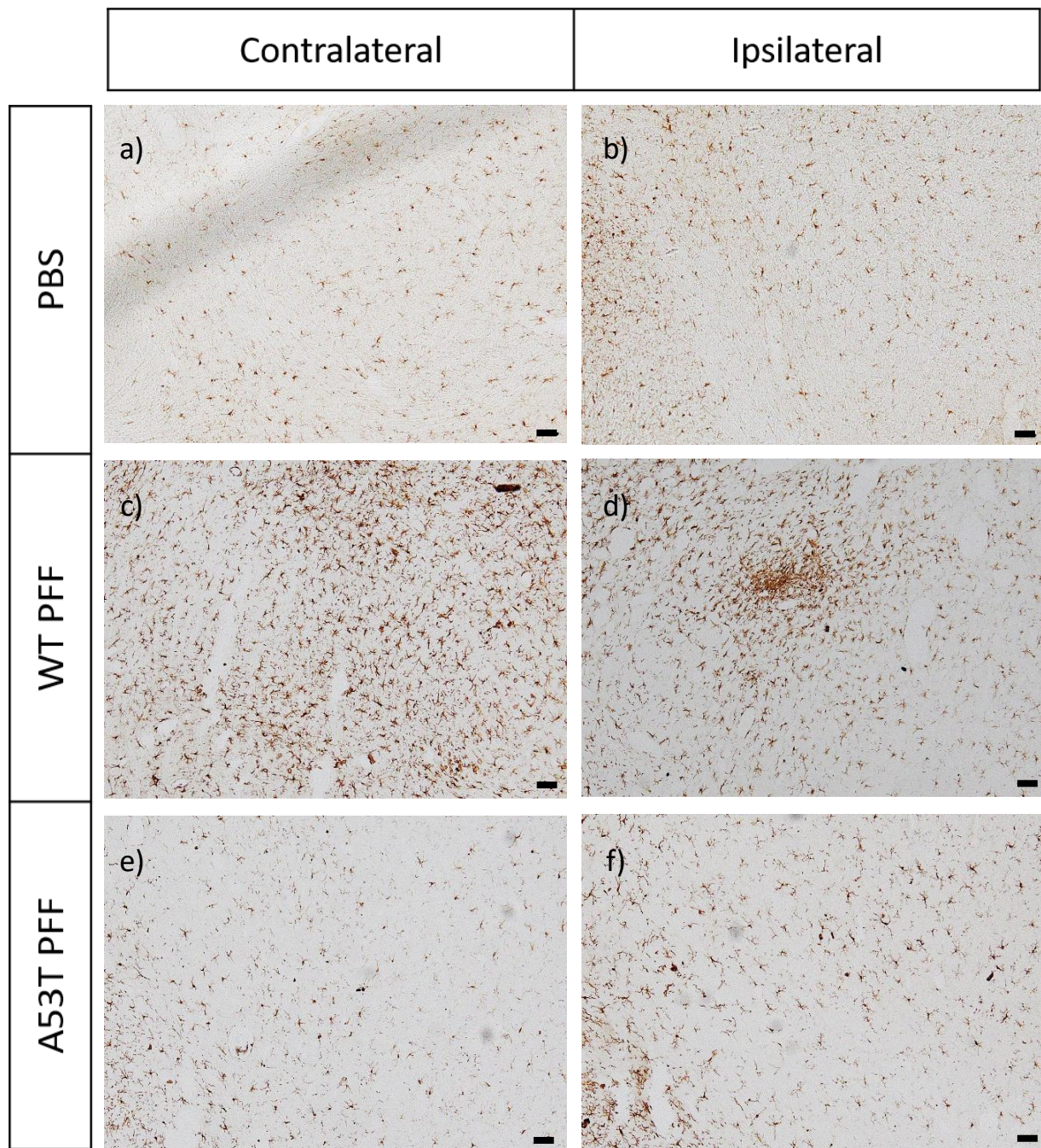


Figure 3.13. Iba1+ microglial cells are increased in the substantia nigra six weeks after intranigral delivery of WT PFF. Brain tissue was cryosectioned and immunohistochemically stained for Iba1 in the substantia nigra to quantify microglial cell bodies. Representative 10x images show Iba1+ microglia in the a) contralateral hemisphere of PBS group, b) ipsilateral hemisphere of PBS group, c) contralateral hemisphere of WT PFF group, d) ipsilateral hemisphere of WT PFF group, e) contralateral hemisphere of A53T PFF group, and f) ipsilateral hemisphere of A53T PFF group. Scale bar=50 μ m.

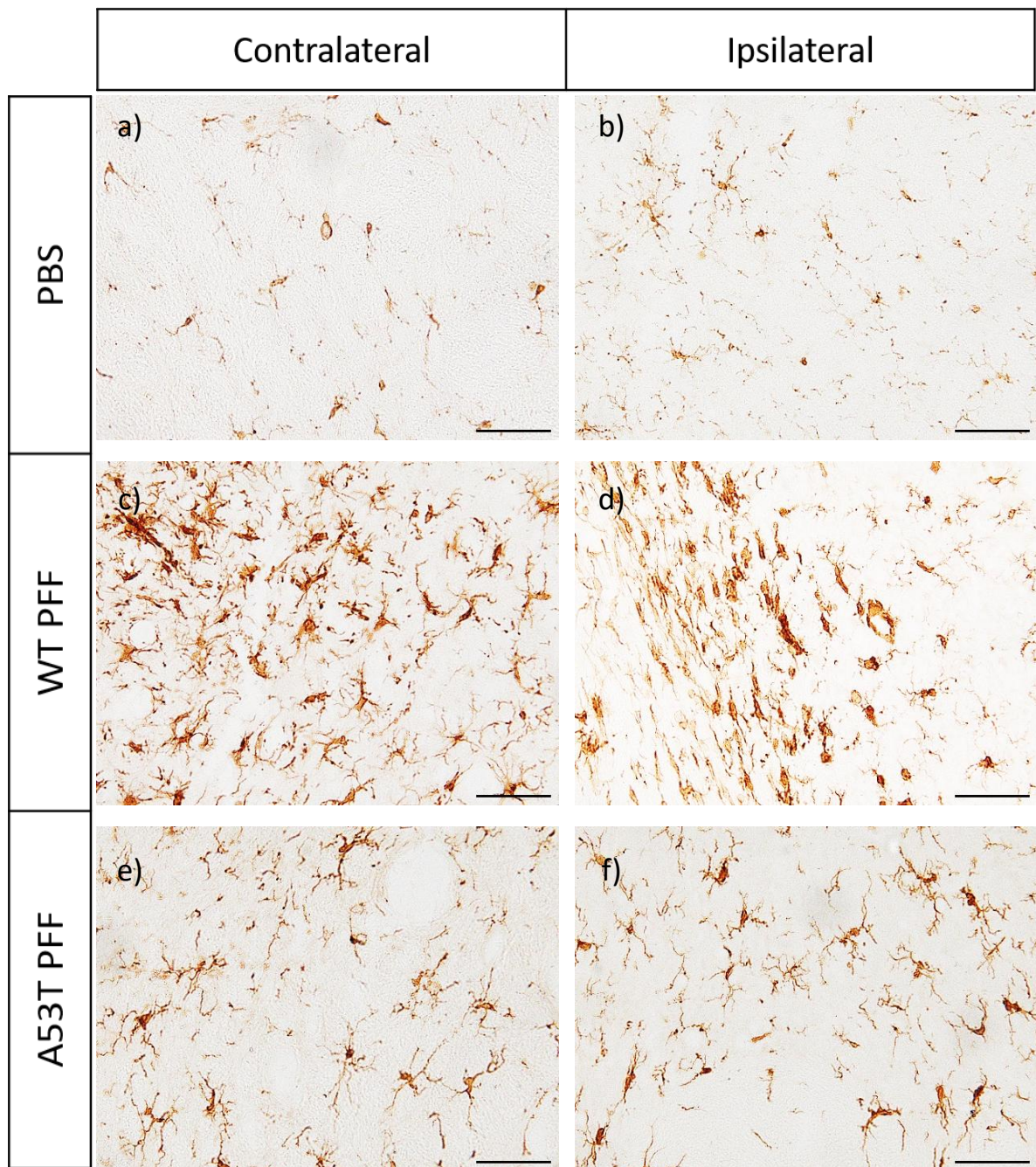


Figure 3.14: Iba1+ microglial cells are increased in the substantia nigra six weeks after intranigral delivery of WT PFF. Brain tissue was cryosectioned and immunohistochemically stained for Iba1 in the substantia nigra to quantify microglial cell bodies. Representative 40x images show Iba1+ microglia in the a) contralateral hemisphere of PBS group, b) ipsilateral hemisphere of PBS group, c) contralateral hemisphere of WT PFF group, d) ipsilateral hemisphere of WT PFF group, e) contralateral hemisphere of A53T PFF group, and f) ipsilateral hemisphere of A53T PFF group. Scale bar=50 μ m.

3.5.6. TH immunohistochemistry

A two-way ANOVA of TH+ cell bodies shows no effect of treatment [$F(2,44)=0.5142$, $p=0.6015$], laterality [$F(1,44)=0.7516$, $p=0.3907$] or interaction between treatment and laterality [$F(2,44)=0.4714$, $p=0.6272$] (Figure 3.15a). A one-way ANOVA shows no effect of treatment on TH+ cell bodies expressed as % of the contralateral hemisphere [$F(2,22)=1.285$, $p=0.2966$] (Figure 3.15b).

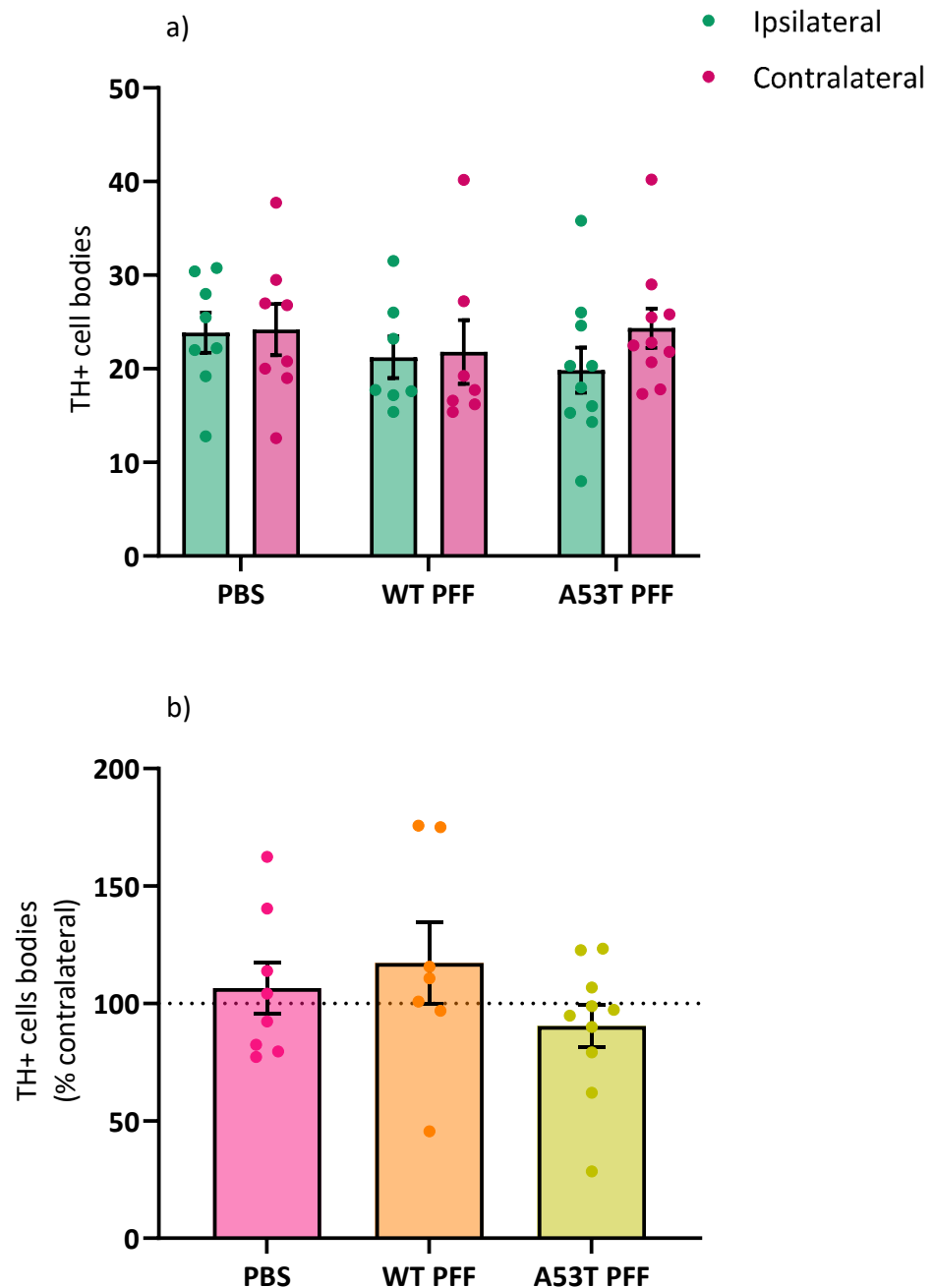


Figure 3.15. Neither WT nor A53T PFFs affect the number of TH+ cell bodies in the substantia nigra six weeks after intranigral delivery. a) Number of TH+ cell bodies in the ipsilateral and contralateral substantia nigra. b) Number of TH+ cell bodies in the ipsilateral hemisphere expressed as a percentage of the contralateral hemisphere. Data is expressed as mean $\pm$ SEM. a) Two-way ANOVA. b) One-way ANOVA followed by Dunnett's test for multiple comparisons. $n=7-10$.

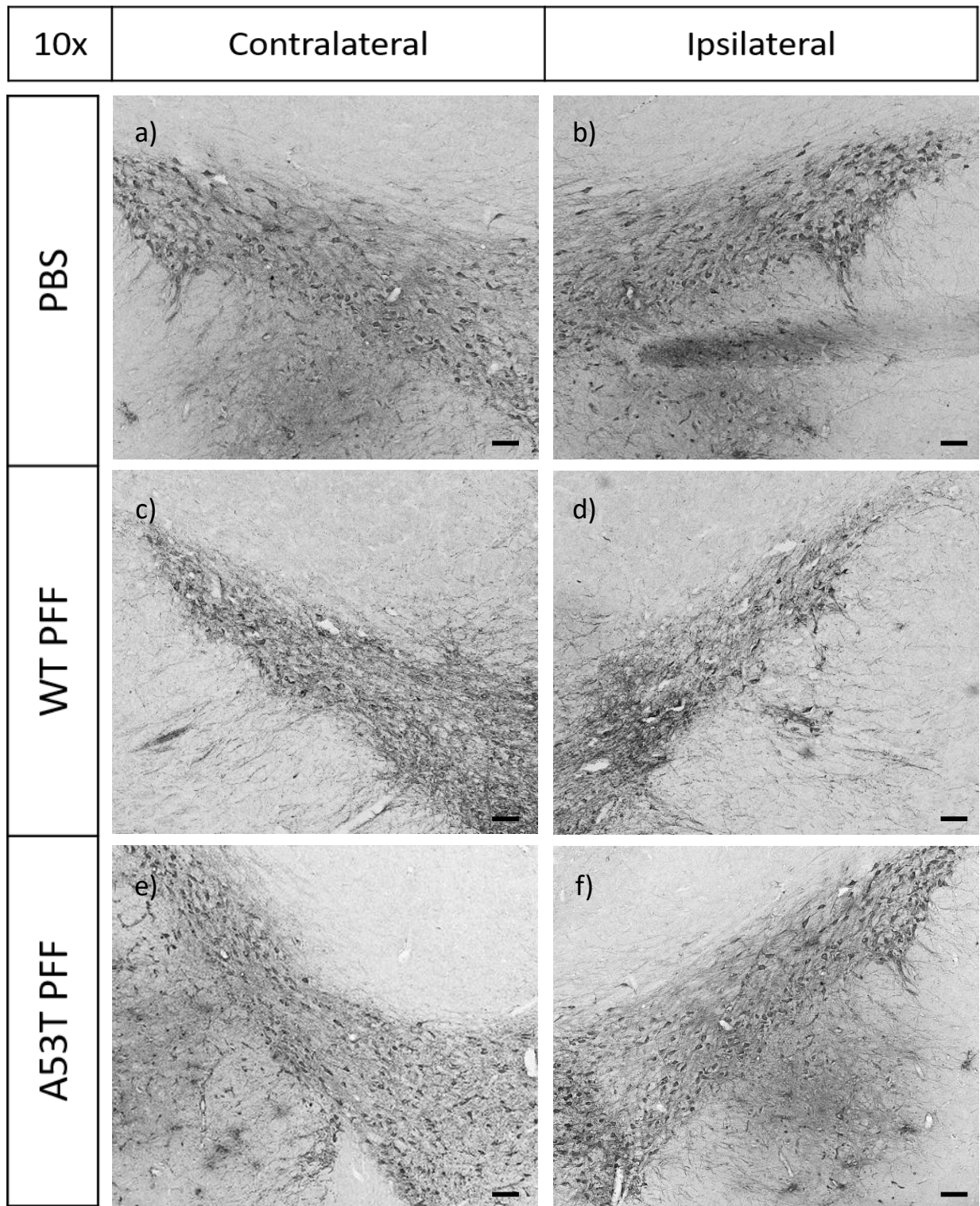


Figure 3.16. TH+ dopaminergic neuronal cell bodies in the substantia nigra remain unaffected six weeks after unilateral intranigral delivery of WT PFF or A53T PFF. Brain tissue was cryosectioned and immunohistochemically stained for TH in the substantia nigra to quantify dopaminergic neuronal cell bodies. Representative 10x images show TH+ dopaminergic neuronal cell bodies in the a) contralateral hemisphere of PBS group, b) ipsilateral hemisphere of PBS group, c) contralateral hemisphere of WT PFF group, d) ipsilateral hemisphere of WT PFF group, e) contralateral hemisphere of A53T PFF group, and f) ipsilateral hemisphere of A53T PFF group. Scale bar=50µm.

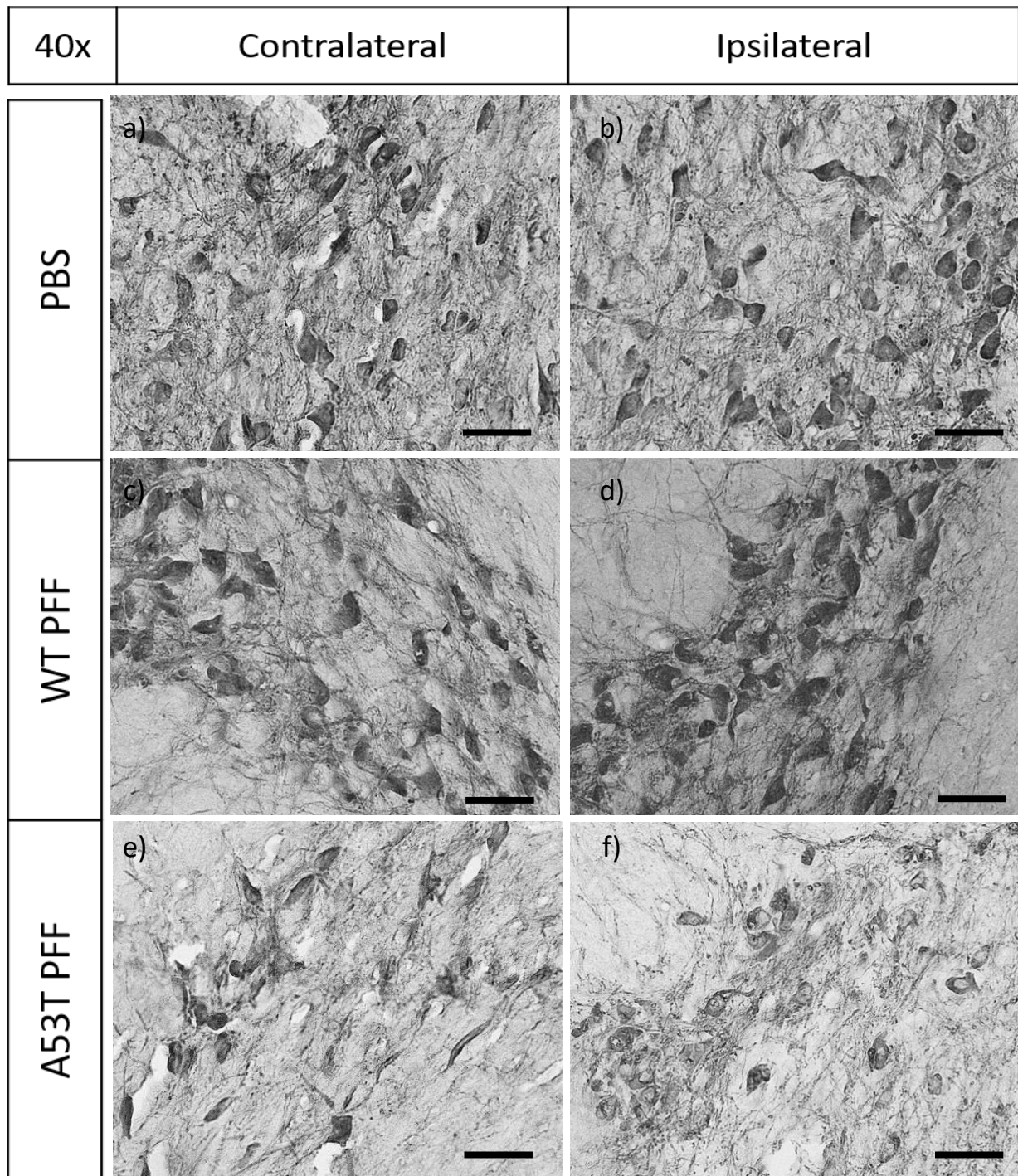


Figure 3.17. TH+ dopaminergic neuronal cell bodies in the substantia nigra remain unaffected six weeks after unilateral intranigral delivery of WT PFF or A53T PFF. Brain tissue was cryosectioned and immunohistochemically stained for TH in the substantia nigra to quantify dopaminergic neuronal cell bodies. Representative 40x images show TH+ dopaminergic neuronal cell bodies in the a) contralateral hemisphere of PBS group, b) ipsilateral hemisphere of PBS group, c) contralateral hemisphere of WT PFF group, d) ipsilateral hemisphere of WT PFF group, e) contralateral hemisphere of A53T PFF group, and f) ipsilateral hemisphere of A53T PFF group. Scale bar=50 μ m.

3.6. Discussion

In this study, a single unilateral intranigral injection of 4µg of human WT PFF or human A53T PFF in a volume of 2µL, lead to limb-use asymmetry observable in the cylinder test four weeks post-delivery. This asymmetry was more prominent in WT PFF-treated animals. Skilled motor performance in the stepping test was not affected by either treatment. Increased pSer129+ αSyn and Iba1 immunoreactivity were evident in the substantia nigra six weeks after intranigral delivery of WT PFF. By contrast, A53T PFF did not affect pSer129+ αSyn or Iba1 immunoreactivity. Neither WT PFF nor A53T PFF provoked changes in the number of TH+ cell bodies in the substantia nigra, indicating the absence of dopaminergic neuronal damage, a finding consistent with the unaltered rotational behaviours measured in the amphetamine-induced rotations test.

As measured in the cylinder test, intranigral delivery of WT PFF and A53T PFF appeared to reduce the number of placements made with the forelimb contralateral to the injected hemisphere, while placements made with the limb ipsilateral to the injected hemisphere appeared unchanged over time. This data, however, should be interpreted with caution, as a bias towards contralateral limb-use was evident in both WT and A53T PFF groups at baseline, thus confounding interpretation of a contralateral motor deficit induced by PFFs. Nonetheless, the reduction in % contralateral limb-use at week four, compared to baseline, suggests that, over time, unilateral WT PFF and A53T PFF treatments affect forelimb function, in this case, by reducing a natural bias towards contralateral limb-use preference. Combined cylinder scores expressed as a percentage of baseline clearly demonstrate that ipsilateral limb-use remained stable four weeks after WT and A53T PFF administration, while the use of the contralateral limb fell to approximately 55% of baseline in the WT group, and 65% of baseline in the A53T group. Further statistical analyses failed to confirm a significant reduction in contralateral limb-use compared to controls. Combined contralateral limb-use of PBS-treated animals at four weeks post-delivery remained within 1% of baseline, implying a behavioural deficit in PFF-treated animals, and not controls.

In the stepping test, ipsilateral combined scores and ipsilateral forward scores remained unchanged over time. Combined contralateral scores and forward contralateral scores appeared reduced at week five compared to baseline in all groups, including controls. While initial interpretation of these findings would suggest otherwise, no relative reduction of contralateral limb function was apparent when contralateral steps were expressed as a percentage of ipsilateral steps, in either scoring category. Moreover, when combined steps and forward steps were expressed as a percentage of baseline, no changes in limb-use were apparent over time. These findings raise the question of whether intranigral injection, irrespective of material injected, has an impact on motor skills, or if the behavioural performance observed in the present investigation is typical of all animals, whether naïve or subject to intranigral injection.

Stepping test data from the present study offer an insight into the importance of implementing appropriate controls to differentiate treatment-associated motor deficits from 1) consequential behaviours resulting from procedural defects, or 2) native behaviours which innately and/or spontaneously occur over time, irrespective of treatment. Care must be taken to identify typical behavioural patterns of naïve rodents without injection, and/or rodents whose surgical procedure involved a sham injection without delivery of disease salient material. Data from the cylinder test, which failed to identify a motor deficit in the control group, supports the likelihood that behavioural changes observed in PBS-treated animals in the stepping test were not reflective of a true motor

deficit. It is possible that the stepping test is not a relevant test for elucidating behavioural deficits associated with prodromal stages of synucleinopathy within the nigra, namely α Syn aggregation and microglial activation, even if overtly evident within the substantia nigra, a region of the brain which is responsible for skilled motor function.

Amphetamine-induced rotations were unaffected by PFF injection, a result which likely demonstrates evidence that neither WT nor A53T PFF, under these conditions, are sufficient to cause damage to the nigrostriatal dopaminergic tract, either directly, or through eliciting microglial activation. In accordance with data from the amphetamine-induced rotations test, TH+ dopaminergic neuronal integrity was maintained five weeks after intranigral delivery of both WT PFF and A53T PFF. This result is not surprising considering the early time point at which TH+ immunoreactivity was assessed, and given the low concentration of volume of PFF injected (4 μ g in 2 μ L). Luk and colleagues have described a high threshold for dopaminergic neuronal loss required to elicit rotational behaviours in male Sprague Dawley rats after an amphetamine challenge. At both 12 and 25-weeks following striatal delivery of mouse recombinant PFFs (8 μ g in 4 μ L), the induced nigral neuronal loss was insufficient to elicit amphetamine-induced rotational asymmetry, or forelimb-use asymmetry in the cylinder test (Paumier et al., 2015).

Given that this 6-week model aimed to induce and represent pathology at early stages of synucleinopathic disease progression, overt nigral degeneration and severe motor dysfunction were not anticipated outcomes in this study. Skilled and spontaneous motor function tests were conducted to assess motoric dysfunction associated with prodromal stages of synucleinopathy. WT PFF-treated animals, which displayed enhanced pSer129+ and Iba1+ immunoreactivity in the substantia nigra six weeks after PFF delivery, also displayed evidence of spontaneous motoric asymmetry in the cylinder test four weeks after intranigral delivery. However, similar spontaneous motor asymmetry was observed in A53T PFF-treated animals, despite the absence of pSer129+ immunoreactivity or changes in Iba1+ microglial cell counts in the nigra. As such, limb-use asymmetry detected in the WT PFF group in the cylinder test cannot necessarily be attributed to α Syn burden or microglial activation, as this motoric asymmetry was apparent in the A53T PFF group, which did not exhibit histopathology in the substantia nigra. As this motor asymmetry is observed only in the PFF groups and not in PBS controls, the outcome cannot be explained by PFF-induced dopaminergic nigral cell loss, for changes in TH+ cell counts were not observed in PFF-treated animals when compared to controls. In early-stage models, normal behavioural patterns which habituate over time can be misinterpreted as pathology-associated motor deficits. It is therefore imperative to conduct behavioural testing using methods which differentiate insult-driven motor dysfunction from spontaneous or habitual behavioural patterns which change over time.

Adult rats have a strong tendency to become habituated to these behavioural tests upon repeated exposure (Olsson et al., 1995; Schallert et al., 2000). As such, the stepping test and cylinder test could not be repeated at later timepoints in this study. In future work, the rotarod test could be employed to measure balance, grip strength, and reaction speed in relation to early-stage motor dysfunction (Monville et al., 2006). Programmes of various rotation speeds, durations, and cycles have been designed to investigate lesion-sensitive behaviours, allowing for a wide range of model applicability. Incremental protocols can be programmed to detect the presence of a lesion, while accelerating protocols provide a more discriminative test to gauge lesion size (Monville et al., 2006). The rotarod test was in fact employed in a series of experiments succeeding those presented in this chapter. These studies utilised an accelerating rotarod protocol to assess the capacity for

pharmacological intervention to mitigate WT PFF-related behavioural disturbances (Appendix II, Figure A2.7). The test was also used to measure imbalance and motoric instability following systemic LPS administration three weeks after intranigral delivery of WT PFFs (Appendix III, Figure A3.7). Although the data from the rotarod test itself is reliable, it remains in question whether the WT PFFs used in these experiments were viable, as, unexpectedly, pSer129+ expression was unobservable six weeks after intranigral delivery (Figures A2.8, A3.8, A4.1-A4.4). As such, results from these experiments were deemed inconclusive, and included in the Appendix (Appendix II, III, IV). As such, a sensitive rotarod protocol may allow for the detection of behavioural phenotypes associated with an early stage α Syn burden more accurately than the stepping or cylinder tests. These tests appear to require severe fracture to fine motor pathways before PFF-associated lesions can become detectable. The staircase test is another method commonly used to measure skilled motor dysfunction in unilateral models of PD (Montoya et al., 1991). While the staircase test has been used to indicate motor dysfunction in an LPS inflammatory model of PD in rats (O'Neill et al., 2020; Yssel et al., 2018), there is minimal research which demonstrates its utility or applicability for early-stage AAV- or PFF-based models of synucleinopathy. The staircase test was initially employed during optimisation phases of this research, but yielded highly variable and ambiguous results (Burke-Mullaly, 2021). Its use was discontinued.

Increased Iba1+ immunoreactivity was observed in the substantia nigra six weeks after delivery of WT PFF. Enhanced microglial reactivity in the ipsilateral nigra was accompanied by an increase in pSer129+ α Syn expression, also localised to the nigra. The immunoreactivity of pSer129+ α Syn in the injected hemisphere of WT PFF-treated animals is a likely indicator of the templated conversion of endogenous rat α Syn into phosphorylated aggregates, which stimulate the local influx and/or activation of midbrain microglia. The presence of Iba1+ microglia was also elevated in the contralateral substantia nigra, where pSer129+ α Syn expression was absent. The increase in Iba1+ cells in the contralateral hemisphere was not accompanied by observable pSer129+ deposits. As such, it cannot be inferred that enhanced Iba1+ microglia in the contralateral hemisphere reflect microglial mobilisation to sites of α Syn deposition, although this may be the case for increased number of Iba1+ microglia in the injected hemisphere. Many have reported findings which support a theory that α Syn propagation through the brain is facilitated by phagocytic migrant microglia (Gao et al., 2023; Lee et al., 2008a; Lee et al., 2008b; Park et al., 2008b; Ponomarev et al., 2005; Sacino et al., 2014; Sanchez-Guajardo et al., 2015). In the present study, sites of highly concentrated microglia align topographically with pSer129+ deposits in the hemisphere ipsilateral to the injection. In the contralateral hemisphere, microglial presence was increased even in the absence of pSer129+ immunofluorescence. As such, it also cannot be suggested that microglia, which have migrated to the contralateral hemisphere, have facilitated the transhemispheric propagation of aggregate constructs.

It can, however, be suggested that WT PFF injection into the substantia nigra triggers microglial influx within the injection site, driving further glial mobilisation beyond the injection region and within the alternate hemisphere. In addition to microglial activation in the substantia nigra, injection of WT PFFs may promote extra-nigral microglial mobilisation, the migration of glial cells through ventricular CSF around and within the brain. Microglial morphology in the substantia nigra was not assessed in the present investigation, but such an analysis would be a welcome future direction to further evaluate the microglial phenotype driven in the CNS in response to intranigral PFF delivery.

While the directional relationship between α Syn phosphorylation and microglial activation is unknown, the evidence that WT PFF promote both, while A53T PFF promote neither, suggests a dependency of microglial activation on α Syn phosphorylation. Dual fluorescence immunostaining of Iba1 with pSer129+ α Syn in these tissue sections would reveal whether pSer129+ immunoreactivity presents evidence of aggregate deposits within microglial cells, and/or extracellular plaques. This would certainly be a valuable future step to further assess likely mechanistic connections between α Syn phosphorylation and microglial activation. Although results were inconclusive, a series of experiments was conducted to assess the added inflammatory weight of systemic LPS administration three weeks following intranigral delivery of WT PFFs (Appendix III). The experiments remain under investigation following absence of observable pSer129 expression in the substantia nigra of WT PFF-treated animals, and thereby uncertainty of viability of the PFF constructs used (Figure A3.8, A4.1-A4.4). Nonetheless, should this experiment be repeated with viable materials (and confirmed pSer129+ expression in the nigra), the experimental design would likely be optimal for elucidating the bi-directional relationship between microglial mobilisation and α Syn phosphorylation.

Evidence of bilateral microglial mobilisation following unilateral injection of α Syn aggregates has been reported in the literature. Xia and colleagues observed reactive ameboid microglia in the bilateral rat substantia nigra two weeks after unilateral striatal injection of PD patient plasma-derived exosomes containing aggregate α Syn. Internalisation of exosomal α Syn by microglia, but not astrocytes or neurons, was also observed in the non-lesioned hemisphere, suggesting a role for microglia in facilitating exosomal α Syn transport through the CNS. Nonetheless, injection of human exosomal α Syn in said report significantly reduced TH expression in striatal dopaminergic neurons (Xia et al., 2019), an outcome not observed within the timeline of the present investigation. Despite unilateral striatal injection, human recombinant WT PFFs led to robust bilateral intraneuronal pSer129+ Lewy body-like pathology within 12 weeks of inoculation in transgenic mice expressing A53T-mutant human α Syn. Immunoreactive α Syn inclusions were detected in the frontal cortex, thalamus, hypothalamus, brainstem nuclei, and within axons of the corpus callosum, anterior commissure, and corticospinal tract. In contrast, pSer129 pathology at four weeks post injection was significantly less pronounced and primarily restricted to the injection site. This report shows the time-dependent fashion through which pathogenic α Syn amplifies and expands through the CNS. Interestingly, findings were replicated with PFFs from a C-terminally truncated form of WT human α Syn, demonstrating an ability of both full length fibrillar α Syn and truncated fibrillar α Syn to seed aggregation in the mouse brain. α Syn aggregation in the injected mice was also accompanied by astrogliosis and microgliosis, and progressive TH degeneration along the nigrostriatal tract (Luk, 2012a). Similar findings were reported by Sacino and colleagues, who demonstrated an association between the development of pSer129+ α Syn inclusions and robust gliosis around the injection sites following bilateral intracerebral injections of human recombinant α Syn PFFs (4 μ g in 2 μ L) into mice. Microglial and astrocytic uptake of α Syn aggregates was evident locally, while distal α Syn inclusion pathology was also observed in neurons in neighbouring brain regions (Sacino et al., 2014).

In Sprague Dawley rats, striatal PFF injection (8 μ g in 4 μ L) led to pSer129+ deposits in the ipsilateral substantia nigra, and extensive pSer129+ pathology across the bilateral striatum and cortex four weeks post PFF delivery. Although α Syn accumulation was only observed in the substantia nigra ipsilateral to the injected striatum, α Syn aggregation was followed by significant bilateral dopamine neuron loss over the course of 12 weeks. Contrary to reports from Sacino and colleagues, α Syn inclusions were not observed in astrocytes or oligodendrocytes, suggesting inclusions developed

specifically and exclusively in neurons (Paumier et al., 2015). This research provides further evidence of bilateral involvement following unilateral injection of PFFs, which may require extended periods of time to observe. In the present model, bilateral microglial mobilisation following unilateral WT PFF injection may signify early-stage inflammatory events associated with a prion-like cascade through which fibrillar α Syn propagates to induce cytotoxicity. Although pSer129 staining was not observed bilaterally in the present unilateral model, double-immunolabeling and confocal microscopy of TH+pSer129+ cells may help to determine the extent to which α Syn aggregates accumulate inside neurons.

The ability of PFF to recruit endogenous α Syn into pathogenic inclusions and undergo extensive phosphorylation at Ser129 is well documented. As this post-translational modification is not characteristic of recombinant PFFs (Fujiwara et al., 2002; Luk et al., 2012a; Luk et al., 2012b; Luk et al., 2009), pSer129+ α Syn expression and accumulation likely reflect modification which occurs intracellularly alongside accumulation of endogenous α Syn (Volpicelli-Daley et al., 2011). It has not yet been clarified whether the pSer129+ α Syn deposits observed in this experiment depict endogenous rat α Syn following phosphorylation as a consequence of delivery of human PFFs, or depict fragments of injected PFFs which have undergone phosphorylation. Although the present study did not attempt to differentiate endogenous protein from injected protein, Blumenstock and colleagues have provided a protocol for how this may be conducted in the future. Fluorescently labelled PFFs were delivered to the mouse striatum, and temporal clearance of the material from the injection site was confirmed before pSer129+ deposits were observed. Furthermore, little evidence of colocalization was seen between PFF injection site and eventual pSer129+ deposits (Blumenstock et al., 2017). This finding has been reinforced by reverse engineering efforts which have shown endogenous α Syn to comprise much larger proportions of intra-cellular inclusions than injected PFF material (Luk et al., 2009). In the same vein, α Syn-null transgenic mice display a total absence of inclusion-related toxicity after PFF injection, supporting the concept that PFFs can drive pathology by sequestration of endogenous α Syn (Luk, 2012b; Volpicelli-Daley et al., 2011).

In stark contrast to observations from WT PFF-treated animals, pSer129+ α Syn immunoreactivity was limited in the A53T PFF group. Similarly, Iba1+ immunoreactivity was not observed at a level above that of control animals. The present study demonstrates that exogenous human A53T PFFs, in isolation, were not sufficient for promoting the phosphorylation of α Syn, or driving changes in microglial cell numbers. While the behaviour of WT PFFs observed *in vitro* appears to be reproducible in animal models, A53T-mutant PFFs appear to perform differently *in vitro* compared to *in vivo* (Kotzbauer et al., 2004; Li et al., 2005; Liu et al., 2005; Tofaris et al., 2006; Zach et al., 2007). Structural features of WT and A53T-mutant protein constructs likely reflect their differing ability to interact with cells, in turn, influencing pSer129+ immunoreactivity.

A common pitfall in PFF research is failure to consistently generate PFFs with the same structural, biochemical, and pathological properties. Disparities between experimental reports which utilise PFFs have led to concerns over reproducibility, dissuading researchers from using models which require months of work to execute before post-mortem results may be determined. Sensitivity of the protocol for fibril formation undoubtedly results in variation both within and across labs. The protocol for PFF production begins with development of monomeric recombinant α Syn protein, which much be specifically formulated for α Syn PFF generation. Typically, monomers are generated through transformation with plasmid encoding human α Syn in E.coli (Earls et al., 2019; Gribaudo et al., 2019; Grozdanov et al., 2019; Harms et al., 2017; Russo et al., 2019; Sarkar et al., 2020). Protein

can be produced from human cDNA samples and amplified through qRT-PCR (Fellner et al., 2013; Stefanova et al., 2001). Generally, purified monomers are centrifuged, diluted, and incubated at 37°C while shaking for 7 days to achieve fibrillar aggregates. On the day of use, PFF aggregates should be fragmented by sonication with a micro probe for a recommended 6 sets of 10 x 0.5 second pulses, with a 10 second pause in between each set to minimise heat generation and protein denaturation (Polinski et al., 2018; Volpicelli-Daley et al., 2014).

The Michael J. Fox Foundation for Parkinson's disease research, together with the collaborative efforts of experts who instigated the development and validation of PFF modelling, has established a set of guidelines and protocols to improve the uniformity of PFF generation, with the goal of enhancing the reproducibility of PFF models. Though these guidelines and protocols are almost exclusively used, heterogeneous results still arise, which are likely the result of variations between PFF preparations across laboratories. Understandably, inadequate PFF generation and sonication to appropriate size will render the constructs too large for cell surface receptor interaction, neuronal internalisation, glial phagocytosis, or propagation throughout the cellular network (Holmes et al., 2013; Mao et al., 2016; Shrivastava et al., 2015). Furthermore, sonicated PFFs are heterogeneous in nature, adding to the challenge of reproducibility (So & Watts, 2023). PFF generation in this research was carried out by the manufacturer, StressMarq Biosciences. Subsequent in-house preparation of the acquired PFFs was identical for both WT and A53T-mutant constructs, and is not thought to have contributed towards differences in results observed between PFF strains. Nonetheless, following consideration that A53T mutant PFFs, which generally have a higher propensity to aggregate than WT, did not induce pSer129+ α Syn expression, it is possible that mal preparation of A53T PFFs could have caused the species to aggregate beyond fibrils, rendering them less effective. It is also possible that the process used by the manufacturer to generate PFFs from monomers was optimised for the generation of WT PFF, and not the A53T-mutant strain, leading to reduced potency or propensity to aggregate. A major shortcoming of the present investigation was the omission of a Thioflavin-T assay to confirm the aggregatory state of constructs immediately before use. With a Thioflavin-t-assay, the experimenter would have been able to confirm whether the constructs were still in oligomeric or fibrillar confirmation at the time of use, and had not degraded or aggregated into smaller or larger constructs. This assay should be employed in all future experiments.

Suzuki and colleagues demonstrate the marked variety of fibrillar α Syn strains which can arise following slight alterations to PFF generation protocols (Suzuki et al., 2020) (Figure 1.4). Naturally, this raises questions about the uniformity of PFF-driven models.

Genetic homology between the rat and human SNCA genomic sequence allows for the use of human α Syn constructs for investigating α Syn-associated pathology in rodent models, and rodent α Syn constructs for investigating pathology in human-derived cell or organoid models. This homology underscores the challenge of distinguishing exogenous α Syn from endogenously expressed protein in models involving overexpression, or delivery of recombinant protein. The absence of pathology following delivery of A53T PFFs to the rat substantia nigra may be related to the fact that human A53T corresponds with WT α Syn in rodents. A threonine at amino acid residue 53 is the normal α Syn sequence found in rodent, and as such, the A53T-mutation may only be relevant in the human condition (Hsu et al., 1998; Stefanis, 2012).

The limited expression of pSer129+ α Syn, and lack of microglial mobilisation in A53T PFF-treated animals, compared to WT PFF-treated animals, also suggests an impact of sequence and/or

structural differences between protein constructs, potentially leading to variations in seeding capabilities, propagation patterns, and microglial activation in rats (Gribaudo et al., 2019; Peelaerts et al., 2015). The A53T-mutation propels the α Syn fibril into a more tightly coiled structure in which the filament-forming region becomes shielded (Guerrero-Ferreira et al., 2018; Lashuel et al., 2013; Wong & Krainc, 2017). Reduced regional exposure likely inhibits its ability to interact not only with neurons, but also microglia, ultimately leading to reduced intracellular deposits and reduced microglial response. Mutations in the α Syn protein may alter the intrinsic seeding capability of recombinant PFF aggregates. Additional cellular chaperones may be required to generate a degenerative phenotype representative of idiopathic PD. While the current model is highly relevant to the human condition in that it utilised PFFs homologous to human α Syn fibrils, it is limited in its lack of genetic overexpression of the protein. Subsequent development of the PFF model could enhance construct validity by exploring the ability for WT or A53T PFF to produce pathology in an animal model with built-in or artificially introduced genetic overexpression.

In the PFF models presented here, pSer129, Iba1, and TH-associated pathology was exclusively assessed within the substantia nigra. In early stage synucleinopathic disease, pathological changes are often localised within the brainstem and midbrain regions. At later stages, neuronal dysfunction and associated protein aggregates can become widespread within the forebrain and throughout the cortices (Braak et al., 2004). Given this trajectory of pathology, a welcome enhancement to the current study would be a comprehensive investigation of pathology in brain regions connecting to the substantia nigra, including the striatum and the LC. Further immunolabeling and thorough analysis of pSer129+ α Syn, Iba1, and TH immunoreactivity in tissue sections collected from LC and striatal regions of PFF-treated animals would provide valuable insight into the directional progression of cellular dysfunction beyond the substantia nigra. Furthermore, a welcome future direction to progress this work would be the temporal investigation of pSer129+ α Syn accumulation and microglial mobilisation over time in the substantia nigra after intranigral delivery of WT PFF. Mapping Iba1+ microglial migration along a six-week trajectory following PFF delivery, to indicate breakdown, cellular uptake, hyper-phosphorylation, and possible propagation, may indicate the point at which PFFs trigger microglia activation. To a similar extent, future studies utilising human protein constructs in rodent models would benefit from an immunohistochemical method capable of differentiating pSer129+ rat α Syn from pSer129+ human α Syn, in order to more precisely identify the timeline along which recombinant PFFs recruit endogenous α Syn into hyper-phosphorylated aggregates.

3.7. Chapter conclusion & next steps

The present investigation demonstrated that delivery of a single unilateral intranigral injection of 2 μ L human WT PFFs or A53T PFFs lead to limb-use asymmetry observable four weeks post-PFF delivery, while skilled motor function remained unaffected. While WT PFFs displayed a propensity to promote the phosphorylation of endogenous α Syn, and increase the number of Iba1+ microglia in the ipsilateral substantia nigra, A53T PFFs, when administered alone, did not. Neither strain of PFF affected TH+ neuronal cell body counts in the substantia nigra six weeks after delivery. No behavioural or histological differences were identified between males and females included in this study.

In line with the aim of generating a unilateral rodent model of synucleinopathy which comprised characteristic nigral neuronal degeneration, in addition to motoric asymmetry and microglial activation, further development of the model was required. PFFs provide a skeleton upon which naïve endogenous α Syn can be recruited for fibrillisation, a property which can be exploited in animal models to reflect mechanisms associated with synucleinopathy in the human condition. The following investigation further explores a capacity for α Syn PFFs, in combination with AAV-mediated genetic expression of α Syn, to induce an α Syn burden sufficient for driving motoric asymmetry, microglial activation, and associated neuronal cell loss in the rat substantia nigra. Both WT and A53T-mutant α Syn remained of interest, as variant strains of α Syn react differently on a background of genetic overexpression. As human forms of synucleinopathies comprise both the overexpression of α Syn and the accumulation of seeded aggregates, an animal model using this combined approach may be more valid for translation to the clinic.

Chapter 4: An assessment of PD-related pathology in rats six weeks following unilateral intranigral delivery of human alpha-synuclein preformed fibrils in combination with human alpha-synuclein-expressing adeno-associated viral vector

4.1. Introduction

α Syn fibrils can self-propagate through the brain, spinal cord, and peripheral nervous system, to provide a template for the conversion of naïve α Syn monomers into polymeric constructs (Karampetsou et al., 2017; Luk et al., 2012b). Fibrillar recruitment of free monomers burdens the brain with the progressive process of α Syn hyper-phosphorylation and aggregation, which can trigger glial activation and resulting neuronal degeneration (Brundin et al., 2010; Buell et al., 2014; Lashuel et al., 2013). Results from *in vivo* investigations using hyper-phosphorylated and phosphorylation-incompetent recombinant PFFs have provided evidence to support a theory that although phosphorylation is not required for the *de novo* formation of pathologic α Syn inclusions, it appears to produce a more severe pathology in rodent models of PD (Karampetsou et al., 2017). The progressive process by which α Syn fibrils accumulate into cytotoxic Lewy body inclusions does, however, appear to require the endogenous genetic expression of α Syn in the brain (Gao et al., 2008; Karampetsou et al., 2017; Luk et al., 2012b). Results from the previous chapter demonstrate an ability for human recombinant WT PFFs to increase phosphorylation of endogenous α Syn and increase microglial cell counts in the substantia nigra, without reducing dopaminergic neuronal cell bodies six weeks following their unilateral intranigral delivery to rats. A53T PFFs did not appear to impart any changes to the phosphorylation state of endogenous α Syn, or drive changes in glial or dopaminergic neuronal cell counts within the nigra.

These results indicate that WT PFFs may be equipped with the ability to promote phosphorylation of endogenous α Syn, while the A53T-mutant constructs appear devoid of this property. Given the finding that brain samples from human cases of synucleinopathy comprise both fibrillar α Syn deposits and the overexpression of the α Syn gene (with genetic variants relating to different forms of synucleinopathy), the question arises whether a genetic background of human α Syn expression in the rat model would be required to drive PFF-related neuropathology. Here, the impacts of combining WT or A53T PFFs with AAV vector-mediated human α Syn gene expression, to drive behavioural impairment, microglial activation, and neurodegeneration are assessed, through unilateral intranigral stereotactic delivery to the rat substantia nigra.

The use of AAV tools to express human α Syn in the rat substantia nigra has largely resolved the primary shortcomings of classic toxin and transgenic rodent models of PD-like neurodegeneration in which Lewy body-like pathology is absent (Van der Perren et al., 2015b). The consensus from the previous decade of AAV research is that delivery of a standard titre of AAV vector expressing human α Syn into the rodent substantia nigra results in key features of the human disease - nigral dopaminergic cell loss, α Syn aggregation in surviving neurons, and motor deficits associated with dopamine depletion. However, the magnitude of these outputs is highly variable, and typically dependent on a timeframe of 10-20 weeks post-AAV vector delivery to the brain (Gombash et al., 2013; Ip et al., 2017; Song et al., 2015b; Taschenberger et al., 2012; von Hovel et al., 2019). However, limited but compelling accounts of dopaminergic degeneration exist following AAV-mediated human WT and A53T-mutant α Syn delivery in rodent models within a timeframe of six weeks.

AAV2-expressing human WT α Syn (2 μ l; 1.2×10^8 transducing units) to the substantia nigra of female Wistar rats reportedly induced α Syn inclusions and TH+ cell loss observable as early as two weeks and up to 14 weeks post-delivery (Taschenberger et al., 2012). A hybrid vector rAAV2/5 expressing human WT α Syn at various titres showed titre-dependent differences between the temporal onset of microglial activation and/or neurodegeneration following injection into the substantia nigra of female Sprague Dawley rats. Low titre-induced WT α Syn expression (2 μ l; 6.2×10^{13} vg/ml) resulted in the transient increase in microglia cell numbers observed as early as four weeks post-delivery, with no nigral degeneration observed. In contrast, high-titre expression (2 μ l; 6.7×10^{13} vg/ml) resulted in TH+ cell loss observed as early as four weeks post-delivery, with a delayed microglial response becoming apparent eight weeks post-delivery. In addition, varying CD4+ and CD8+ T-lymphocyte infiltration kinetics suggested a dependency of the adaptive immune response on the degree of neurodegeneration, whereby increased infiltration was associated with timepoints marked by increased levels of cell death (Sanchez-Guajardo et al., 2010).

α Syn-associated nigral dopaminergic degeneration has been observed in Sprague Dawley rats, wherein rAAV2/8 expressing human WT α Syn (2 μ l; 1.4×10^{13} vg/ml) was delivered to the substantia nigra of one hemisphere, while an empty vector (2 μ l; 1.16×10^{14} vg/ml) was delivered to the other. Six weeks following delivery, TH+ immunoreactivity was reduced only in the AAV-WT α Syn-injected hemisphere. Extensive ipsilateral α Syn expression was observed at both two and six weeks post-delivery within the substantia nigra pars compacta, substantia nigra pars reticula, ventral tegmental nucleus, and the ventral mesencephalic reticular formation (McFarland et al., 2009a). Gorbatyuk and colleagues report evidence of cytoplasmic α Syn aggregates within four weeks of unilateral intranigral delivery of rAAV2/5-encoding human WT α Syn (1.5 μ l; 0.9×10^{13} vg/ml) in Sprague Dawley rats, but loss of TH+ immunoreactivity was not evident until 26 weeks post-delivery (Gorbatyuk et al., 2008). Interestingly, the same group did observe dopaminergic degeneration within eight weeks of delivery of AAV5-WT α Syn, suggesting a change in serotype might enhance the efficacy of α Syn expression and downstream effects on nigrostriatal integrity (Gorbatyuk et al., 2010). Cytoplasmic inclusions and swollen α Syn+ dystrophic neurites were apparent in the nigra between 3- and 8-weeks post intranigral injection of rAAV- α Syn to Sprague Dawley rats (2 μ l; 8.2×10^{11} vg/ml), eventually leading to TH+ cell loss in the nigra and TH+ fibre denervation in the striatum (Kirik et al., 2002).

AAV-mediated overexpression of A53T-mutant α Syn has also been reported. Koprach and colleagues observed limb-use asymmetry, along with α Syn inclusions in TH+ neurons at three weeks following nigral delivery of 2 μ L of AAV1/2-A53T α Syn at various titres - a low titre (5.1×10^{11} vg/ml), medium titre (1.7×10^{12} vg/ml), and high titre (5.1×10^{12} vg/ml). Interestingly, while nigral dopaminergic degeneration, striatal dopamine depletion, and behavioural impairment progressed steadily over the course of six weeks in response to the medium and high titre, these deficits were not evident by 6-weeks in the low-titre group (Koprach et al., 2011; Koprach et al., 2010). While high viral titres may drive progressive pathology against which disease-modifying therapies can be investigated, low titres might prove more useful for investigating mechanisms of endogenous compensation in early disease states where pathology remains malleable.

Female Sprague Dawley rats presented with insoluble α Syn inclusions co-localized with TH+ cells in the substantia nigra six weeks after intranigral delivery of AAV1/2 A53T α Syn (2 μ L, 5.1×10^{12} vg/ml). Vector delivery also reduced nigral TH+ fibres by approximately 50% across both the substantia nigra and striatum of the injected hemisphere (Koprach et al., 2010). Other work conducted in Wistar rats, which displayed pSer129+ α Syn immunoreactivity (Figure A1.1, A1.2), failed to show TH+ cell loss at

six weeks following intranigral delivery of AAV-A53T α Syn (Figure A1.1, A1.4), despite injecting equal volumes of the same vector serotype at comparable titres into the substantia nigra (Appendix I). Reports of the behavioural implications of AAV-driven α Syn overexpression remain equivocal. Injection of AAV2/6 human WT α Syn into the substantia nigra of female Sprague Dawley rats has induced TH+ cell loss within eight weeks of delivery. Degeneration was evidenced by behavioural deficits observed in the amphetamine-induced rotations test and stepping test at three weeks, and again in both tests, and the cylinder test, at eight weeks. A similar experiment was conducted using rAAV2/6 human WT α Syn (1.7×10^{14} vg/ml; 2.8×10^7 transducing units), the intranigral injection of which impaired contralateral forepaw function after 13 weeks, as assessed through the cylinder test and apomorphine-induced rotations test (Gaugler et al., 2012). Gombash and colleagues have also explored the impact of viral titre relative to time of TH+ neuronal loss and behavioural impairment in an AAV-model of synucleinopathy. Contralateral motoric impairments in the stepping and cylinder tests were apparent in rats initially after eight weeks of low titre rAAV2/5-mediated α Syn expression (1.0×10^{13} vg/ml), which gradually worsened along a trajectory of progressive striatal TH+ fibre loss over a timeline of 16 weeks (Gombash et al., 2013). In contrast, other have shown that at a timepoint of four weeks, animals with high titre rAAV2/5-mediated α Syn expression (1.0×10^{14} vg/ml) displayed no behavioural impairments, despite TH+ cell loss in response to the enhanced viral load. Together, evidence suggests a highly varying ability for AAV-mediated α Syn expression to influence skilled or spontaneous motor behaviour (Jimenez-Ferrer et al., 2017; Kirik et al., 2002; Yamada et al., 2004). Many have attempted to identify the causes of variability between reported outcomes in animal models of AAV-mediated α Syn expression, with the intent of standardising the use of AAVs in the research field (Gombash et al., 2013; Kirik et al., 2002; Maingay et al., 2006; Sanchez-Guajardo et al., 2010).

Given that human synucleinopathies comprise both the overexpression of α Syn and the accumulation of seeded aggregates, animal models which incorporate both recombinant PFFs and AAV-mediated α Syn expression may drive pathology with higher resemblance to human conditions. In line with findings reported in the previous chapter, Peelearts and colleagues reported accumulation of pSer129+ Lewy body- and Lewy neurite-like inclusions in nigral dopaminergic neurons, but no significant loss of TH+ cells after nigral injection of fibrillar WT human α Syn. In female Wistar rats, unilateral intranigral administration of WT human α Syn PFFs ($10\mu\text{g}$ in $2\mu\text{L}$) in combination with AAV2/7 A53T human α Syn vector (3.0×10^{10} vg/ml in $1\mu\text{L}$) through a single injection, resulted in more severe degeneration of TH+ nigral cell bodies and striatal fibres at 16 weeks, than either fibrils or vectors alone when delivered independently, or than vector when delivered in combination with α Syn ribbons or oligomers. Assessment of spontaneous forepaw use with the cylinder test revealed a progressive motor deficit from eight weeks onwards, following injection of α Syn fibrils, which was accelerated by treatment combination with a vector. While TH+ nigral cell death was also observed following inoculation of rAAV in combination with α Syn oligomers and ribbons, striatal TH volume proved sensitive to depletion following rAAV- α Syn overexpression with inoculation of fibrils only. These findings supports a theory that α Syn fibrils drive neuronal degradation and dopaminergic depletion more robustly than other conformers when used *in vivo*. Interestingly, ribbons, but not fibrils, in combination with rAAV-mediated α Syn overexpression, produced a sparse α Syn phosphorylation pattern in oligodendrocytes (Peelaerts et al., 2015).

In a seven-week paradigm with female Sprague Dawley rats, unilateral double-injection of human recombinant PFFs to the medial and lateral substantia nigra ($5\mu\text{g}$ in $2.5\mu\text{L}$ per injection; $10\mu\text{g}$ in

total), four weeks after initial delivery of AAV6 human WT α Syn to the same sites (total of 8.8×10^8 genome copies (gc)) injected to each animal; 2 μ L per site), resulted in nigral dopaminergic depletion greater than that of either PFF or AAV when delivered independently. Behaviourally, limb-use asymmetry was observed in combination groups as early as three weeks post PFF-injection (seven weeks post-AAV). An extended paradigm of 28-weeks post initial AAV-injection was required to observe dopaminergic neuronal loss in response to independent treatments (Thakur et al., 2017). Bilateral delivery of PFFs (concentration not specified) to the rostro-medial striatum in the Sprague-Dawley rats three weeks after initial bilateral delivery of AAV2/6-encoding human WT α Syn (titre not specified), to the medial prefrontal cortex resulted in depletion in working memory and attention. In line with similar research, cognitive impairment was not apparent after independent compound administration. While AAV alone did promote increased α Syn immunoreactivity, Lewy body-like inclusions representative of the human condition were only observed in the combination model. Furthermore, combination treatment displayed affected dendritic morphology in the region of delivery (Espa et al., 2019).

It should be noted that in these studies, the rate of α Syn spread and timeline for the progression of PD-like neurodegeneration varies across models, making reproducibility difficult. Nonetheless, the PFF+AAV rodent model can offer distinct advantages in promoting seeding and propagation of α Syn, and synucleinopathy pathogenesis. In contrast to the slow progression of pathology in models driven by PFF-alone or AAV-alone, the amplification of α Syn and acceleration of pathology in combination models may provide for a more robust and reproducible model. In a shorter study, striatal delivery of 40 μ g of α Syn PFFs divided over three injection sites in combination with rAAV-mediated α Syn expression, drove the accumulation of insoluble rodent α Syn throughout the nigrostriatal pathway after eight weeks. This further demonstrates that exogenous α Syn fibrils seed the assembly of endogenous α Syn. Moreover, fibrils exhibit a higher resistance to enzymatic degradation by proteinase K compared to ribbons, reflecting a dependency on fibrils to drive pathology (Peelaerts et al., 2015).

Perhaps the study providing the strongest rationale in support of a combination approach was that conducted by Luk and colleagues in 2012. Transgenic mice expressing A53T-mutant human α Syn develop abnormal posture, seizures and paralysis, and abundant Lewy body and Lewy neurite-like α Syn pathology at about 12 months of age. To investigate whether disease-associated aggregated α Syn could seed prodromal pathology *in vivo*, brainstem homogenate from 12-month-old symptomatic mice was isolated for injection into the neocortex and striatum of pre-symptomatic 2–5-month-old transgenic mice. When examined 90 days post-injection, pSer129+ α Syn accumulation was apparent throughout the brain of lysate-injected mice, while absent in PBS-injected mice, and mice injected with lysate from pre-symptomatic animals. These findings indicate a direct association between pathogenic α Syn protein and progressive pathology, independent of transgene-related symptomatology. pSer129+ α Syn inclusions were also absent 90 days following injection of the same lysate into α Syn knockout mice, thus indicating the dependency of *de novo* formation of Lewy body-like inclusions on host expression of α Syn. Together, these findings show pathological α Syn in symptomatic brain lysate from transgenic mice to be capable of initiating and accelerating α Syn pathology in this transgenic mouse line. Furthermore, intracerebral inoculation of pre-symptomatic transgenic mice with human WT α Syn PFFs also elicited robust pSer129+ aggregate pathology, comparable to that of lysate-treated mice. TH+ immunostaining in the substantia nigra was reduced in both lysate- and PFF-injected pre-symptomatic transgenic mice, but remained unchanged

following injection of pre-symptomatic-mouse lysate or PBS. Comparatively, pathology in pre-symptomatic mice injected with either symptomatic mouse-lysate or WT PFFs was more pronounced and regionally diffuse than that of noninjected transgenic mice which developed symptoms with age. This model also recapitulates the biochemical and post-translational modifications that are characteristic of α Syn pathogenesis, in demonstrating that, while the solubility of β Syn remains unchanged over time, soluble endogenous murine α Syn becomes insoluble once recruited and chaperoned into Lewy body-like inclusions. Finally, progressive α Syn pathology from lysate- or PFF-injected mice was accompanied by astrogliosis and microgliosis, a finding which supports the theory suggesting fibrillar α Syn-mediated inflammation to be a contributing factor in synucleinopathic neurodegeneration (Luk et al., 2012a).

Thakur and colleagues observed pSer129+ α Syn accumulation, TH+ cell loss, and behavioural deficits in rats following delivery of AAV α Syn through two injections into medial and lateral regions of the substantia nigra, and another two injections of PFF into the same sites four weeks later. Similar pathology was achieved following AAV delivery to both the substantia nigra and ventral tegmental area, followed four weeks later by subsequent PFF injections into both regions (Thakur et al., 2017). In a further combination model, adult rats received an intranigral injection of rAAV6- α Syn (3 μ L; 3.6×10^{14} vg/ml), followed two weeks later by two striatal injections of human recombinant α Syn PFFs (1 x 2 μ L injections containing 5 μ g protein per site; 10 μ g protein total). This combination paradigm resulted in humanoid synucleinopathy-like pathology, manifesting as Lewy-body and Lewy-neurite-like aggregates within the substantia nigra and across the greater midbrain (Jimenez-Ferrer et al., 2017).

Taken together, these findings demonstrate that the introduction of prion-like protein seeds can greatly enhance neurodegeneration in conditions where α Syn expression is amplified artificially. Relative to other α Syn conformers, fibrils have been reported to impose the largest neurotoxic burden on the nigrostriatal pathway, especially in an environment of heightened α Syn expression. Research has yet to generate a combination model comprising simultaneous intranigral inoculation of AAV and PFFs together, giving rise to representative of prodromal pathology in synucleinopathy. In combination models generated thus far, the implications of fibrillar α Syn seeding, when combined with AAV-associated α Syn expression, on inflammation-associated neurodegeneration has not been studied. Furthermore, the implications of the A53T-mutation have not been directly assessed in a combination model in which both PFFs and AAV-mediated α Syn expression contain the mutation. Thus, generation of a combination model exhibiting α Syn overexpression and fibrillar seeding using WT and A53T-mutant constructs in rats is of interest for modelling progressive neurodegenerative effects of early stage α Syn aggregation. Combination models may depict the human condition more accurately *in vivo*.

Comparative analysis between combination models exclusively expressing WT and A53T-mutant α Syn may provide insight into pathological differences between idiopathic and familial PD in prodromal stages, and provide models in which disease-modifying treatments can be tested.

To further develop the rat model generated in the previous chapter, human WT and A53T-mutant fibrillar constructs were used to introduce pathogenic seeds into the rat midbrain, along with α Syn-expressing AAV vectors, to deliver localised strain-specific expression of the protein. This may provide the genetic background upon which pathogenic properties of PFFs can be more readily observed. A hybrid AAV1/2 capsid combines the high tropism of AAV2 for TH+ dopamine neurons

with the ability for AAV1 to diffuse expression widely through brain tissue, ultimately mediating robust transduction within dopaminergic neurons of the substantia nigra (Koprach et al., 2010). For these reasons, AAV1/2 serotype was selected for optimal utilisation in A53T investigations. In WT investigations, AAV serotype 5 was selected to compliment previous research conducted which assessed the skilled and spontaneous motor phenotypes associated with AAV-mediated WT α Syn in a rodent model of PD (Burke-Mullaly, 2021).

4.2. Aims and objectives

The primary aim of this study was to explore the impact of unilateral intranigral delivery of human recombinant PFFs in combination with AAV vector expressing human α Syn on nigral α Syn phosphorylation, microglial activation, dopaminergic neuronal integrity, and associated motor function six weeks after stereotactic delivery into the rat substantia nigra. With an aim to assess early-stage pathogenic processes which likely precede nigral neurodegeneration and striatal dopamine depletion, development of this model may better reflect prodromal stages of synucleinopathic disease progression. Both WT and A53T-mutant α Syn PFFs were utilised to further assess the role of mutant forms of α Syn in driving synucleinopathic pathology in the rat.

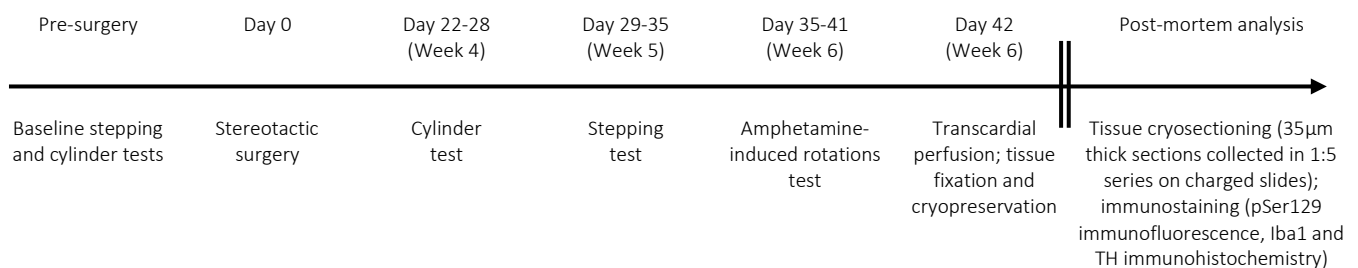
The objective of this study was to deliver WT or A53T-mutant α Syn PFFs, in combination with WT or A53T α Syn-expressing AAV, respectively, unilaterally into the rat substantia nigra by stereotactic injection, and assess the efficacy of these constructs in combination to increase microglial cell counts and reduce dopaminergic neuronal cell counts in the nigra within six weeks. Skilled motor function and limb-use asymmetry were assessed through application of the stepping and cylinder tests, respectively. Rotational behaviour in the amphetamine-induced rotations test was assessed to functionally evaluate the presence of a unilateral nigral lesion. pSer129 immunofluorescence, Iba1 immunohistochemistry, and TH immunohistochemistry were subsequently conducted on cryopreserved midbrain tissue sections to assess α Syn burden, microglial cell counts, and dopaminergic neuronal cell counts, respectively, in the substantia nigra. Comparisons were made between the WT combination and A53T combination treatments to gauge their capacity to drive disease pathology, and further consider the relevance of the A53T mutation in ongoing model development.

4.3. Experimental design

16 adult (7–8-month-old) Wistar rats (10 females, 6 males) underwent behavioural training and baseline testing before being randomly assigned to a treatment group. All animals received two intranigral stereotactic injections of 2µL per injection. Animals in the WT combination treatment cohort received one 2µL injection of human WT αSyn PFFs and one 2µL injection of AAV5-human WT αSyn. Animals of the A53T-mutant combination treatment cohort received one 2µL injection of human recombinant A53T-mutant αSyn PFFs and one 2µL injection of AAV1/2-human A53T αSyn. Difference between WT vector and A53T vector titre was due to product availability and manufacturing. Equal vector titres should be used in all future experiments. The coordinates for AAV infusion in males were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for AAV infusion in females were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. The coordinates for PFF infusion in males were as follows; AP: -4.9 from bregma, ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for PFF infusion in females were as follows; AP: -4.9 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. In weeks four, five, and six following PFF+AAV delivery, rats were subjected to the cylinder test, stepping test, and amphetamine-induced rotations test, respectively. Six weeks post-PFF+AAV delivery, animals were euthanised by sodium pentobarbital overdose and transcardial perfusion/fixation, in preparation for immunofluorescence and histochemical analysis of pSer129+ αSyn, Iba1+ microglia, and TH+ neuronal cell bodies in the substantia nigra. As naïve controls were omitted from this experiment, the unaffected limb and hemisphere were used as internal controls for behavioural and histological analysis, respectively.

Treatment groups (n = 6-10):

1. Human WT αSyn PFF + AAV5-CBA-human WT αSyn (two unilateral injections of 2µl)
 - n = 6 (5 females, 1 male)
 - PFF concentration: 2mg/ml (4µg in 2µL)
 - AAV titre = 1×10^{13} vg/mL (2µl)
2. Human A53T αSyn PFF + AAV1/2-CMV-human A53T αSyn (two unilateral injections of 2µl)
 - n = 10 (5 females, 5 males)
 - PFF concentration: 2mg/ml (4µg in 2µL)
 - AAV titre: $>5 \times 10^{12}$ vg/ml (2µL)



4.4. Methods

All methods were conducted as described in chapter 2.

4.5. Results

4.5.1. Cylinder test

A two-way repeated measures ANOVA of ipsilateral placements shows no effect of treatment [$F(1,14)=0.013$, $p=0.9112$], time [$F(1,14)=0.0040$, $p=0.9506$], or interaction between treatment and time [$F(1,14)=0.18$, $p=0.6783$] (Figure 4.1a). A two-way repeated measures ANOVA of contralateral placements shows an effect of treatment [$F(1,14)=5.2$, $p=0.0385$], time [$F(1,14)=55$, $p<0.0001$], and an interaction between treatment and time [$F(1,14)=4.9$, $p=0.0445$]. Post hoc comparisons reveal reduced combined contralateral placements in WT PFF+AAV-treated animals ($p<0.0001$) and A53T PFF+AAV-treated animals ($p=0.0008$) at four weeks post-delivery compared to baseline. Post hoc comparisons also reveal lower scores for combined contralateral placements in the A53T group compared to WT group at baseline ($p=0.0050$) (Figure 4.1b). A two-way repeated measures ANOVA of % contralateral placements shows an effect of treatment [$F(1,14)=4.8$, $p=0.0450$] and time [$F(1,14)=13$, $p=0.0029$], and no interaction between treatment and time [$F(1,14)=0.079$, $p=0.7832$]. Post hoc comparisons reveal reduced % contralateral placements in WT ($p=0.0276$) and A53T-treated animals ($p=0.0167$) four weeks post-delivery compared to baseline (Figure 4.1c).

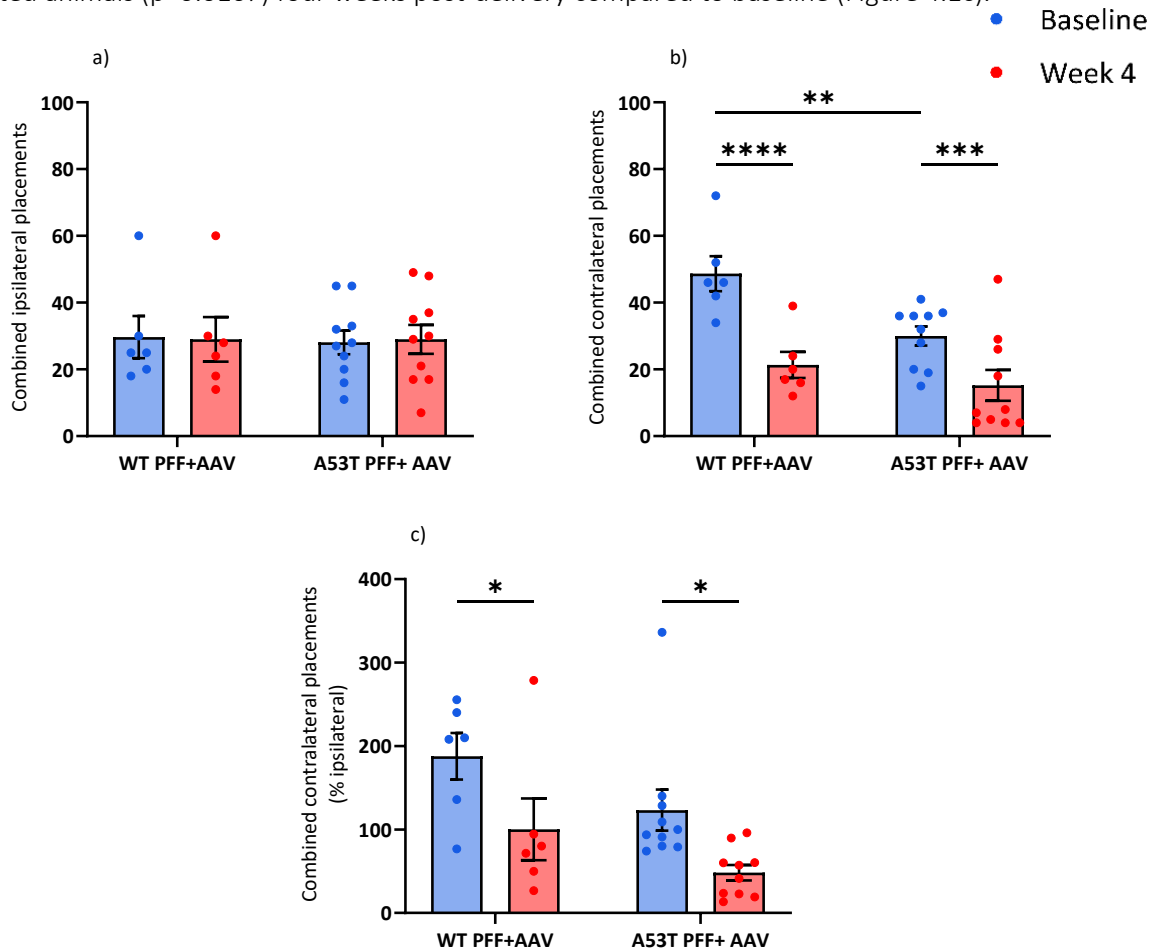


Figure 4.1. WT PFF+AAV and A53T PFF+AAV reduce the number of placements made with the forelimb contralateral to the injection four weeks after intranigral delivery. a) Combined placements made with the limb ipsilateral to the injection, b) combined placements made with the limb contralateral to the injection, c) combined contralateral placements expressed as a percentage of combined ipsilateral placements. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=6-10$. *= $p\leq 0.05$, **= $p\leq 0.01$, ***= $p\leq 0.001$, ****= $p\leq 0.0001$.

A two-way repeated measures ANOVA of ipsilateral wall placements shows no effect of treatment [$F(1,14)=0.0028$, $p=0.9588$], time [$F(1,14)=3.4$, $p=0.0858$], or interaction between treatment and time [$F(1,14)=0.076$, $p=0.7872$] (Figure 4.2a). A two-way repeated measures ANOVA of contralateral wall placements shows an effect of treatment [$F(1,14)=9.0$, $p=0.0096$], time [$F(1,14)=31$, $p<0.0001$], and an effect of interaction between treatment and time [$F(1,14)=5.9$, $p=0.0291$]. Post hoc comparisons reveal reduced contralateral wall placements in WT PFF+AAV-treated animals ($p=0.0002$) and A53T PFF+AAV-treated animals ($p=0.0229$) at four weeks post-delivery compared to baseline. Post hoc comparisons also reveal lower contralateral scores at baseline in the A53T group compared to the WT group ($p=0.0007$) (Figure 4.2b). A two-way repeated measures ANOVA of % contralateral wall placements shows an effect of treatment [$F(1,14)=8.7$, $p=0.0104$] and time [$F(1,14)=12$, $p=0.0037$], and no interaction between treatment and time [$F(1,14)=1.2$, $p=0.2953$]. Post hoc comparisons reveal a reduction in % contralateral wall placements at 4-weeks post-WT PFF+AAV administration compared to baseline ($p=0.0119$). Baseline scores for % contralateral wall placements are lower in the A53T PFF+AAV group than those in the WT PFF+AAV group ($p=0.0047$) (Figure 4.2c).

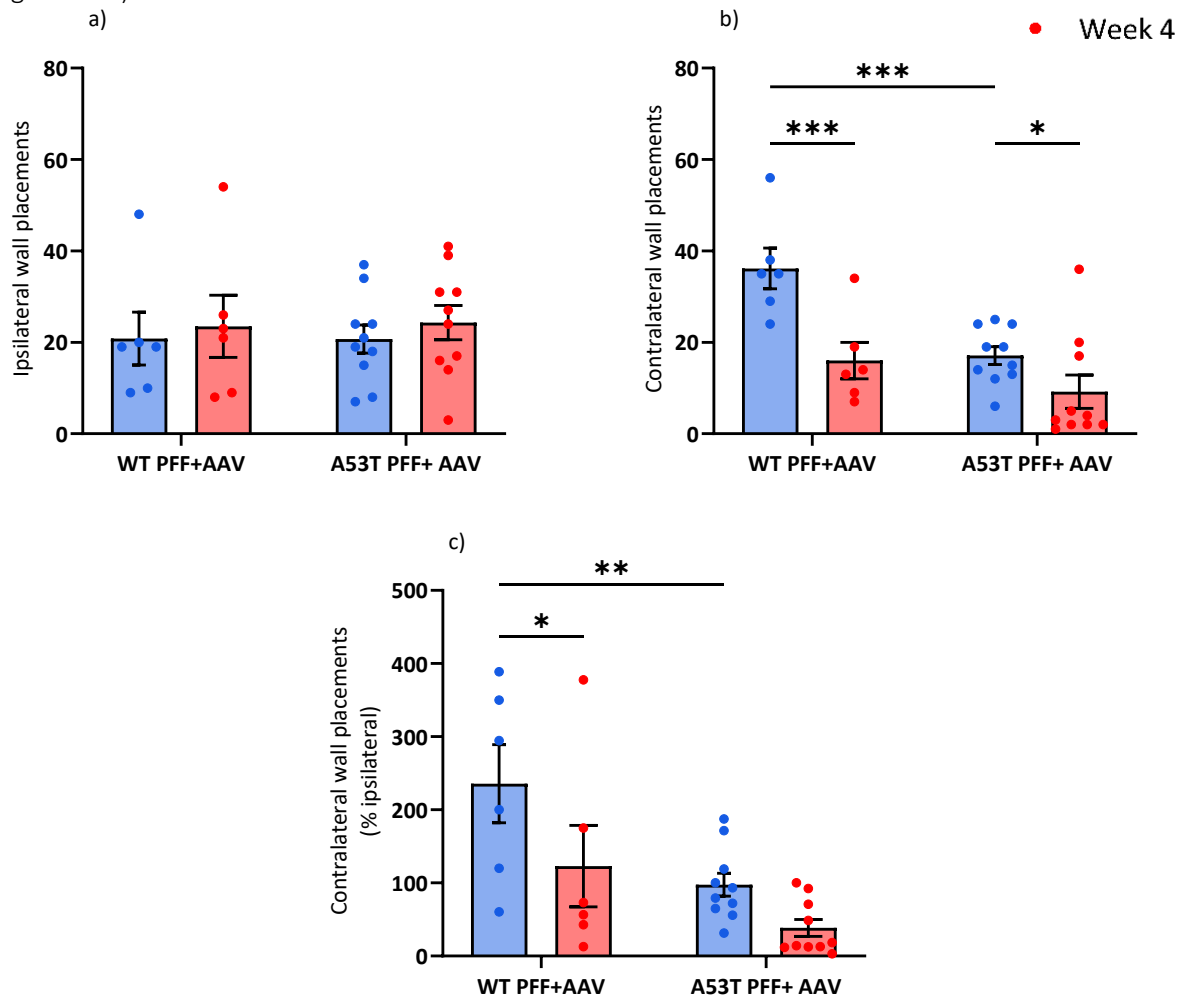


Figure 4.2. WT PFF+AAV and A53T PFF+AAV reduce the number of wall placements made with the forelimb contralateral to the injection four weeks after intranigral delivery. a) Wall placements made with the limb ipsilateral to the injection, b) wall placements made with the limb contralateral to the injection, c) contralateral wall placements expressed as a percentage of ipsilateral wall placements. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=6-10$. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

To account for within-animal variability, scores for ipsilateral and contralateral cylinder placements at 4-weeks are presented as a % of respective baseline scores. In both treatment groups, average scores for total ipsilateral placements made in the cylinder at four weeks remain within 5% of baseline scores (WT=99.78±11.23; A53T=103.73±10.83), while average scores for total contralateral placements at five weeks drop to approximately 45% of baseline scores (WT=43.92±6.36; A53T: 46.36±11.88). A two-way ANOVA of combined placements expressed as a % of baseline shows an effect no effect of treatment [$F(1,28)=0.076$, $p=0.7851$], an effect of laterality [$F(1,28)=24$, $p<0.0001$], and no interaction between treatment and laterality [$F(1,28)=0.0043$, $p=0.9482$]. Post hoc comparisons reveal reduced contralateral placements compared to ipsilateral placements in both the WT ($p=0.0050$) and A53T ($p=0.0004$) groups when data is expressed as a % of baseline (Figure 4.3a). Two-way ANOVA of wall placements expressed as % of baseline shows no effect of treatment [$F(1,28)=0.028$, $p=0.8692$], an effect of laterality [$F(1,28)=16$, $p=0.0004$], and no interaction between treatment and laterality [$F(1,28)=0.018$, $p=0.8935$]. Post hoc comparisons reveal reduced contralateral placements compared to ipsilateral placements in both WT ($p=0.0130$) and A53T ($p=0.0034$) groups, when data is expressed as % of baseline (Figure 4.3b).

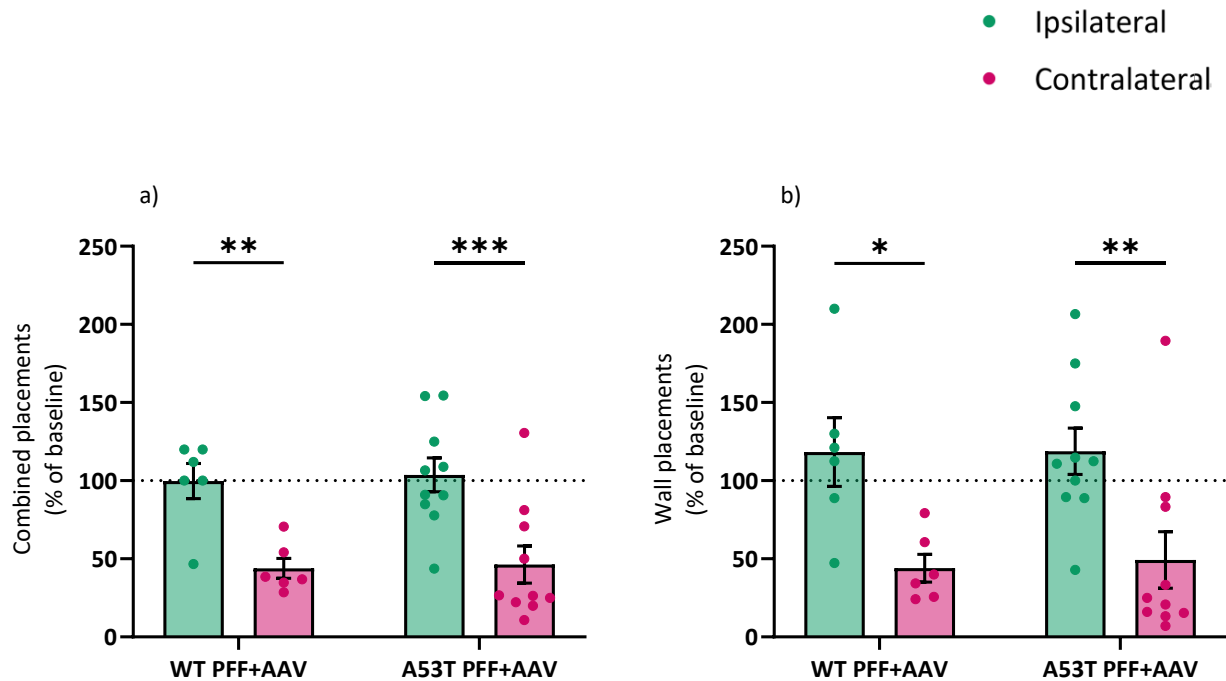


Figure 4.3. WT PFF+AAV and A53T PFF+AAV reduce contralateral forelimb-use in the cylinder test four weeks after intranigral delivery. a) Combined placements expressed as a percentage of baseline, b) wall placements expressed as a percentage of baseline. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=6-10$. *= $p\leq 0.05$, **= $p\leq 0.01$, ***= $p\leq 0.001$.

4.5.2. Stepping test

A two-way repeated measures ANOVA of combined ipsilateral steps shows no effect of treatment [$F(1,14)=0.069$, $p=0.7970$], time [$F(1,14)=1.3$, $p=0.2675$], or interaction between treatment and time [$F(1,14)=0.13$, $p=0.7247$] (Figure 4.4a). A two-way repeated measures ANOVA of combined contralateral steps shows no effect of treatment [$F(1,14)=2.9$, $p=0.1118$], an effect of time [$F(1,14)=44$, $p<0.0001$], and no interaction between treatment and time [$F(1,14)=0.65$, $p=0.4346$]. Post hoc comparisons reveal reduced combined contralateral steps in WT PFF+AAV ($p=0.0024$) and A53T PFF+AAV (<0.0001) groups at week 5 compared to baseline (Figure 4.4b). A two-way repeated measures ANOVA of % contralateral steps shows no effect of treatment [$F(1,14)=2.9$, $p=0.1096$], an effect of time [$F(1,14)=56$, $p<0.0001$], and no interaction between treatment and time [$F(1,14)=3.4$, $p=0.0881$]. Post hoc comparisons reveal reduced % contralateral steps in both WT PFF+AAV ($p=0.0029$) and A53T PFF+AAV (<0.0001) groups at week 5 compared to baseline (Figure 4.4c).

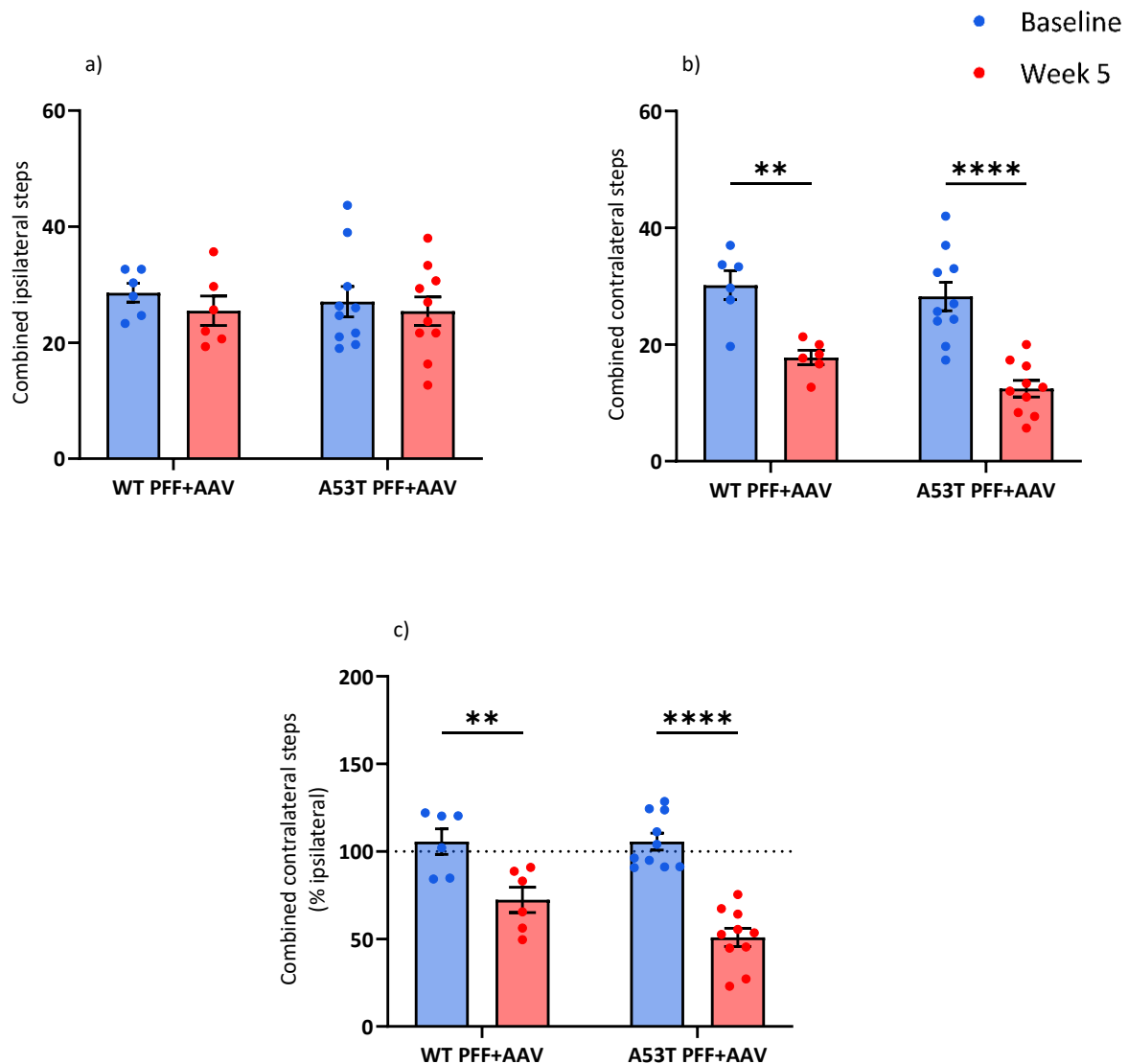


Figure 4.4. WT PFF+AAV and A53T PFF+AAV reduce the number of steps made with the forelimb contralateral to the injection five weeks after intranigral delivery. a) Combined steps made with the limb ipsilateral to the injection, b) combined steps made with the limb contralateral to the injection, c) combined contralateral steps expressed as a percentage of combined ipsilateral steps. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=6-10$. **= $p \leq 0.01$, ****= $p \leq 0.0001$.

A two-way repeated measures ANOVA of forward ipsilateral steps shows no effect of treatment [$F(1, 14)=0.086$, $p=0.7741$], time [$F(1, 14)=3.0$, $p=0.1035$], or interaction between treatment and time [$F(1, 14)=0.42$, $p=0.525$] (Figure 4.5a). A two-way repeated measures ANOVA of forward contralateral steps shows no effect of treatment [$F(1, 14)=1.0$, $p=0.3323$], an effect of time [$F(1, 14)=38$, $p<0.0001$], and no interaction between treatment and time [$F(1, 14)=0.11$, $p=0.7466$]. Post hoc comparisons reveal reduced forward contralateral steps 5-weeks after WT PFF+AAV ($p=0.0011$) and A53T PFF+AAV ($p=0.0003$) delivery compared to baseline (Figure 4.5b). Two-way repeated measures ANOVA of % contralateral forward steps shows no effect of treatment [$F(1, 14)=1.9$, $p=0.1911$], an effect of time [$F(1, 14)=20$, $p=0.0006$], and no interaction between treatment and time [$F(1, 14)=1.2$, $p=0.2871$]. Post hoc comparisons reveal reductions in % contralateral steps five weeks after delivery of WT PFF+AAV ($p=0.0035$) and A53T PFF+AAV ($p=0.0165$) compared to baseline (Figure 4.5c).

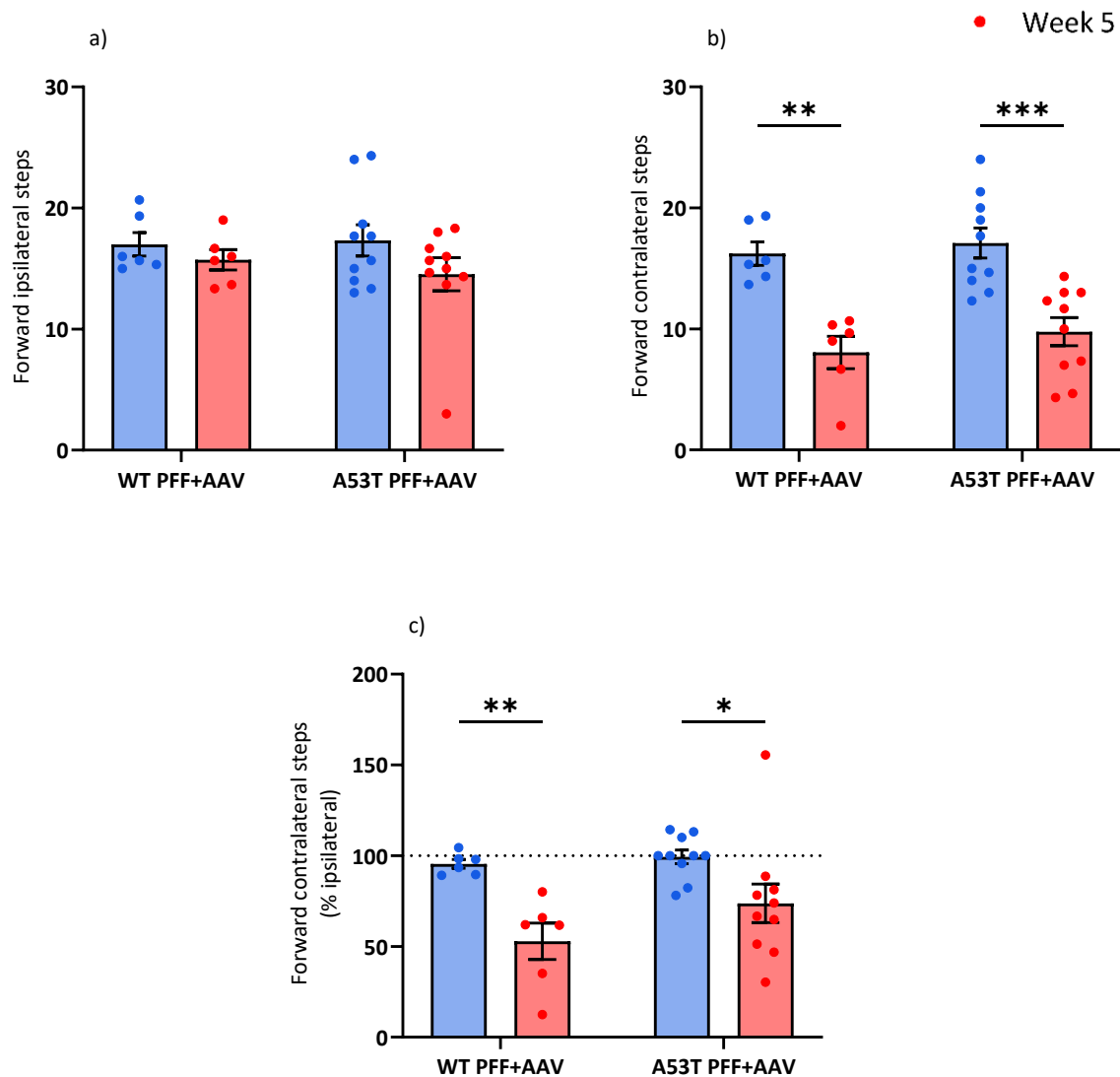


Figure 4.5. WT PFF+AAV and A53T PFF+AAV reduce the number of forwards steps made with the forelimb contralateral to the injection five weeks after intranigral delivery. a) Forward steps made with the limb ipsilateral to the injection, b) forward steps made with the limb contralateral to the injection, c) forward contralateral steps expressed as a percentage of forward ipsilateral steps. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=6-10$. *= $p\leq 0.05$, **= $p\leq 0.01$, ***= $p\leq 0.001$.

The average ipsilateral stepping score five weeks after intranigral injection remain within a 17% range of baseline combined steps (WT PFF+AAV=89.42±7.609; A53T PFF+AAV=89.42±7.609) (Figure 4.6a), and within approximately a 15% range of baseline forward steps alone (WT PFF+AAV=93.35±5.251; A53T PFF+AAV=77.93±10.45) (Figure 4.6c). Combined contralateral steps are reduced to approximately 50-60% of baseline in both groups (WT PFF+AAV=62.42±9.802; A53T PFF+AAV=46.09±5.513). Forward steps also drop to approximately 50% of baseline scores in both groups (WT PFF+AAV=50.34±9.320; A53T PFF+AAV=59.35±7.290). A two-way ANOVA of combined steps expressed as a % of baseline shows no effect of treatment [$F(1, 28)=0.19$, $p=0.6675$], an effect of laterality [$F(1, 28)=19$, $p=0.0001$], and no interaction between treatment and laterality [$F(1, 28)=1.9$, $p=0.1750$]. Post hoc comparisons reveal reduced scores for contralateral steps as % of baseline compared to ipsilateral steps as % of baseline in A53T group ($p<0.0001$) (Figure 4.6a). A two-way ANOVA of forward steps expressed as a % of baseline shows no effect of treatment [$F(1, 28)=0.12$, $p=0.7325$], an effect of laterality [$F(1, 28)=11$, $p=0.0025$], and no interaction between treatment and laterality [$F(1, 28)=1.7$, $p=0.1983$]. Post hoc comparisons reveal reduced scores for contralateral steps as a % of baseline compared to ipsilateral steps as a % of baseline in the WT group ($p=0.0066$) (Figure 4.6b).

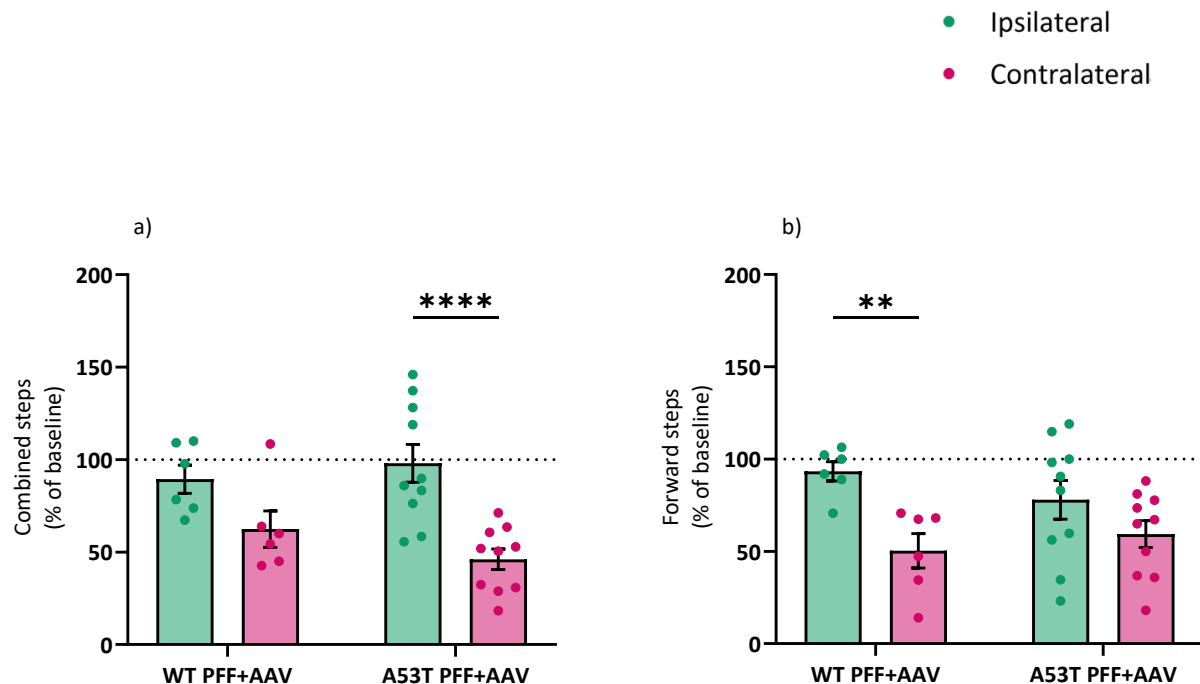


Figure 4.6. Reduced combined contralateral steps in A53T PFF+AAV-treated animals and reduced forward contralateral steps in WT PFF+AAV-treated animals when steps are presented as a % of baseline. a) Combined steps expressed as a percentage of baseline, b) forward steps expressed as a percentage of baseline. Data is expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=6-10$. **= $p\leq 0.01$, ****= $p\leq 0.0001$.

4.5.3. Amphetamine-induced rotations test

An unpaired t-test of rotational scores in the amphetamine-induced rotations test shows no differences in the number of ipsilateral rotations between WT and A53T groups ($p=0.33$) (Figure 4.7a). An unpaired t-test shows greater contralateral rotations in A53T PFF+AAV-treated animals compared to WT ($p=0.0247$) (Figure 4.7b). A two-way ANOVA of number of rotations in each direction (laterality) shows an effect of treatment [$F(1,26)=6.163$, $p=0.0198$], no effect of laterality [$F(1,26)=0.8264$, $p=0.3717$], and no interaction between treatment and laterality [$F(1,26)=1.032$, $p=0.3190$]. Post hoc comparisons reveal greater contralateral rotations in A53T-treated animals compared to contralateral rotations in WT-treated animals ($p=0.0202$) (data presented in Figures 4.7a and 4.7b). An unpaired t-test shows greater total rotations in A53T PFF+AAV-treated animals compared to WT ($p=0.0054$) (Figures 4.7c). No differences between net ipsilateral rotations of WT and A53T groups were observed ($p=0.4132$) (Figure 4.7d).

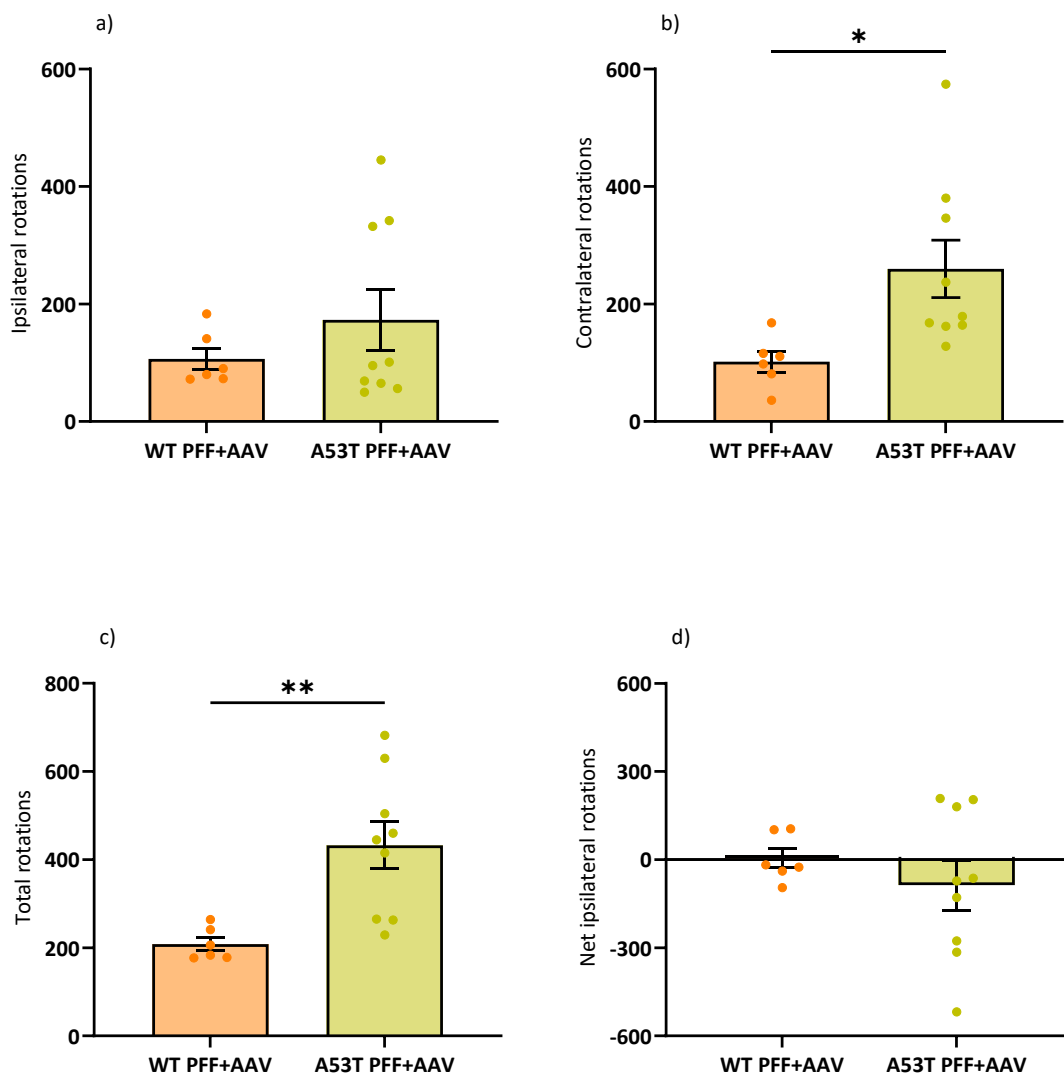


Figure 4.7. A53T PFF+AAV increases total rotations and rotations in the direction contralateral to the lesion in the amphetamine-induced rotations test six weeks after intranigral delivery. a) Number of ipsilateral rotations, b) number of contralateral rotations, c) total number of total rotations, d) net ipsilateral rotations. Test duration of 90 minutes. Data are expressed as mean $\pm$ SEM. Unpaired t-test. $n=6-10$. *= $p \leq 0.05$, **= $p \leq 0.01$.

4.5.4. pSer129 immunofluorescence

A two-way ANOVA of pSer129 immunofluorescence shows an effect of treatment [$F(1,24)=5.249$, $p=0.0310$], laterality [$F(1,24)=42.18$, $p<0.0001$], and an interaction between treatment and laterality [$F(1,24)=4.549$, $p=0.0434$]. Post hoc comparisons reveal increased pSer129 immunofluorescence in the ipsilateral hemisphere of WT-treated animals compared to the contralateral hemisphere ($p=0.0081$), and in the ipsilateral hemisphere of A53T-treated animals compared to the contralateral hemisphere ($p<0.0001$). Post hoc comparisons reveal greater pSer129 immunofluorescence in the ipsilateral hemisphere of the A53T group compared to their WT counterparts ($p=0.0046$) (Figure 4.8). Mann-Whitney U-test does not distinguish between the WT and A53T groups when pSer129 immunofluorescence is expressed as a % of contralateral hemisphere ($p=0.5728$) (Figure 4.8b).

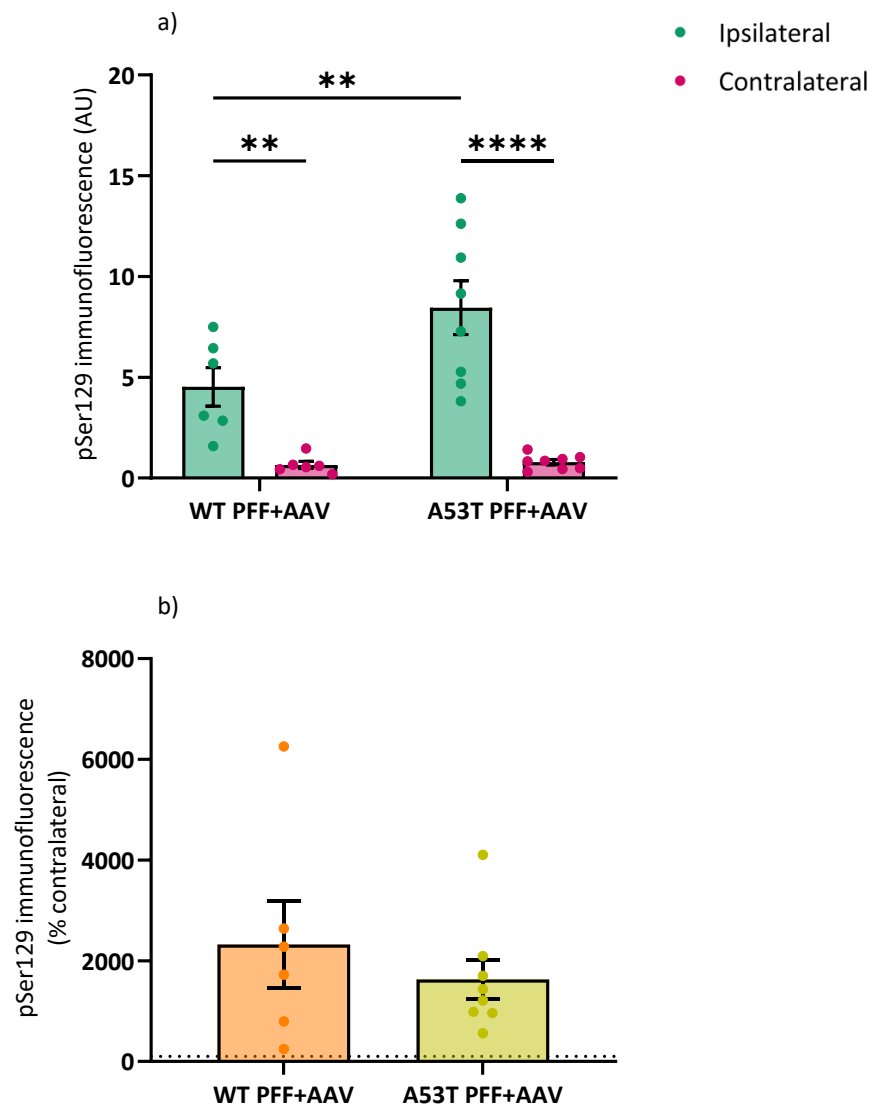


Figure 4.8. WT PFF+AAV and A53T PFF+AAV increases pSer129+ α Syn immunoreactivity in the ipsilateral hemisphere six weeks after intranigral delivery. a) Mean grey value of pSer129 immunofluorescence in the ipsilateral and contralateral substantia nigra, b) Mean grey value of pSer129 immunofluorescence in the ipsilateral hemisphere expressed as a percentage of the contralateral hemisphere. Data is expressed as mean $\pm$ SEM. a) Two-way ANOVA with Fisher's test for multiple comparisons, b) Mann-Whitney U test. $n=6-10$. **= $p\leq 0.01$, ****= $p\leq 0.0001$.

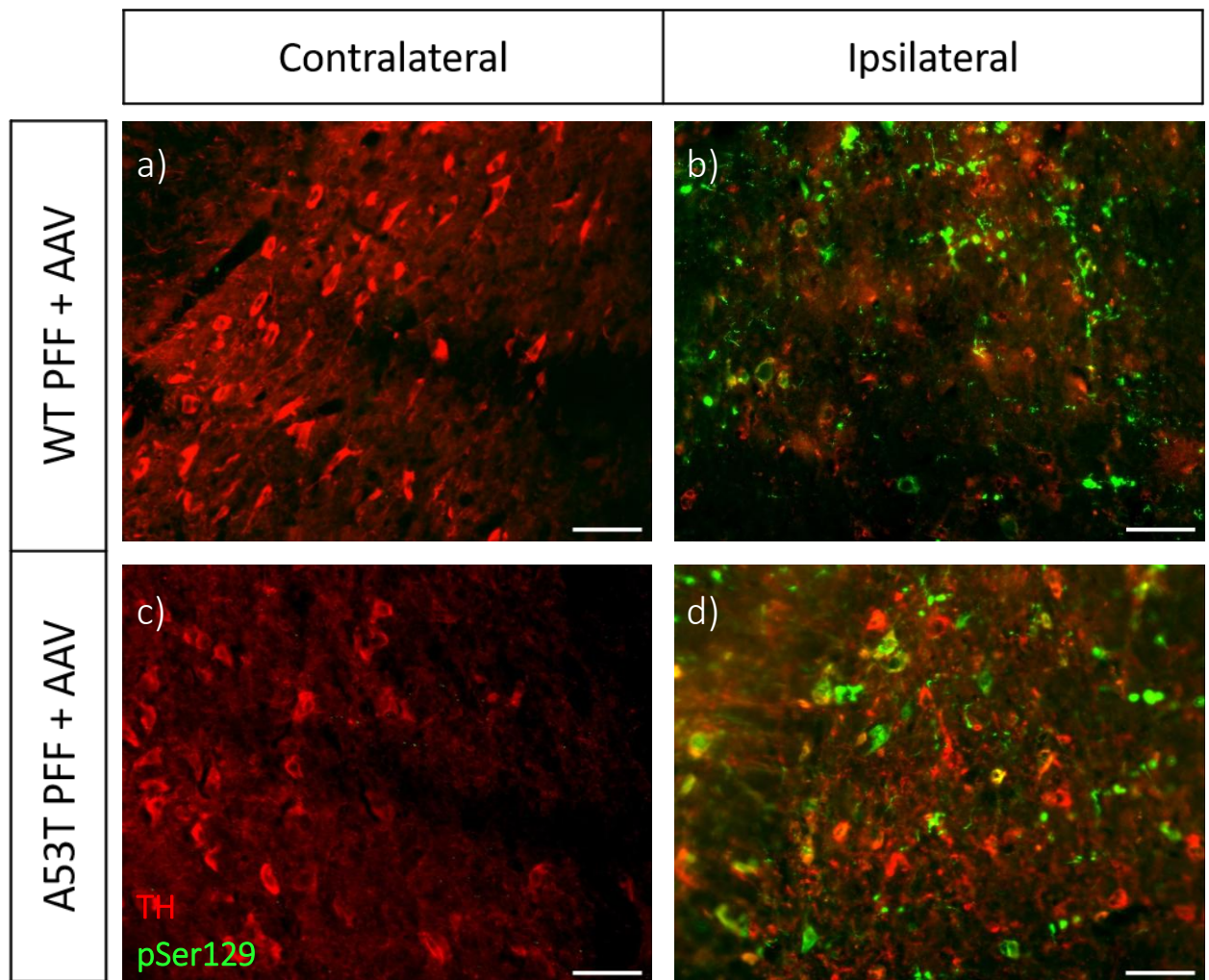


Figure 4.9. pSer129+ α Syn immunofluorescence is increased in the ipsilateral substantia nigra six weeks after intranigral delivery of WT PFF+AAV and A53T PFF+AAV. Brain tissue was cryosectioned and fluorescently stained for pSer129+ α Syn and TH to confirm location in the substantia nigra. Representative 20x images show pSer129+ staining in green and TH+ staining in red in the a) contralateral hemisphere of WT PFF + AAV, b) ipsilateral hemisphere of WT PFF + AAV, c) contralateral hemisphere of A53T PFF + AAV, and d) ipsilateral hemisphere of A53T PFF + AAV. Scale bar=50 μ m.

4.5.5. Iba1 immunohistochemistry

A paired t-test shows an increase in Iba1+ cells in the ipsilateral hemisphere of A53T PFF+AAV-treated animals compared to the contralateral hemisphere ($p=0.0001$). Only 1 animal brain was available for Iba1 immunostaining in the WT PFF+AAV group, precluding further statistical analysis from being conducted on this dataset. Results from this animal are included in the Figure for reference, whilst not included in the analysis (Figure 4.10a). While statistical analysis cannot be used to compare % Iba1+ cell counts of WT-treated animals to A53T-treated animals, Iba1+ cell counts in the ipsilateral hemisphere of A53T-treated animals are increased by approximately 300% of contralateral hemisphere (316 ± 38.45) (Figure 4.10b).

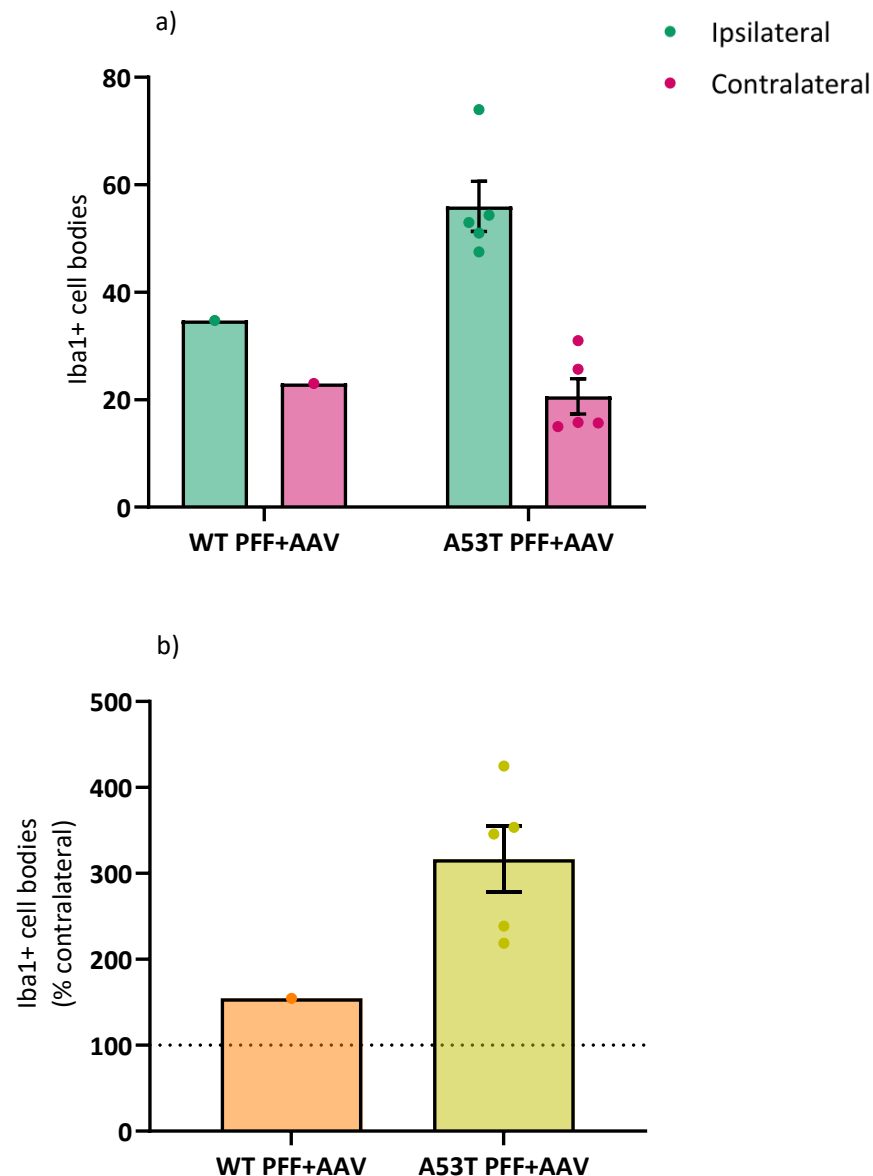


Figure 4.10. A53T PFF+ AAV increases the number of Iba1+ microglia in the ipsilateral hemisphere six weeks after intranigral delivery. a) Number of Iba1+ cells in the ipsilateral and contralateral substantia nigra. b) Number of Iba1+ cells in the ipsilateral hemisphere expressed as a percentage of Iba1+ cells on the contralateral hemisphere. A single WT-treated animal is included in the figure but excluded from statistical analysis. a) Unpaired t-test comparing cell counts in ipsilateral and contralateral hemisphere of A53T PFF+AAV group. $n=1-5$. ***= $p \leq 0.001$.

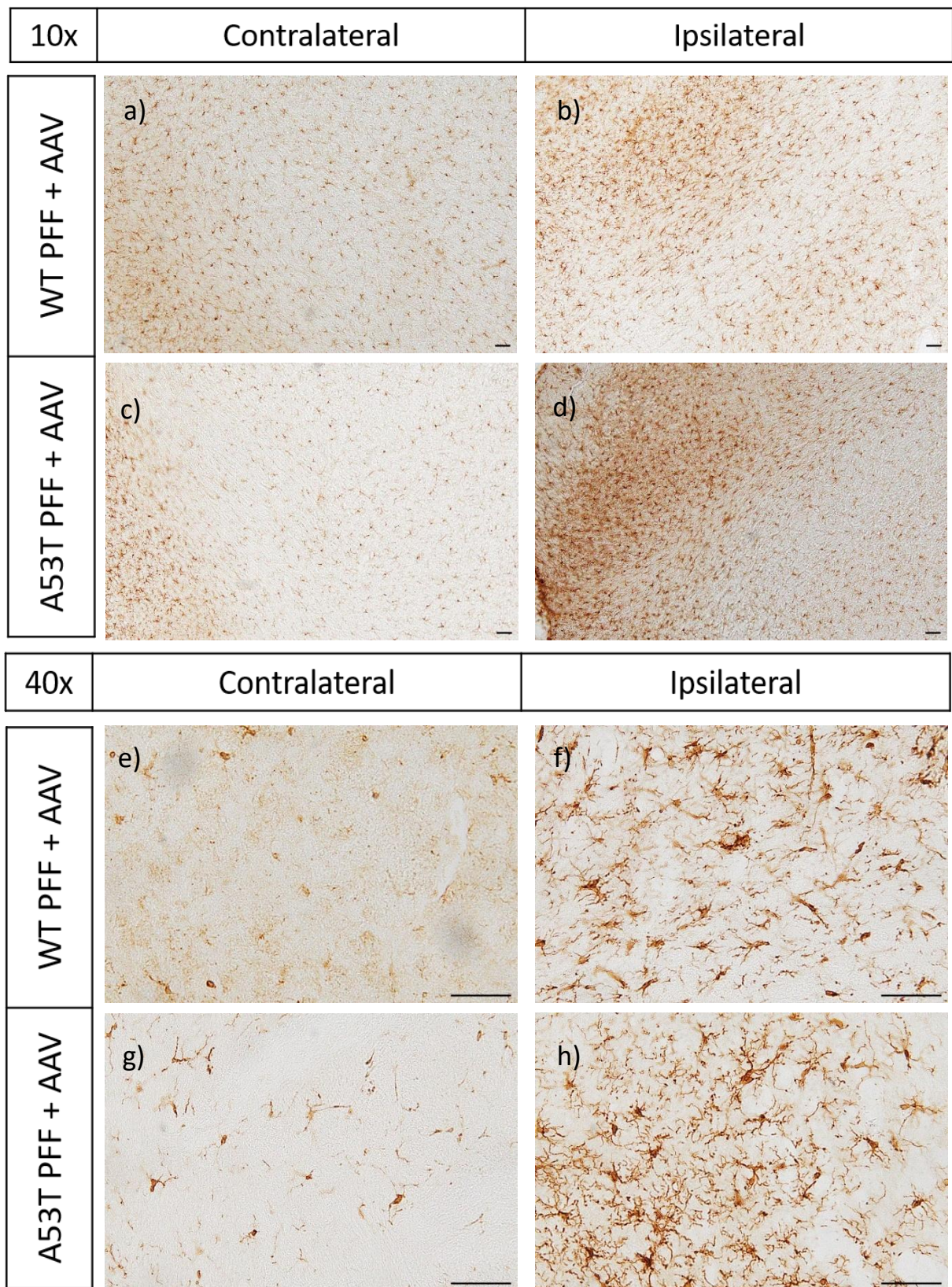


Figure 4.11: Iba1+ microglial cells are increased in the ipsilateral substantia nigra six weeks after intranigral delivery of A53T PFF+AAV. Brain tissue was cryosectioned and immunohistochemically stained for Iba1+ in the substantia nigra to quantify microglial cell bodies. Representative 10x (top panels) and 40x (bottom panels) images show Iba1+ immunoreactivity in the a,e) contralateral hemisphere of WT PFF+AAV, b,f) ipsilateral hemisphere of WT PFF+AAV, c,g) contralateral hemisphere of A53T PFF + AAV, and d,h) ipsilateral hemisphere of A53T PFF+AAV. Scale bar=50µm.

4.5.6. TH Immunohistochemistry

A two-way ANOVA of TH+ cell bodies shows an effect of treatment [$F(1, 28)=9.3$, $p=0.0050$] and laterality [$F(1, 28)=8.4$, $p=0.0072$], and no interaction between treatment and laterality [$F(1, 28)=2.6$, $p=0.1163$]. Post hoc comparisons reveal a reduction in TH+ cell bodies in the ipsilateral hemisphere of A53T-treated animals compared to the contralateral hemisphere ($p=0.0010$), and compared to the ipsilateral hemisphere of WT-treated animals ($p=0.0026$) (Figure 4.12a). While TH+ cell counts in the ipsilateral hemisphere of A53T-treated animals fall to about 75% of the contralateral hemisphere, a student's unpaired t-test shows no differences between A53T- and WT-treated groups ($p=0.0994$) (Figure 4.12b).

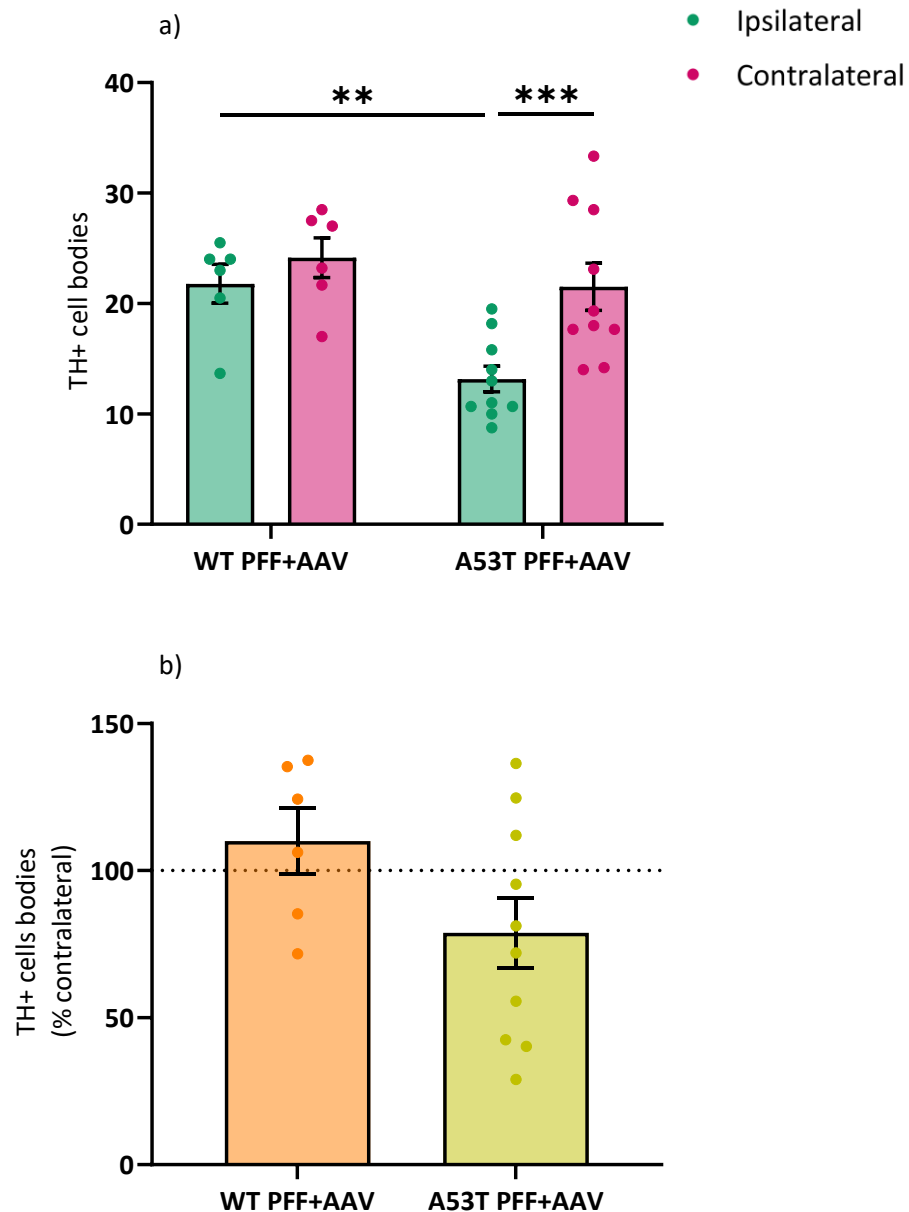


Figure 4.12. A53T PFF+AAV reduces TH+ cell bodies in the ipsilateral substantia nigra six weeks after intranigral delivery. a) Number of TH+ cells in the ipsilateral and contralateral substantia nigra. b) Number of TH+ cell bodies in the ipsilateral hemisphere expressed as a percentage of TH+ cells on the contralateral hemisphere. Data is expressed as mean $\pm$ SEM. a) Two-way ANOVA with Fisher's test for multiple comparisons. b) Unpaired t-test. $n=6-10$. **= $p \leq 0.01$, ***= $p \leq 0.001$.

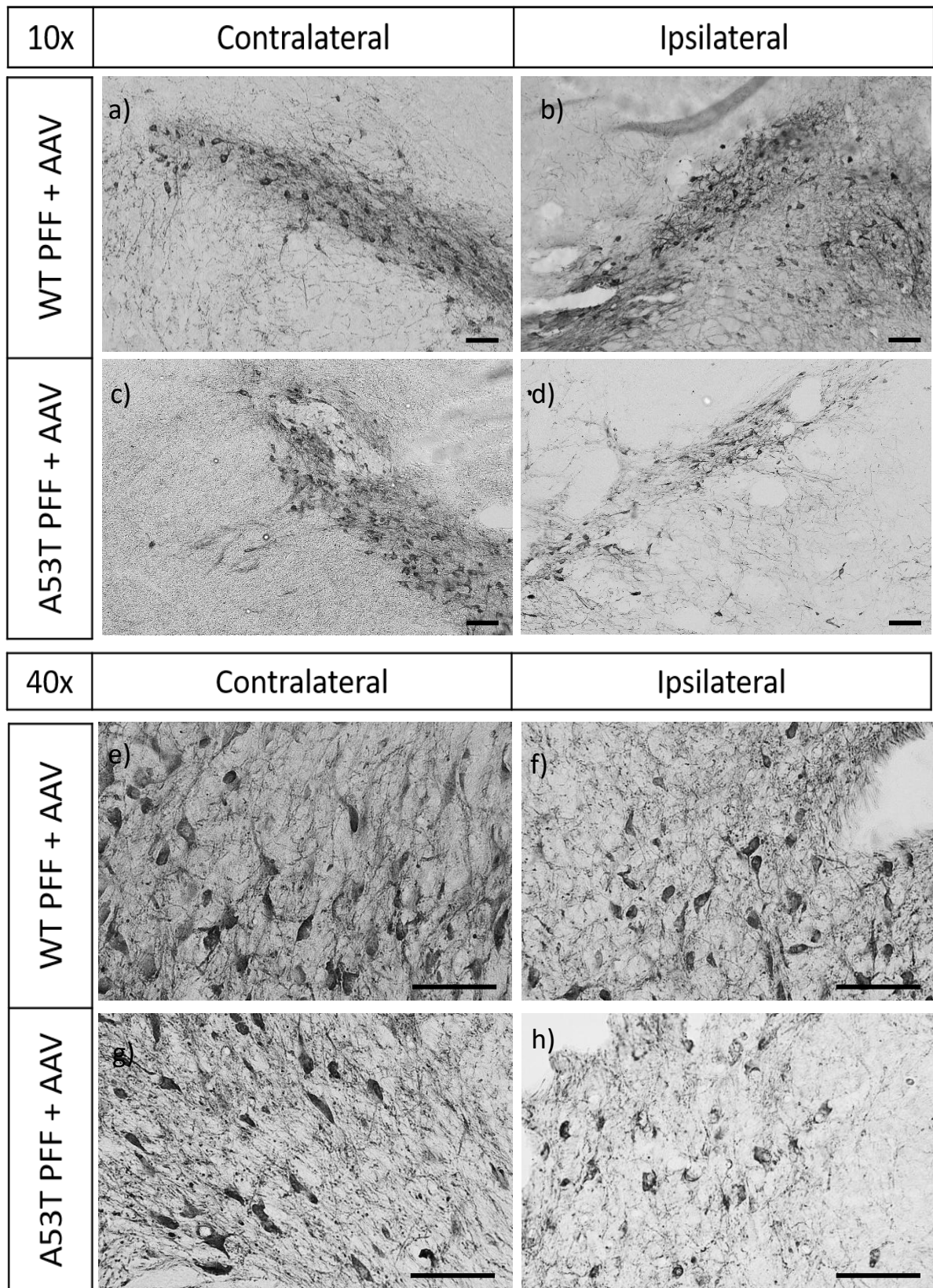


Figure 4.13. TH+ dopaminergic neuronal cell bodies are reduced in the ipsilateral substantia nigra six weeks after intranigral delivery of A53T PFF+AAV. Brain tissue was cryosectioned and immunohistochemically stained for TH in the substantia nigra to quantify dopaminergic neuronal cell bodies. Representative 10x (top panels) and 40x (bottom panels) images show TH+ immunoreactivity in the a,e) contralateral hemisphere of WT PFF + AAV, b,f) ipsilateral hemisphere of WT PFF + AAV, c,g) contralateral hemisphere of A53T PFF + AAV, and d,h) ipsilateral hemisphere of A53T PFF + AAV. Scale bar=50µm.

4.6. Discussion

In the previous study, it was shown that WT PFFs alone can seed phosphorylation of endogenous α Syn and increase the number of Iba+ microglia in the ipsilateral substantia nigra six weeks after unilateral intranigral injection. These cellular changes were not complimented by changes in the number of TH+ dopaminergic neurons in the substantia nigra. The addition of AAV vector-expressing WT α Syn further drove α Syn phosphorylation and Iba1+ immunoreactivity, but did not affect the number of TH+ dopaminergic neurons. A53T PFFs alone did not seed phosphorylation of endogenous α Syn, or induce changes to Iba1+ microglial or TH+ dopaminergic neuronal cell numbers in the substantia nigra. The addition of AAV vector expressing A53T-mutant α Syn promoted α Syn phosphorylation, and was sufficient to increase the number of Iba1+ microglia and reduce the number of TH+ dopaminergic neurons in the ipsilateral substantia nigra.

In the cylinder test, use of the limb ipsilateral to the lesion was unaffected by treatment with either WT PFF+AAV or A53T PFF+AAV. While baseline scores of A53T-treated animals differed from those of WT, both groups displayed reduced contralateral limb-use while exploring the cylinder four weeks after intranigral injection compared to baseline. It is difficult to determine the root cause of baseline variability in behavioural tasks. In the cylinder test, animals were exposed to the testing environment for the first time on the day that baseline measures were scored. For this reason, habituation to the task is not contributing issue. Baseline scores for all animals were recorded on the same day, which minimizes the likelihood that variability resulted from altered environmental conditions (i.e. day-to-day variation of room temperature, humidity, background noise, or lighting conditions). The experimenter remained consistent between animals, with every attempt made to control for environmental variability, thus exposing all animals to the same conditions. Cylinder test scores can be confounded if rats receive distracting olfactory cues in the testing environment. All females are tested in separate cylinders to males to prevent olfactory crossover, and the apparatus are thoroughly wiped with 70% EtOH between animals to minimize distraction. In some animal experiments, variability in baseline test scores result simply from natural animal variability. Care was taken to control for all conditions within the circle of influence of the experimenter.

Expressing the number of contralateral placements as a percentage of ipsilateral placements is a measure used to assess bias towards the use of one limb over the other in this test. A bias for use of the contralateral limb during baseline cylinder exploration is evident in the WT group, confounding interpretation of results. Nonetheless, the extent of the reduction in contralateral limb-use relative to ipsilateral limb-use at week four indicates that, over time, unilateral WT PFF+AAV treatment impacts contralateral forelimb function, such that the natural bias for contralateral limb-use is reduced. A53T-treated animals also showed a contralateral bias in the cylinder test at baseline, but this behaviour was clearly disrupted by week four, at which point relative contralateral limb-use dropped from an average of 120% to 48% of ipsilateral limb-use. Scores for wall placements in isolation illustrate similar patterns of stable ipsilateral limb-use, partnered with reduced contralateral limb-use in both WT and A53T-treated groups, albeit interpretation is somewhat confounded by uneven baseline scores. The baseline bias towards contralateral limb-use in WT-treated animals is also evident in scores for wall placements alone, though the bias appears to be reduced four weeks after treatment. A53T-treated animals displayed equal preference for limb-use in wall placement in the cylinder test at baseline. Four weeks after treatment, contralateral wall placements dropped to approximately 40% of ipsilateral placements, indicating a trend towards reduced contralateral forelimb-use when rearing in the cylinder, albeit this result did not reach

statistical significance. In both groups, combined ipsilateral placement scores at week four remained within 1% of baseline scores, indicative of preserved ipsilateral function. Combined contralateral placement scores, however, dropped to approximately 45% of baseline. Wall placement behaviours in isolation show similar patterns of stable (if not increased) ipsilateral function over time, with reduced contralateral limb-use associated with both treatment groups. These results indicate the selective impact of unilateral PFF+AAV delivery on contralateral, but not ipsilateral, limb motor function. WT and A53T treatments could not be further distinguished with the data obtained from this behavioural test.

In the stepping test, combined ipsilateral limb function remained unaffected over time following intranigral delivery of PFF+AAV. Reduced functional use of the limb contralateral to the hemisphere in which PFF+AAV was delivered was apparent in the stepping test five weeks after intranigral delivery of both WT PFF+AAV and A53T PFF+AAV treatments. The balanced use of the left and right limbs displayed at baseline was affected five weeks after delivery of both WT and A53T treatments. At week five, animals in both groups displayed a reduced ability to step with their contralateral limb. Scores for forward stepping in isolation were also indicative of unilateral akinesia imposed by WT and A53T PFF+AAV administration. To control for intra-animal performance variability over time, week five stepping scores from each limb were expressed as a percentage of respective baseline scores. In both groups, the average ipsilateral stepping score at week five remained within a 17% range of average baseline scores for both combined stepping and forward stepping alone. Contralateral limb-use at week five, however, dropped to approximately 50% of baseline scores in both scoring categories in both treatment groups. While both treatments produced a motor deficit in the limb contralateral to the injection, no differences between WT and A53T treatments were observed in this behavioural test.

The amphetamine-induced rotations test was employed to identify changes in motor function attributable to dopaminergic neuronal degeneration in the substantia nigra six weeks after delivery of either WT PFF+AAV or A53T PFF+AAV. Traditionally, rats with an induced unilateral lesion portray increased number of rotations in the direction ipsilateral to the lesion, following systemic amphetamine administration. Compared to the WT combination, the number of ipsilateral rotations in the A53T group was trending towards an increase. More apparent, however, was the increase in contralateral rotations in the A53T combination group in response to a systemic amphetamine challenge, compared to its WT counterpart. Having driven more observable changes in both ipsilateral and contralateral rotations relative to WT, it is reasonable to suggest that A53T PFF+AAV treatment induced more pronounced cellular and/or biomolecular changes along the pathways which underlie behaviour induced by the amphetamine injection. These changes are likely explained by alteration to the quantity of soluble dopamine in the striatum and the quality of nigral nerve terminals projecting to the striatum. The outcomes observed in this experiment may indicate evidence of a partial lesion associated with early stage α Syn pathology, where contralateral rotational behaviour reflects a transient rise in striatal dopaminergic content just before the onset of overt nigral degeneration and neuronal death, which may have progressed after the test was conducted.

Prior to overt dopamine depletion, antithetical fluctuations in striatal dopamine content are apparent in early stages of PD in human patients (Domenger & Grimm, 2019), and rodent models of synucleinopathy (Daniel et al., 2015; Decressac et al., 2012a; Ip et al., 2017; Koprach et al., 2011; Rodriguez-Perez et al., 2018; Song et al., 2015b). Findings from research with four-month-old

transgenic mice overexpressing α Syn have shown surges in striatal dopamine content before the onset of characteristic depletion of axonal terminals in the substantia nigra or striatum (Lam et al., 2011). While amphetamine administration at the point of overt nigral degradation would likely be characterised by ipsilateral rotational behaviour, the point of striatal dopamine surge would likely be characterised by an increase in contralateral rotation behaviour, should the amphetamine rotation test have been conducted in this study. This boosted in striatal dopamine turnover has also been observed before degeneration of dopaminergic neuronal cells in the nigra in Sprague Dawley rats, four weeks four weeks after intranigral delivery of AAV expressing A53T- α Syn. DAT binding has also been shown to fluctuate before eventual dopamine depletion (Koprich et al., 2011).

Our findings of increased contralateral rotations following amphetamine administration support a hypothesis that early stages of α Syn pathology, when induced via A53T PFF+AAV, may be associated with a paradoxical increase in striatal dopamine just prior to characteristic depletion. It is unknown, however, how long this transient period of contralateral rotation lasts before reports from the amphetamine-induced rotations task reflect overt neurodegenerative pathology. At the cellular level, proliferation of dopamine receptors has frequently been proposed as the mechanism responsible for behavioural super-sensitivity and recovery of function (Bjorklund & Dunnett, 2019; Labandeira-Garcia et al., 1996). At the time the test was conducted, rats in the present study may have been undergoing a paradoxical stage of neuropathy, where increased contralateral rotation reflected a transient rise in unilateral striatal dopaminergic content. This output was not directly measured in the present investigation, but results suggest it could be a possibility explanation. In future assessments, amendments to the timeline of behavioural examination can be made to reflect various stages along the trajectory of dopamine fluctuation which may occur before dopaminergic degeneration and cell death.

Unilateral behavioural deficits were complimented by extensive pSer129+ immunoreactivity in the ipsilateral substantia nigra of both WT and A53T combination-treated animals. In comparison to WT PFF alone, WT PFF+AAV treatment resulted in greater distribution of pSer129+ immunoreactivity in the substantia nigra. pSer129+ inclusions were co-localised with TH+ neurons in the nigra, and distributed throughout the midbrain in ribbon-like assemblies. Strikingly, although A53T PFFs did not drive phosphorylation of α Syn when delivered alone, extensive pSer129+ immunoreactivity was observed throughout the substantia nigra and midbrain following delivery of the A53T PFF+AAV combination. Both intraneuronal pSer129 expression and ribbon-like structures, which appear extracellularly, were evident across the substantia nigra and midbrain at greater levels than those observed in the WT PFF+AAV combination group.

pSer129+ immunofluorescence in the ipsilateral substantia nigra was accompanied by an increase in the number of Iba1+ microglia, and a reduction in the number of TH+ neurons, six weeks following delivery of A53T PFF+AAV combination treatment. Though WT combination did not induce TH+ neuronal cell loss, a conclusion cannot be made with respect to a treatment-induced microglial response, as only one animal was available for Iba1+ immunostaining. In that one animal, an increased number of Iba1+ microglia was apparent in the substantia nigra of the injected hemisphere, while microglial cell counts in the noninjected hemisphere appeared comparable to naïve animals. Our findings align with those reported by Karampetsou and colleagues, who show a propensity for both WT and pSer129+ α Syn PFFs to drive seeding of endogenous α Syn, but only pSer129+ PFFs to trigger Lewy body-like aggregation, nigral dopaminergic neuronal loss, and fine motor impairment at 60 days post intra-striatal administration in mice (Karampetsou et al., 2017).

Together, these findings suggest an association between Iba1+ microgliosis with TH+ cell loss and the presence of pSer129+ α Syn.

The observation of contralateral behavioural changes in the stepping and cylinder tests following delivery of the WT PFF+AAV combination treatment, in the absence of dopaminergic degeneration, indicates that these contralateral limb-use measures do not directly relay the consequences of dopaminergic cell death. Classic rodent toxin models of PD-like neurodegeneration typically demonstrate the dependency of motor dysfunction on TH+ cell loss. Conversely, α Syn models have demonstrated evidence of the onset of motor disturbance before overt nigral neurodegeneration or Lewy body pathology (Decressac et al., 2012b; Gombash et al., 2013; Koprach et al., 2011). Injection of α Syn PFFs into the rat striatum has been reported to produce overt striatal accumulation of pSer129+ inclusions 4, 8, and 24 weeks-post-injection without driving striatal neuronal cell loss (Paumier et al., 2015). These findings further support a theory that α Syn aggregates are not directly toxic (at least within this timeframe and under these conditions), or alternatively, that various cellular populations show varying levels of vulnerability to cytotoxic α Syn accumulation.

Prior to the present investigation, it was confirmed that, when delivered alone, the AAV vector tools were nontoxic to TH+ dopaminergic neurons in the rodent substantia nigra (Koprach et al., 2010) (Appendix I). Unilateral delivery of AAV1/2 expressing human A53T α Syn induced pSer129+ immunoreactivity (Figure A1.1, A1.2) and increased Iba1+ microglial cell counts (Figure A1.1, A1.3), although pathology observed six weeks following intranigral delivery was less apparent compared to that observed in the A53T PFF+AAV combination model in the present study. Moreover, TH loss was not apparent in AAV A53T-injected animals (Figure A1.1, A1.4). It can therefore be concluded that the TH-loss observed in the current study is dependent on both the presence of A53T PFFs and the overexpression of A53T-mutant α Syn.

While microglial activation is not apparent following A53T PFF delivery, and relatively mild following A53T AAV delivery, a notable increase in the number of Iba1+ microglial cells in the nigra is apparent, following delivery of both PFF and AAV together, suggesting a role for α Syn induced pSer129+ immunoreactivity in glial activation, with knock-on consequences for dopaminergic neuronal loss and behavioural changes observed in this model. Addition of AAV to the PFF model to produce a two-hit model of synucleinopathy might prove more useful for exploring progressive pathology, given the possibility that PFFs may be engulfed, metabolised, or manipulated, before their associated effects can be observed, while AAVs expression is strongly believed to remain consistent for an extended period of time.

An interesting alternative approach to the PFF+AAV combination model describes a PFF model combined with a secondary inflammatory trigger, like IFN or LPS. This secondary hit represents an immune system burden contracted by patients with synucleinopathy, which has been shown in the clinic to further exacerbate disease progression. The model falls in line with a cellular theory that microglia in the amyloidogenic brain become “primed” following interaction with protein aggregates. Moreover, findings from the investigations reported in Chapter 3 suggest that while microglial activation associated with intranigral WT PFF delivery may not be sufficient to trigger changes in TH+ dopaminergic cells in the substantia nigra, stimulating α Syn-primed microglia with the bacterial endotoxin LPS may drive a more potent glial response, with downstream consequences for neuronal integrity and motor function. An experimental protocol was conducted to model this combination in vivo in the lab, where WT PFFs were administered unilaterally to the rat substantia

nigra. In substitution for boosting pathology with an AAV, pathology was boosted with a systemic LPS injection. Commencing four hours post-LPS delivery, the β 2AR agonist, formoterol, was administered to further assess a potential for pharmacological intervention to influence α Syn accumulation, microglial activation, dopaminergic neuronal cell counts, and motor behaviours in LPS-challenged animals following WT PFF delivery (Appendix III). Unexpectedly, pSer129+ immunofluorescence was not detected in brain sections of WT PFF-treated animals from this investigation, and therefore the viability of PFFs used could not be confirmed (Figure A3.8). Various histological approaches were employed to unveil the presence of α Syn, if any, in the substantia nigra, but no approach provided confirmatory results (Appendix IV, Figures A4.1-4.4). The experiments remain inconclusive, but nonetheless lay the foundation of an experimental design which can be repeated in future to compare various two-hit models of synucleinopathy (Appendix III).

A limitation of this study was the non-matching vector serotypes used between treatment cohorts. To promote the expression of WT α Syn in combination with intranigral delivery of WT PFFs, AAV5 was used. To promote expression of A53T α Syn in combination with delivery of A53T PFFs, AAV1/2 was used. Different serotypes were chosen for a variety of reasons. A hybrid AAV1/2 capsid combines the high tropism of AAV2 for TH+ dopamine neurons with the ability for AAV1 to diffuse expression widely through brain tissue, ultimately mediating robust transduction within dopaminergic neurons of the substantia nigra (Koprach et al., 2010). For these reasons, AAV1/2 serotype was selected for optimal utilisation in A53T-mutant α Syn investigations. AAV serotype 5 was selected for WT α Syn investigation to compliment previous research conducted prior to the current investigation (Burke-Mullaly, 2021). Although A53T-mutant PFFs were expected to seed A53T α Syn gene expression to a greater extent than the WT counterparts, the use of different stereotypes may have contributed, in part, to varying degrees of pSer129+ and/or TH+ immunoreactivity. The serotype 1 component of the AAV1/2 construct used in the A53T treatment cohort may have afforded the A53T α Syn-expressing vector with greater transducing efficiency in midbrain neurons, compared to AAV5 (McFarland et al., 2009b; Richichi et al., 2004). In the last decade, several studies have been conducted to compare transduction efficiencies of various AAV serotypes, where serotype 1 has demonstrated superior coverage of expression, at the level of the substantia nigra compared to others, including serotypes 5 (Koprach et al., 2010; McFarland et al., 2009b).

A further limitation of this study was the uneven distribution of males and females across treatment groups. In datasets collected from the stepping test, cylinder test, and amphetamine-induced rotations test, the WT PFF+AAV group contained 1 male and 5 females, while the A53T PFF+AAV group contains 5 males and 5 females. In datasets collected for the analysis of pSer129, Iba1, and TH immunostaining, the WT PFF+AAV group contained 0-1 males and 1-5 females, while the A53T PFF+AAV group contained 2-5 males and 3-5 females. This was a result of limited tissue availability during post-mortem tissue processing. Although the data is too limited to draw conclusions, the males and females included in the present analyses both contribute to the high variability shown across behavioural and histological datasets. Of specific note, both males and females contributed to the high average % contralateral score measured in WT-treated animals in the stepping test at baseline (Figure 4.1c and 4.2c). Cylinder test results from males within the A53T PFF+AAV treatment cohort show a behavioural trend which might suggest their enhanced susceptibility to a contralateral motor deficit six weeks post A53T AAV+PFF delivery, compared to females. This trend is not evident in the stepping test or amphetamine test. Analysis of pSer129+ immunofluorescence, Iba1+

microglial cell numbers, and TH+ cell body numbers from males alone, show comparable patterns of cellular changes to those of females alone, irrespective of treatment group. Together, these findings suggests that the variation observed in these datasets is not sex-based, and that the uneven distribution of males and females included in the analysis is not thought to contribute significantly to variation either between or within treatment groups.

4.7. Chapter conclusion and next steps

The present investigation demonstrated an ability for recombinant human WT α Syn PFFs, in the presence of WT α Syn genetic expression, to drive α Syn phosphorylation and microglial activation in the rat substantia nigra six weeks following intranigral delivery. These cellular changes, however, were insufficient to reduce the number of nigral dopaminergic neuronal cells six weeks after unilateral intranigral delivery. Human recombinant A53T-mutant PFFs, when delivered in the presence of A53T-mutant α Syn AAV vector, proved capable of enhancing pSer129+ α Syn distribution and Iba1+ microglial activation in the substantia nigra, compared to PFF delivery alone. Nigral TH+ cell loss, which was not apparent following delivery of AAV-A53T α Syn or A53T PFFs alone, was evident six weeks following delivery of the A53T PFF+AAV combination treatment. These results indicate an increased capacity for recombinant A53T-mutant α Syn PFFs to seed pathology in the rat brain, through the recruitment and phosphorylation of vector-driven expression of α Syn.

Limb-use asymmetry in skilled and spontaneous motor tasks were apparent four and five weeks post-delivery, respectively, of both WT PFF+AAV and A53T PFF+AAV. The observed reduction in contralateral behaviour following delivery of WT combination treatment, in the absence of dopaminergic degeneration, indicates that the apparent functional deficit was not a downstream consequence of dopaminergic neuronal cell death. In line with the current hypothesis, α Syn-induced microglial activation, which occurred before TH+ cells loss, may play a role in driving symptoms of prodromal synucleinopathy, including motor dysfunction, as observed in the stepping and cylinder tests. Targeting microglial activation and subsequent inflammation may prove useful for ameliorating the nigral degeneration and motor deficits observed in this model. Following these findings, an experiment testing a pharmacological intervention aimed at reducing microglial activation was designed to further test this working hypothesis.

Chapter 5: An investigation of the effects of the beta2 adrenergic receptor agonist, formoterol, on PD-related pathology in rats following intranigral delivery of A53T-mutant human alpha-synuclein preformed fibrils in combination with alpha-synuclein-expressing adeno-associated viral vector

5.1. Introduction

Historically, the onset and progression of synucleinopathies have been attributed to the pathogenic accumulation of misfolded α Syn inside neurons, ultimately resulting in the formation of Lewy bodies. The accumulation of intracellular Lewy body deposits in the brain has long been regarded as the principal hallmark of disease pathology which drives neurodegeneration (Braak et al., 1998). In recent years, chronic microglial activation has been regarded as a detrimental consequence of α Syn aggregation in prodromal stages of synucleinopathy, and is thought to precede Lewy body accumulation and subsequent nigral neurodegeneration. The overactivation of microglia leads to upregulation of pro-inflammatory cytokines which are deemed cytotoxic to vulnerable neurons within the degenerating substantia nigra (Duffy et al., 2018; Sanchez-Guajardo et al., 2015; Wong & Krainc, 2017).

In rodent models, chemical, pharmacological, or genetic ablation of noradrenergic neurons of the LC, and consequent loss of noradrenaline supply to forebrain regions, including the striatum, has been shown to drive downstream consequences for motor dysfunction, microglial activation, production of pro-inflammatory cytokines, and may contribute to the loss of dopaminergic neurons in the substantia nigra (Heneka et al., 2002; Jiang et al., 2015; Rommelfanger et al., 2007; Rommelfanger et al., 2004). Noradrenergic neurons in the LC play a role in maintaining a quiescent CNS immune state. α Syn-induced damage to noradrenergic neurons renders the brain ill-equipped for combatting a cyclical loop of α Syn-induced inflammation, in turn contributing to neurodegeneration, which further perpetuates subsequent neuroinflammation. Given exacerbated activation of the CNS immune system accelerates neuronal death, targeting the noradrenergic system to regulate microglial activation and inflammation in the brain may help to slow or prevent α Syn-related neurodegeneration.

It has been established that activation of the β_2 ARs expressed on microglia drives anti-inflammatory processes in the brain (Dello Russo et al., 2004; McNamee et al., 2010). This has been supported by evidence from rat inflammatory models of PD, which demonstrate robust neuroprotective effects of targeting β_2 ARs, either directly through β_2 AR agonists, or indirectly through NRIs and α_2 AR agonists (O'Neill & Harkin, 2018; O'Neill et al., 2020; Yssel et al., 2018). However, the potential of such compounds to ameliorate α Syn-induced microglial activation and associated neuropathology in clinically relevant models of synucleinopathy remains unknown.

Noradrenergic activation of β_2 AR receptors drives activation of adenylyl cyclase, which leads to increased intracellular cAMP and activation of PKA. In turn, activated PKA phosphorylates cAMP-response element binding (CREB) protein and induces *de novo* synthesis of the NF κ B inhibitor protein (I κ B α), thus stabilising cytosolic levels of I κ B α . I κ B α blocks nuclear translocation of NF κ B, a transcription factor which drives the transcription of inflammatory cytokines including IL-1 β , IL-6, and TNF α . Inhibition of NF κ B translocation to the nucleus precludes the over production of pro-inflammatory cytokines and associated mediators, known to be toxic to neurons (Ryan et al., 2013).

In rats, administration of the β_2 AR agonist clenbuterol (0.5mg/kg i.p.) suppressed LPS-induced Nf κ B activity and expression of TNF α , whilst concurrently elevating the temporal expression of I κ B α (Ryan et al., 2013). Clenbuterol (0.5mg/kg; i.p.) has also been reported to induce IL-10, IL-1 receptor antagonist, and IL-1 receptor type II expression in cortical tissue of rats (Ryan et al., 2011). Moreover, the long acting β_2 AR agonist salmeterol attenuates IL-1 β -induced CD54 expression in human airway smooth muscle cells, thus displaying immunoregulatory properties beyond the CNS (Kaur et al., 2008). Continuous subcutaneous infusion of the β_2 AR agonist salmeterol through an osmotic pump implant (1 or 10 μ g/kg/day for two weeks) beginning 2 days prior to induction of an MPTP lesion in mice, prevents MPTP-associated dopaminergic neurotoxicity three weeks after MPTP administration. Data from the same study has shown that salmeterol administration (10 μ g/kg/day for two weeks), beginning three months after a single systemic injection of LPS (5mg/kg i.p.), protected against motor deficits in the rotarod test, and prevented the loss of TH+ cells in the substantia nigra (Qian et al., 2011).

Neuroprotection afforded by glial β_2 AR activation is thought to involve the β_2 AR/ β -arrestin2/TAK1-dependent phosphorylation of MAPK and NF κ B in microglia, resulting in downregulation of pro-inflammatory cytokines (Qian et al., 2011). In human monocyte and mesangial cultures, β_2 AR stimulation by isoprenaline attenuated LPS-induced TNF α and IL-8 production (Farmer & Pugin, 2000; Nakamura et al., 2003). Similarly, in an inflammatory rat model of PD, β_2 AR agonist drugs formoterol and clenbuterol conferred protection against LPS-induced IL-1 β and CD68 expression, and associated TH+ cell loss (O'Neill & Harkin, 2018; O'Neill et al., 2020; Yssel et al., 2018). *In vitro* studies reported that noradrenaline, at sub-micromolar concentrations, can attenuate LPS-induced TNF α and NO production, and inhibit microglial superoxide production. Noradrenaline-associated reductions in microglial-derived pro-inflammatory mediator production also protected against neuronal cell death in LPS-challenged mesencephalic neuron-glia co-cultures. Subsequent investigations proposed the anti-inflammatory and neuroprotective effects of noradrenaline to be mediated exclusively by β_2 ARs *in vitro* (Jiang et al., 2015). Together, these findings highlight the likelihood that enhancing noradrenergic tone through pharmacological β_2 AR stimulation may regulate the inflammatory response, with the potential to slow and/or limit progressive neurodegeneration.

In addition to stalling a microglial-associated pro-inflammatory phenotype, β_2 AR activation by noradrenaline and downstream CREB phosphorylation drives the upregulation of astrocytic anti-inflammatory and neurotrophic factors, including IL-10, BDNF, and NGF (Braun et al., 2014; Furukawa et al., 1989; Juric et al., 2008). β_2 AR-associated upregulation of BDNF and its receptor TrKB, promotes neuronal survival, neurogenesis, and synaptic plasticity (Rabie et al., 2018; Sayed et al., 2020; Song et al., 2015a). In 6-OHDA-lesioned marmoset monkeys, intra-cerebral delivery of cerebral dopamine neurotrophic factor (CDNF) (20 μ g) increases DAT binding activity, while intra-cerebral delivery of glial cell line-derived neurotrophic factor (GDNF) (20 μ g) increases the number of TH+ neurons, suggesting a neurorestorative potential for astrocyte-derived neurotrophic factors (Garea-Rodriguez et al., 2016). Similar results were achieved in rats, in which striatal injection of mesencephalic astrocyte-derived neurotrophic factor (MANF) or GDNF protected against 6-OHDA-induced TH+ neuronal cell loss in the substantia nigra, and loss of TH+ fibres in the striatum (Voutilainen et al., 2009).

In human-derived neuronal and primary rat hippocampal cultures treated with amyloid- β , to model Alzheimer's disease, β_2 AR activation has been shown to boost NGF and BDNF production, protecting

against amyloid- β -associated degeneration (Counts & Mufson, 2010; Liu et al., 2015). NGF mRNA and protein expression were upregulated in primary astrocytic cultures exposed to clenbuterol at 1, 10, and 100 μ M for 4 hours. In neuronal and astrocytic co-cultures, clenbuterol-driven NGF production afforded neuronal protection against glutamate-induced excitotoxicity (Culmsee et al., 1999). Junker and colleagues corroborated these findings in cultured hippocampal neurons, showing neuroprotection by astrocytic NGF production driven by clenbuterol. Interestingly, subsequent *in vitro* studies demonstrated that additional treatment with the non-selective β AR antagonist propranolol, and specific β_2 AR antagonist ICI 118551 (10 μ M) blocked the neuroprotective actions of the β_2 AR agonist salmeterol (0.001-1 μ M), indicating neuroprotective actions afforded by β_2 AR activation. Furthermore, β_1 AR antagonist metoprolol (10-100 μ M) failed to alter the neuroprotective effects of clenbuterol, ultimately indicating that neuronal protection against glutamate-induced excitotoxicity was attributed to glial β_2 AR stimulation specifically (Junker et al., 2002). In a mouse model of cerebral ischaemia, pre-treatment with clenbuterol (0.3mg/kg i.p.) 5 hours prior to occlusion of the middle cerebral artery reduced the infarct area compared to vehicle, likely as a result of increased astrocyte activation and production of neuroprotective growth factors. This neuroprotective effect was blocked by co-administration with the β_2 AR antagonist butoxamine (5mg/kg i.p.) and the $\beta_{1/2}$ AR antagonist propranolol (5mg/kg i.p.), but not with the β_1 AR antagonist metoprolol at any dose applied (0.5-5mg/kg i.p.). These findings further indicate that neuroprotection afforded by clenbuterol occurs exclusively via the activation of β_2 ARs (Junker et al., 2002).

In neurodegenerative disease, degeneration of LC neuronal population and depletion of noradrenergic supply constrains the brain to a state of forfeit, unable to uphold the immunoregulatory or neurotrophic support provided by midbrain and striatal noradrenergic afferents. Noradrenergic deficit subsequently curtails noradrenaline-mediated production of glial-derived neurotrophic factors, thus rendering the vulnerable nigrostriatal neuronal population devoid of growth factor support (Heneka et al., 2002; Hertz et al., 2004). Furthermore, noradrenergic depletion is likely to impact the susceptibility of nigral neurons to toxicity induced directly by the accumulation and propagation of α Syn, in cases of synucleinopathy. In addition to conferring neuroprotection directly, astrocytic BDNF production restimulates a phosphatidyl inositol-3 kinase (PI3k) and protein kinase B (Akt)-mediated positive feed forward loop which can promote mitophagy and inhibit α Syn aggregation (Khidr et al., 2023; Palasz et al., 2020). Mover phosphorylated CREB has been shown to directly activate a significant number of target genes that possess the CRE motif “TGACGTCA,” including the TH gene (Chen et al., 2021; Xu et al., 2020). Thus, β_2 AR may directly influence TH transcription and dopamine synthesis, and play a role in preventing nigrostriatal dopamine depletion in synucleinopathic disease states.

Though only loosely explored, there is evidence to indicate a link between β_2 AR activation and SNCA transcription. WT mice displayed reduced SNCA mRNA and α Syn protein expression in the substantia nigra following treatment with the β_2 AR agonist clenbuterol. This downregulation was abrogated by genetic deletion of β_2 AR, and pharmacological antagonism by propranolol (Mittal et al., 2017). Clenbuterol treatment also ameliorated mitochondria-associated superoxide production and TH+ cell death in PD patient-derived neuronal precursor cells carrying SNCA triplication. Interestingly, the antioxidant and neuroprotective benefits provided by clenbuterol in this model were recapitulated by genetic knockdown of the SNCA gene (Flierl et al., 2014; Mittal et al., 2017). Furthermore, clenbuterol protected against MPTP-associated dopaminergic nigral degeneration in

mouse models of PD. In a rat primary cortical neuronal model of synucleinopathy, high-throughput screening coupled with ELISA, β_2 AR agonists salbutamol, clenbuterol, and metaproterenol were linked with downregulation of SNCA transcription and reduced α Syn translation. Mechanistically, Mittal and colleagues propose β_2 AR activation by noradrenaline, or noradrenergic agonists, to drive acetylation of H3K27 along the SNCA promoter and enhancer sites, thus influencing transcription of SNCA and its translation into α Syn protein. Perhaps the most compelling evidence to support the current hypothesis and rationalise future work in this field comes from a series of population studies of asthma patients who repeatedly used drugs which target β_2 ARs. Published in 2017, a population study in Norway reported reduced risk rate of PD in salbutamol-treated asthmatics compared to corticosteroid-treated asthmatics, and increased PD risk rates in those prescribed propranolol, a β_2 AR antagonist used mainly for treating hypertension (Mittal et al., 2017). An Israeli population study reported an association between short-acting, long-acting, and ultra-long-acting β_2 AR agonist use and reduced risk of PD, and between nonselective β_2 AR antagonist use and increased PD risk (Gronich et al., 2018). These findings have been challenged by French and American studies which show these associations to be confounded by other behavioural, health, and/or environmental factors (de Germaey et al., 2020; Searles Nielsen et al., 2018). Patterson and colleagues report negligible effects of β_2 AR agonism on α Syn mRNA or protein expression in rodents and neuronal cultures, and suggest that the limited changes are due either to β_2 AR desensitization, or enhanced metabolism of the administered drug following repeated administration of β_2 AR agonists (Patterson et al., 2022). These data, albeit preliminary, provide rationale for the continued study of the modulation of SNCA translation by β_2 AR stimulation with respect to synucleinopathy progression.

Formoterol is blood brain barrier permeant which can enter the CNS parenchyma to exert potent regulatory effects through interaction with glial cells. Formoterol is a long-acting selective β_2 AR agonist. It displays higher lipophilicity, and higher affinity, selectivity, and potency at β_2 AR than most short-acting or non-selective β_2 AR agonists (Linden et al., 1996). In the clinic, formoterol is regularly prescribed in conjunction with the anti-inflammatory corticosteroid budesonide as an FDA-approved dose-metred inhaler for the treatment of asthma and airflow obstruction in COPD patients (Rosenhall et al., 2002). In the periphery, systemic formoterol treatment induces bronchodilation, vasodilation, and smooth muscle relaxation. With a rapid onset of action and minimal adverse effects profile, its safety and efficacy when used over prolonged periods of time are well regarded (Rosenhall et al., 2003).

The use of formoterol to curtail inflammation and neurodegeneration in the diseased brain remains only loosely explored. In an inflammatory rat model of PD, formoterol attenuated the loss of nigral TH+ dopaminergic neurons and striatal TH+ dopaminergic nerve terminals, prevented the depletion of midbrain and striatal dopamine content, reduced microglial activation, and ameliorated unilateral motor deficits induced by intranigral injection of LPS. Clenbuterol also produced protective effects, but was unable to fully attenuate LPS-induced TH+ striatal denervation, dopamine depletion, or microgliosis (O'Neill et al., 2020). The superior neuroprotective efficacy of formoterol relative to clenbuterol in this study can likely be explained by formoterol's greater ability to penetrate the CNS barrier and target microglia (Tanaka et al., 2002), and greater binding affinity for β_2 ARs over β_1 ARs (Anderson, 1993).

In the same LPS rat model, treatment with atomoxetine, a selective NRI, exhibited efficacy in suppressing LPS-associated nigral dopaminergic neuronal degeneration, striatal dopaminergic nerve fibre degeneration, nigrostriatal dopamine depletion, and microglial activation, and provided partial

protection against unilateral motor impairments (Yssel et al., 2018). Co-treatment of atomoxetine with the β_2 -adrenoceptor antagonist ICI 118551 blocked the protective effects of atomoxetine, in support of the proposed β_2 AR-dependent pathway through which anti-inflammatory and neuroprotective actions are afforded (O'Neill et al., 2020).

Pre-treatment of mesencephalic neuron-glia co-cultures with formoterol over a range of concentrations (10^{-7} – 10^{-12} M) for 30 minutes prior to stimulation with LPS imparted protection against LPS-induced toxicity *in vitro* (Qian et al., 2011). In Wistar rats, formoterol treatment ameliorated nigral and striatal dopamine depletion and nigral TH+ cell loss, repressing NF κ B and subsequent production of TNF α , and alleviating motor dysfunction, as measured through open field, grip strength-meter, and rotarod tests, in a rotenone-induced toxin model of PD. Rats received rotenone (1.5 mg/kg; s.c.) every other day for three weeks with or without formoterol treatment (25 μ g/kg/day; i.p.) 1 hour after rotenone administration, starting from day 11. In parallel, Western blot analysis of striatal homogenate showed a tight inverse correlation between downregulation of β_2 AR expression and upregulation of striatal α Syn protein ($r = -0.99$, $p = 0.0003$). In this model, β_2 AR agonism by formoterol was suggested to mitigate α Syn accumulation through activation of mitophagy, the molecular process of selective degradation and clearance of defective mitochondria resulting from the complex I inhibitor rotenone. This study demonstrates a neuron survival cascade afforded by β_2 AR activation through enhancing mitophagy and mitochondrial biogenesis to remove α Syn aggregates. Moreover, β_2 AR activation by formoterol protects dopaminergic neuronal integrity through PI3k/Akt/CREB signalling-mediated BDNF production and synthesis of TH (Khidr et al., 2023). Taken together, exploratory research from toxin, genetic, and inflammatory models of PD support a hypothesis that β_2 AR activation, by formoterol or other pharmacological β_2 AR agonists, exerts neuroprotective actions in the brain by inhibiting microglial activation and pro-inflammatory mediator release, and promoting an anti-inflammatory neurotrophic phenotype.

Specifically, repeated i.p. administration of formoterol at a dose of 0.1mg/kg once daily for seven days immediately following a systemic LPS challenge (250 μ g/kg i.p.), has been shown to attenuate Iba1+ microgliosis and TH+ cell loss, alleviate CD68 and IL-1 β expression, and mitigate associated motor deficits in a six-week LPS-induced inflammatory rodent model of PD (O'Neill et al., 2020). Lesser known, however, is the translatability of these benefits to models of synucleinopathy driven by AAV-mediated α Syn overexpression and/or fibrillar protein constructs. In the previous chapter, an increase in Iba1+ microglia and decrease in TH+ neuronal cell bodies was reported in the injected substantia nigra in rats six weeks after A53T AAV+PFF delivery. β_2 AR activation has been shown to regulate the microglial response, promote astrocytic neurotrophic production, and regulate α Syn gene and protein expression, three likely contributing factors to synucleinopathic disease. There is therefore a rationale for investigating the potential for the β_2 AR agonist formoterol to alleviate motor dysfunction, microglial activation, and dopaminergic neuronal degeneration in clinically relevant models of α Syn-related pathology.

5.2. Aims and objectives

The primary aim of this study was to determine if pharmacological stimulation of β_2 AR alleviates α Syn accumulation, microglial activation, dopaminergic degeneration, and motor impairment six weeks after unilateral delivery of A53T-mutant α Syn PFFs and AAV1/2 vector expressing human A53T-mutant α Syn into the rat substantia nigra.

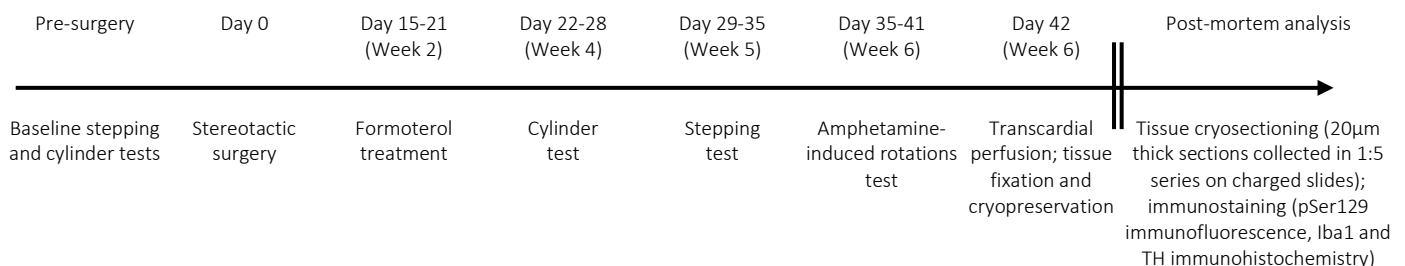
A53T-mutant α Syn PFFs, in combination with AAV1/2 vector expressing human A53T-mutant α Syn, were delivered into the rat substantia nigra by a single stereotactic unilateral injection. Two weeks after intranigral delivery of A53T PFF+AAV, the β_2 AR agonist formoterol was administered once daily for seven days, to assess its potential to reduce α Syn accumulation, microglial activation, dopaminergic neurodegeneration, and behavioural impairment following A53T PFF+AAV delivery. Skilled motor function and limb-use asymmetry were assessed through application of the stepping and cylinder tests, respectively. Rotational behaviour in the amphetamine-induced rotations test was assessed to functionally evaluate the presence of a unilateral nigral lesion. pSer129 immunofluorescence, Iba1 immunohistochemistry, and TH immunohistochemistry were subsequently conducted on cryopreserved midbrain tissue sections to assess α Syn deposition, microglial cell counts, and dopaminergic neuronal cell counts, respectively, in the substantia nigra.

5.3. Experimental design

36 adult (7–8-month-old) Wistar rats (20 male, 16 female) underwent behavioural training and baseline testing before being randomly assigned to a treatment group. All animals received a single intranigral stereotactic injection of 4μL containing 2μL of human recombinant A53T-mutant αSyn PFFs and 2μL of AAV1/2-human A53T αSyn. The coordinates for delivery to the substantia nigra in males were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for delivery to the substantia nigra in females were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. Two weeks post-surgery, rats received repeated injections of either formoterol (0.1mg/kg, i.p., once daily for 7 days) or sterile NaCl (0.89% NaCl, i.p., once daily for 7 days). All i.p. injections were administered in volume of 1ml/kg. In weeks four, five, and six following PFF+AAV delivery, rats were subjected to the cylinder test, stepping test, and amphetamine-induced rotations test, respectively. Six weeks post-surgery, animals were euthanised by sodium pentobarbital overdose and transcardial perfusion/fixation, in preparation for immunofluorescence and histochemical analysis of pSer129+ αSyn, Iba1+ microglia, and TH+ neuronal cell bodies in the substantia nigra. As naïve controls were omitted from this experiment, the unaffected limb and hemisphere were used as internal controls for behavioural and histological analysis, respectively.

Treatment groups (n = 18):

1. Human A53T αSyn PFF + AAV1/2-CMV-human A53T αSyn (one unilateral injection of 4μl)
+ Sterile NaCl (0.89% NaCl, i.p.)
 - n = 18 (8 females, 10 males)
 - PFF concentration: 2mg/ml (4μg injected)
 - AAV titre: >5x10¹² vg/ml (1x10¹⁰vg injected)
2. Human A53T αSyn PFF + AAV1/2-CMV-human A53T αSyn (one unilateral injection of 4μl)
+ Formoterol (0.1mg/kg, i.p.)
 - n = 18 (8 females, 10 males)
 - PFF concentration: 2mg/ml (4μg injected)
 - AAV titre: >5x10¹² vg/ml (1x10¹⁰vg injected)



5.4. Methods

All methods were conducted as described in chapter 2, with the following minor amendment. During rat tissue processing, cryopreserved brains were sectioned at a thickness of 20µm instead of 35µm. This amendment was introduced to improve the quality of immunohistochemical staining and clarity of images generated.

5.5. Results

5.5.1. Cylinder test

A two-way repeated measures ANOVA of combined ipsilateral placements shows no effect of treatment [$F(1,34)=1.637$, $p=0.2094$], an effect of time [$F(1,34)=11.64$, $p=0.0017$], and no interaction between treatment and time [$F(1,34)=0.1300$, $P=0.7206$]. Post hoc comparisons reveal reduced combined ipsilateral limb placements in vehicle ($P=0.0381$) and formoterol ($p=0.0116$) treated animals at four weeks post-PFF+AAV delivery compared to baseline (Figure 5.1a). A two-way ANOVA of combined contralateral placements shows no effect of treatment [$F(1,34)=2.266$, $p=0.1415$], an effect of time [$F(1,34)=120.9$, $p<0.0001$], and no interaction between treatment and time [$F(1,34)=0.002953$, $p=0.9570$]. Post hoc comparisons reveal reduced combined contralateral limb placements in vehicle ($p<0.0001$) and formoterol ($p<0.0001$) treated animals at four weeks post-PFF+AAV delivery compared to baseline (Figure 5.1b). A two-way repeated measures ANOVA of combined % combined contralateral placements shows no effect of treatment [$F(1,34)=0.04782$, $p=0.8282$], an effect of time [$F(1,34)=0.6102$, $p=0.4401$], and no interaction between treatment and time [$F(1,34)=24.09$, $p<0.0001$]. Post hoc comparisons reveal reduced % contralateral placements in vehicle ($p=0.0003$) and formoterol ($p=0.0062$) treated animals at four weeks post-PFF+AAV delivery compared to baseline (Figure 5.1c).

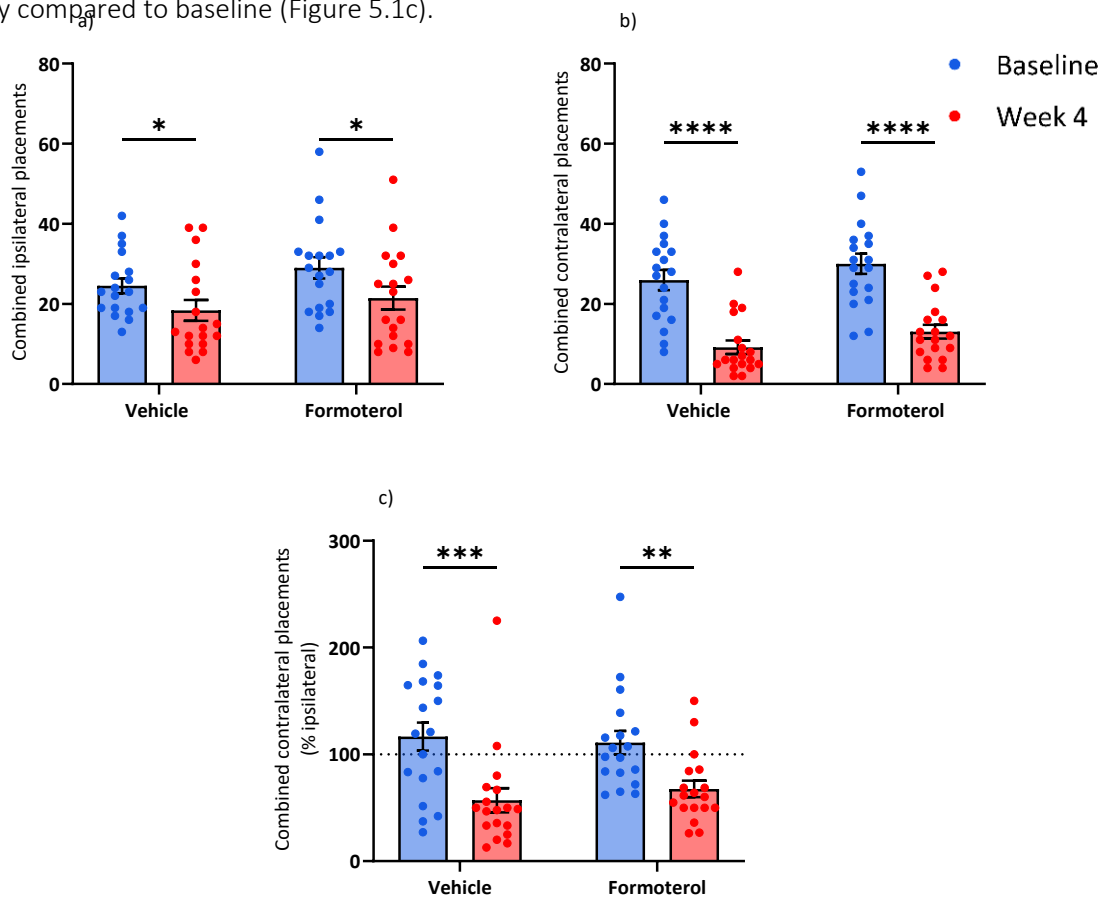


Figure 5.1. Formoterol does not alleviate the reduction in combined placements observed in the cylinder test four weeks after intranigral delivery of A53T PFF+AAV. a) Combined placements made with the limb ipsilateral to the injection, b) combined placements made with the limb contralateral to the injection, c) combined contralateral placements expressed as a percentage of combined ipsilateral placements. Test duration of 5 minutes. Data are expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=18$. * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$, **** $p\leq0.0001$.

A two-way repeated measures ANOVA of ipsilateral wall placements shows no effect of treatment [$F(1,34)=1.124$, $p=0.2965$], an effect of time [$F(1,34)=9.102$, $p=0.0048$], and no interaction between treatment and time [$F(1,34)=0.9885$, $p=0.3271$]. Post hoc comparisons reveal reduced ipsilateral wall placements in formoterol treated animals at four weeks post-PFF+AAV delivery compared to baseline ($p=0.0076$) (Figure 5.2a). A two-way repeated measures ANOVA of contralateral wall placements shows no effect of treatment [$F(1,34)=1.461$, $p=0.2350$], an effect of time [$F(1,34)=60.19$, $p<0.0001$], and no interaction between treatment and time [$F(1,34)=1.825$, $p=0.1856$]. Post hoc comparisons reveal reduced contralateral wall placements in vehicle ($P<0.0001$) and formoterol ($P<0.0001$) treated animals at four weeks post-PFF+AAV delivery compared to baseline (Figure 5.2b). A two-way repeated measured ANOVA of % contralateral wall placements shows no effect of treatment [$F(1,33)=0.06246$, $p=0.8042$], an effect of time [$F(1,33)=18.08$, $p=0.0002$], and an interaction between treatment and time [$F(1,33)=4.861$, $p=0.0346$]. Post hoc comparisons reveal reduced % contralateral wall placements in vehicle-treated animals four weeks post-PFF+AAV delivery compared to baseline ($p<0.0001$) (Figure 5.2c).

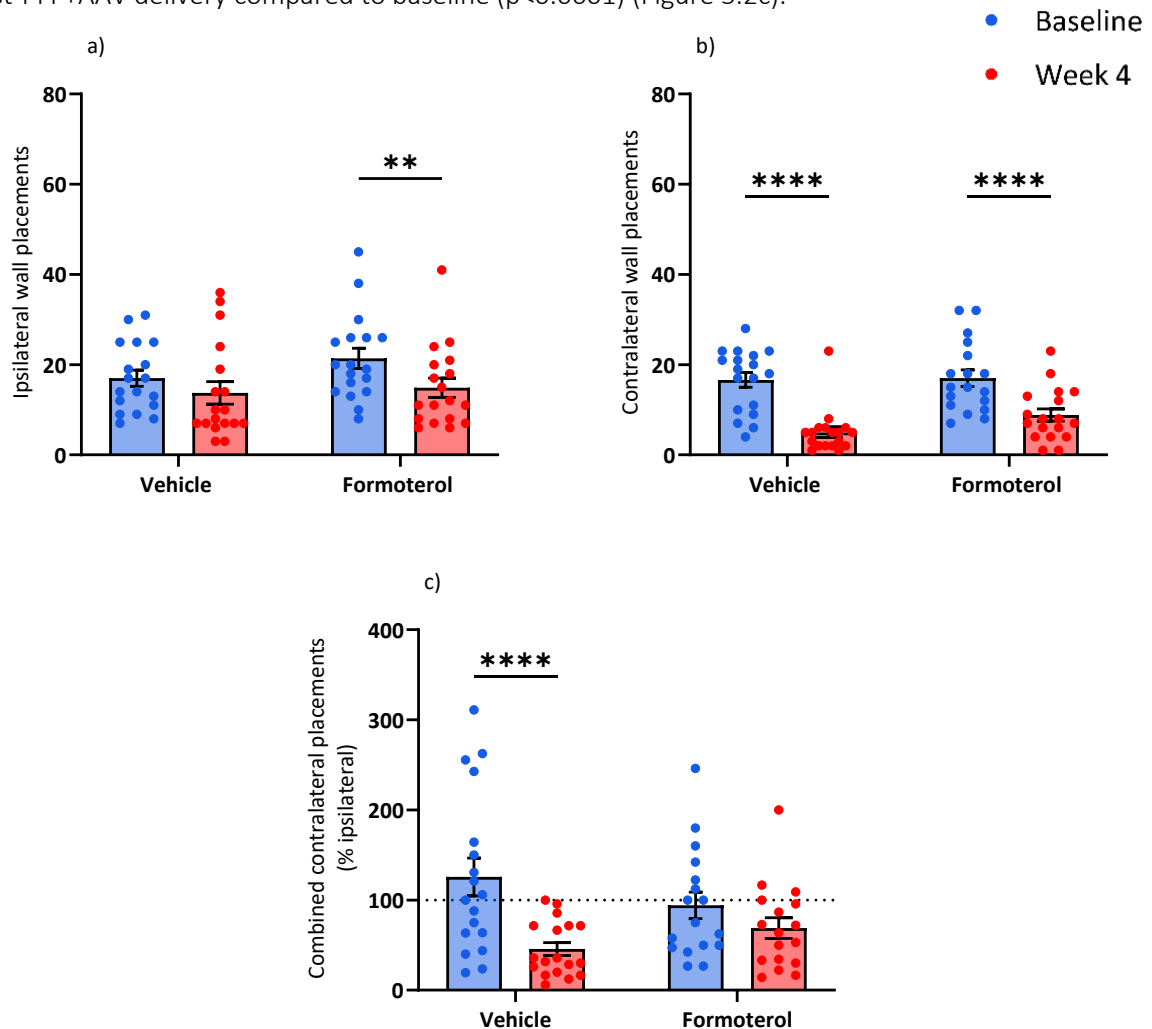


Figure 5.2. Formoterol partially mitigates the reduction in wall placements observed in the cylinder test four weeks after intranigral delivery of A53T PFF+AAV. a) Wall placements made with the limb ipsilateral to the injection, b) wall placements made with the limb contralateral to the injection, c) contralateral wall placements expressed as a percentage of ipsilateral wall placements. Test duration of 5 minutes. Data are expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=18$. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$.

Independent Mann Whitney U-tests show no impact of formoterol treatment on combined ipsilateral placements [$U=107.5$, $p=0.0861$] (Figure 5.3a), combined contralateral placements [$U=158$, $p=0.9064$] (Figure 5.3b), or ipsilateral wall placements [$U=147.5$, $p=0.8642$] when scores from four weeks post-PFF+AAV delivery are expressed as % of baseline (Figure 5.3c). A Mann Whitney U-test reveals increased contralateral wall placements when expressed as % baseline in formoterol-treated animals compared to vehicle-treated animals [$U=82.50$, $p=0.0190$] (Figure 5.3c).

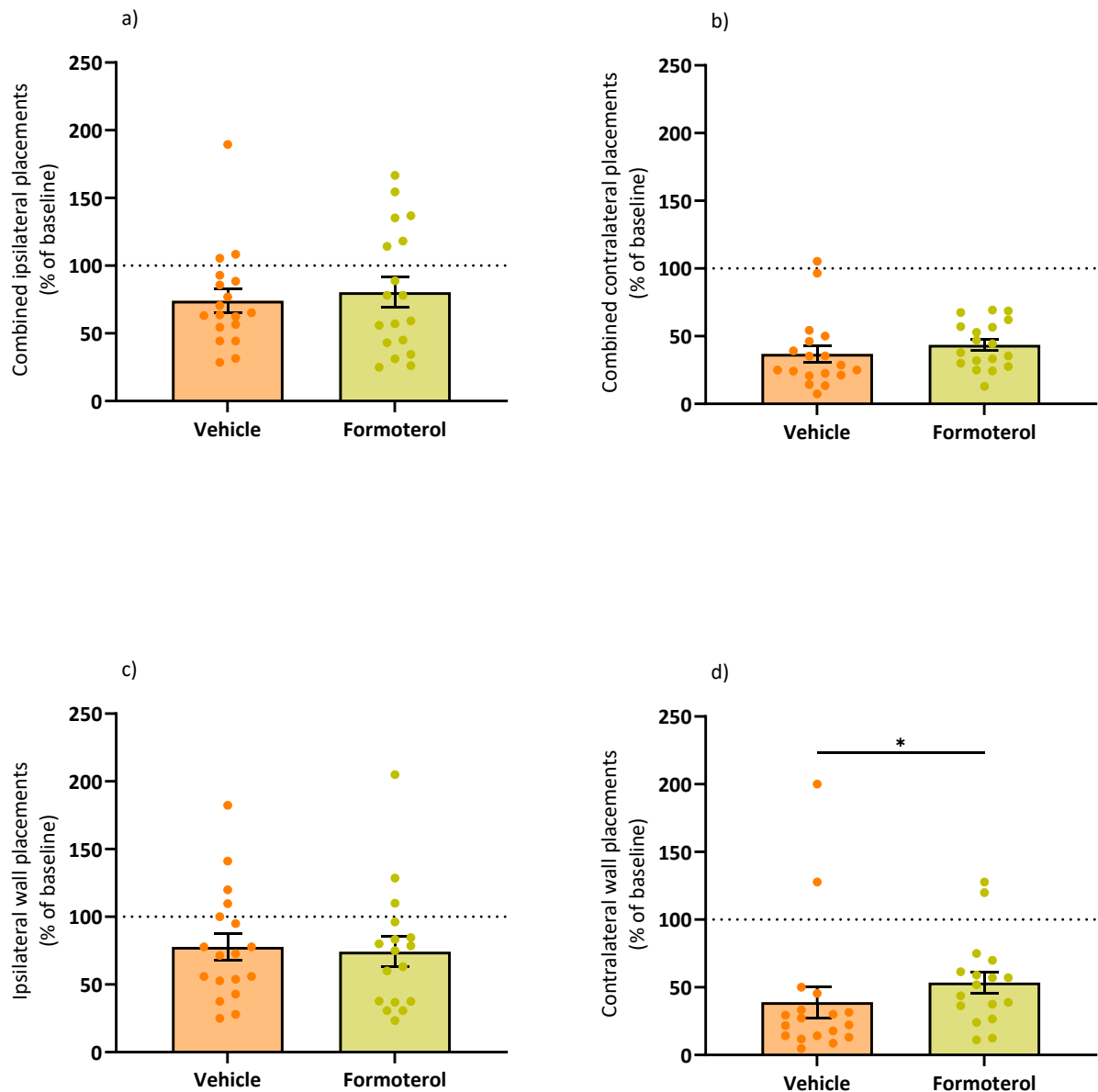


Figure 5.3. Formoterol-treated animals display increased wall placements made with the limb contralateral to the injection (% of baseline) compared to vehicle-treated controls. a) Combined ipsilateral placements, b) combined contralateral placements, c) ipsilateral wall placements, and d) contralateral wall placements, expressed as a % of baseline. Test duration of 5 minutes. Data are expressed as mean $\pm$ SEM. Mann Whitney U-test. $n=18$. *= $p\leq 0.05$.

A two-way ANOVA of combined cylinder placements expressed as a % of baseline shows no effect of treatment [$F(1,68)=0.6820$, $p=0.4118$], an effect of laterality [$F(1,68)=21.88$, $p<0.0001$], and no interaction between treatment and laterality [$F(1,68)=0.0001466$, $p=0.9904$]. Post hoc comparisons reveal reduced combined contralateral placements (% baseline) compared to combined ipsilateral placement (% baseline) in vehicle ($p=0.0015$) and formoterol ($p=0.0015$) treated animals (Figure 5.4a). A two-way ANOVA of wall placement scores expressed as a % of baseline shows no effect of treatment [$F(1,66)=0.2893$, $p=0.5925$], an effect of laterality [$F(1,66)=8.568$, $p=0.0047$], and no interaction between treatment and laterality [$F(1,66)=0.7913$, $p=0.3770$]. Post hoc comparisons reveal reduced contralateral wall placements (% of baseline) compared to ipsilateral wall placements (% baseline) in vehicle-treated animals ($p=0.0079$) (Figure 5.4b).

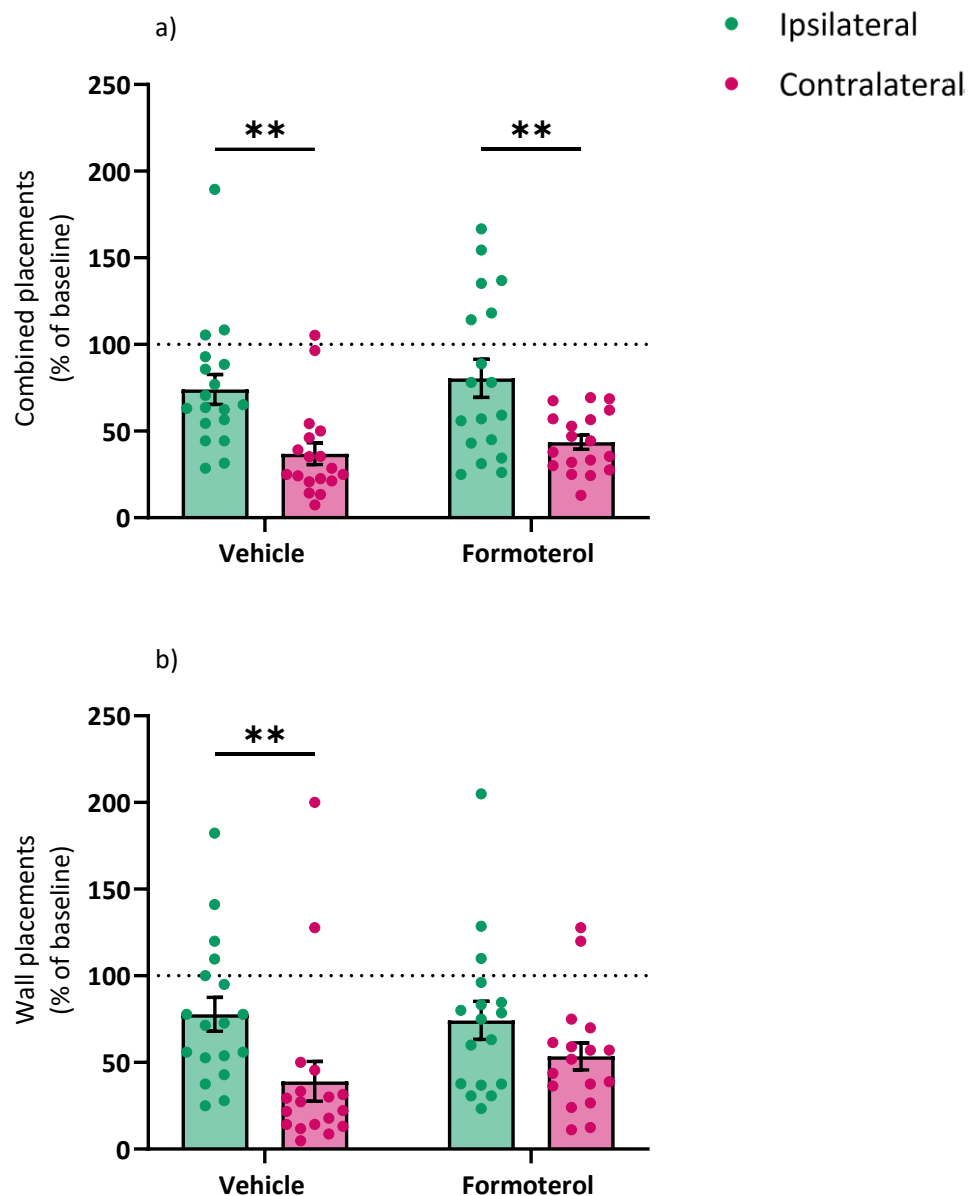


Figure 5.4. Formoterol does not impact combined placements or wall placements made with the limb contralateral to the injection four weeks after intranigral delivery of A53T PFF+AAV. a) Combined placements expressed as a percentage of baseline, b) wall placements expressed as a percentage of baseline. Test duration of 5 minutes. Data are expressed as mean $\pm$ SEM. Two-way ANOVA followed by Fisher's test for multiple comparisons. $n=18$. **= $p\leq 0.01$.

5.5.2. Stepping test

A two-way repeated measures ANOVA of combined ipsilateral steps shows no effect of treatment [$F(1, 34)=0.4723$, $p=0.4966$], an effect of time [$F(1, 34)=13.77$, $p=0.0007$] and no interaction between treatment and time [$F(1, 34)=0.5115$, $p=0.4794$]. Post hoc comparisons reveal increased ipsilateral steps in vehicle ($p=0.0036$) and formoterol ($p=0.0415$) treated animals at five weeks post-PFF+AAV delivery compared to baseline (Figure 5.5a). A two-way repeated measures ANOVA of combined contralateral steps shows no effect of treatment [$F(1, 34)=0.1771$, $p=0.6765$], an effect of time [$F(1, 34)=26.19$, $p<0.0001$] and no interaction between treatment and time [$F(1, 34)=0.09211$, $p=0.7634$]. Post hoc comparisons reveal reduced contralateral stepping in vehicle ($p=0.0017$) and formoterol ($p=0.0005$) treated animals at five weeks post-PFF+AAV delivery compared to baseline (Figure 5.5b). A two-way repeated measures ANOVA of % combined contralateral steps shows no effect of treatment [$F(1, 34)=0.9355$, $p=0.3403$], an effect of time [$F(1, 34)=34.53$, $p<0.0001$], and no interaction between treatment and time [$F(1, 34)=1.155$, $p=0.2901$]. Post hoc comparisons reveal significant reductions in % combined contralateral steps in vehicle ($p<0.0001$) and formoterol ($p=0.0018$) treated animals at five weeks post-PFF+AAV delivery compared to baseline (Figure 5.5c).

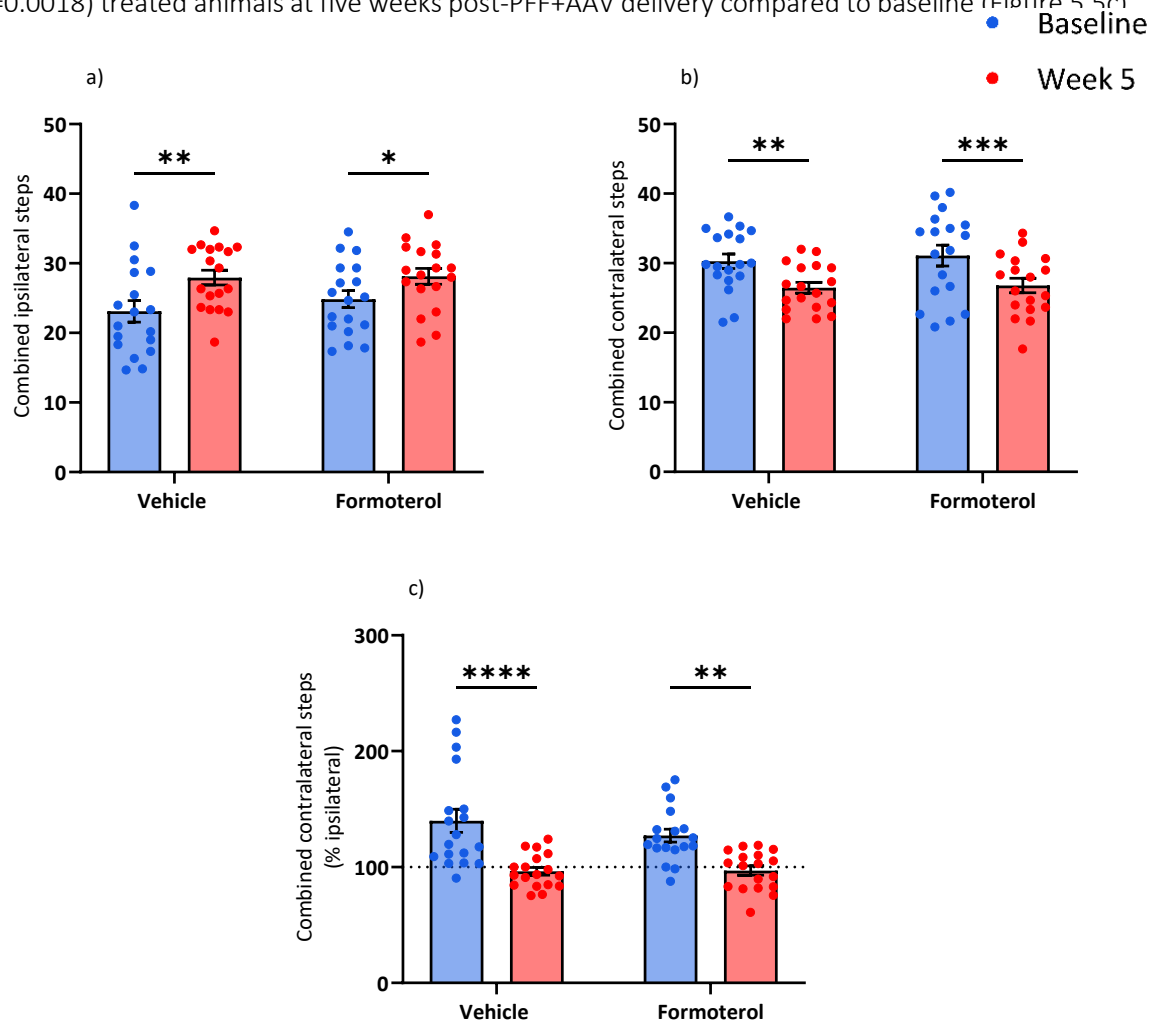


Figure 5.5. Formoterol does not affect combined stepping test performance observed five weeks post-A53T PFF+AAV delivery to the substantia nigra. a) Combined steps made with the limb ipsilateral to the injection, b) combined steps made with the limb contralateral to the injection, c) combined contralateral steps expressed as a percentage of combined ipsilateral steps. Data are expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=18$. $*$ = $p\leq 0.05$, $**$ = $p\leq 0.01$, $***$ = $p\leq 0.001$, $****$ = $p\leq 0.0001$.

A two-way repeated measures ANOVA of forward ipsilateral steps shows no effect of treatment [$F(1, 34)=0.5036$, $p=0.4828$], time [$F(1, 34)=1.720$, $p=0.1984$], or interaction between treatment and time [$F(1, 34)=1.311$, $p=0.2603$] (Figure 5.6a). A two-way repeated measures ANOVA of forward contralateral steps shows no effect of treatment [$F(1, 34)=0.8514$, $p=0.3627$], an effect of time [$F(1, 34)=70.25$, $p<0.0001$], and no interaction between treatment and time [$F(1, 34)=0.1443$, $p=0.7064$]. Post hoc comparisons reveal reduced forward contralateral steps in vehicle ($p<0.0001$) and formoterol ($p<0.0001$) treated animals at five weeks post-PFF+AAV delivery compared to baseline (Figure 5.6b). A two-way repeated measures ANOVA of % forward contralateral steps shows no effect of treatment [$F(1, 34)=0.09607$, $p=0.7585$], an effect of time [$F(1, 34)=13.79$, $p=0.0007$], and no interaction between treatment and time [$F(1, 34)=1.484$, $p=0.2316$]. Post hoc comparisons reveal reduced % forward contralateral steps in vehicle treated animals ($p=0.0014$) at five weeks post-PFF+AAV delivery compared to baseline (Figure 5.6d).

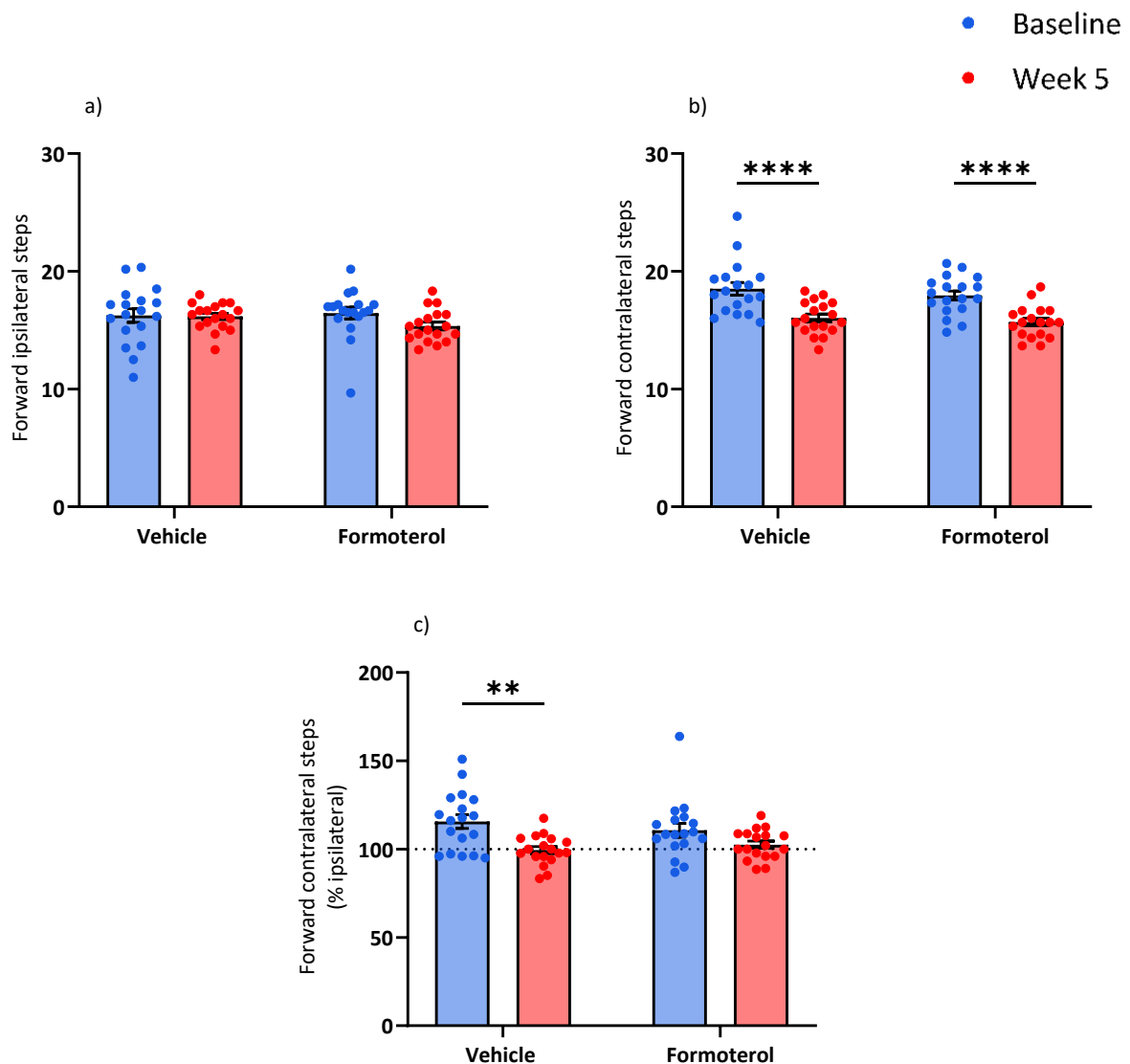


Figure 5.6. Formoterol does not affect forward stepping test performance observed five weeks post-A53T PFF+AAV delivery to the substantia nigra. a) Forward steps made with the limb ipsilateral to the injection, b) forward steps made with the limb contralateral to the injection, c) forward contralateral steps expressed as a percentage of forward ipsilateral steps. Data are expressed as mean ± SEM. Two-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=18$. **= $p\leq 0.01$, ****= $p\leq 0.0001$.

Independent students' t-tests show no effects of formoterol on combined ipsilateral steps [$t(34)=0.9271$, $p=0.3604$], combined contralateral steps [$t(34)=0.04784$, $p=0.9621$], forward ipsilateral steps [$t(34)=0.9836$, $p=0.3323$], or forward contralateral steps [$t(34)=0.1761$, $p=0.8613$] when scores from five weeks post-PFF+AAV delivery are expressed as a percentage of baseline (Figure 5.7).

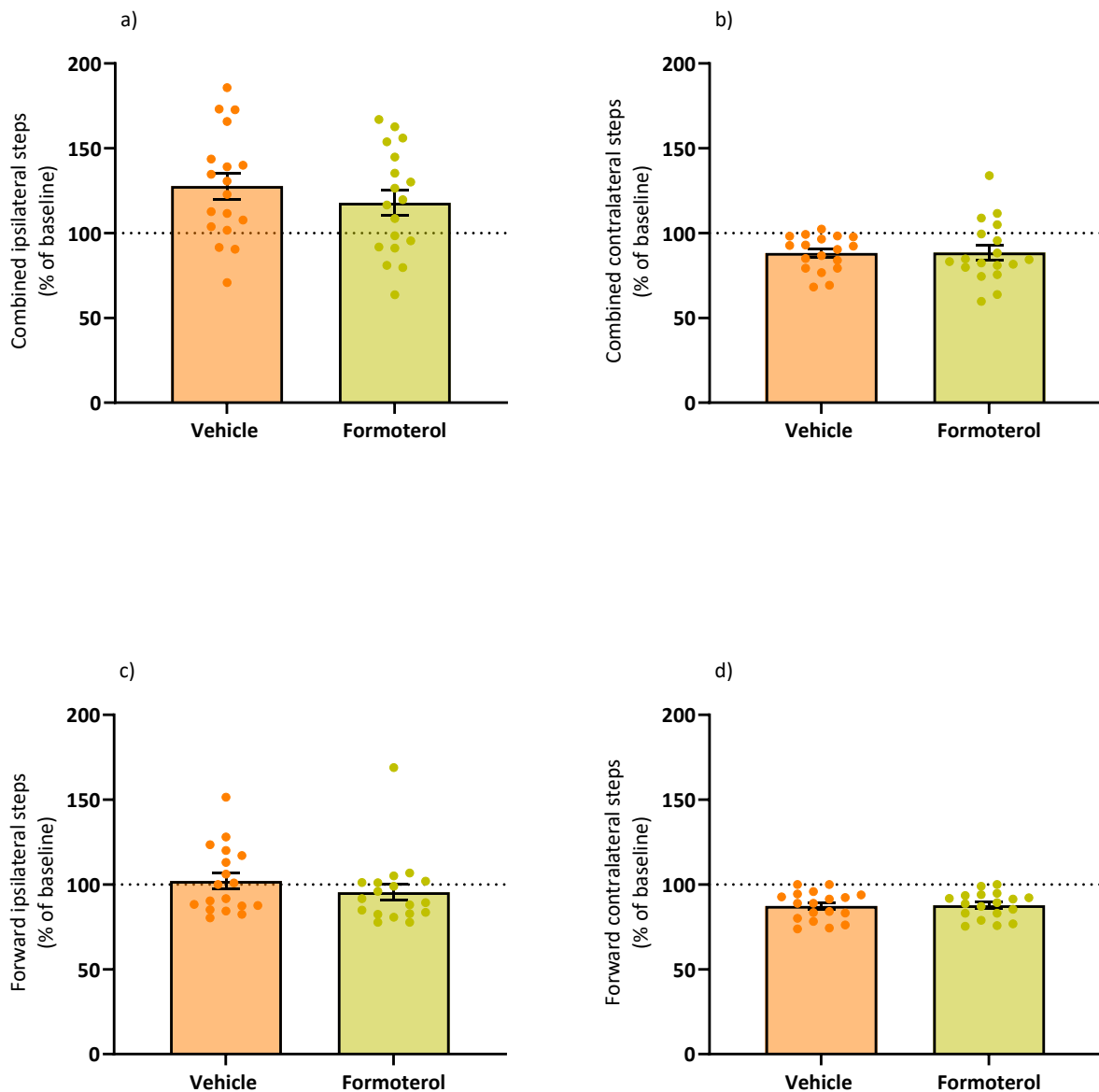


Figure 5.7. Formoterol does not impact stepping test performance four weeks after intranigral delivery of A53T PFF+AAV. a) Combined ipsilateral steps, b) combined contralateral steps, c) forward ipsilateral steps, d) forward contralateral steps, expressed as % of baseline. Data are expressed as mean $\pm$ SEM. Unpaired t-test. $n=18$.

A two-way ANOVA of combined steps expressed as % of baseline shows no effect of treatment [$F(1, 68)=0.6737$, $p=0.4146$], an effect of laterality [$F(1, 68)=34.92$, $p<0.0001$], and no interaction between treatment and laterality [$F(1, 68)=0.7414$, $p=0.3922$]. Post hoc comparisons reveal greater combined ipsilateral steps (% baseline) than combined contralateral steps (% baseline) in vehicle ($p<0.0001$) and formoterol ($p=0.0007$) treated animals (Figure 5.8a). A two-way ANOVA of forward steps expressed at % of baseline shows no effect of treatment [$F(1, 68)=0.7193$, $p=0.3993$], an effect of laterality [$F(1, 68)=9.638$, $p=0.0028$], and no interaction between treatment and laterality [$F(1, 68)=0.9579$, $p=0.3312$]. Post hoc comparisons reveal reduced contralateral forward steps (% baseline) compared to ipsilateral forward steps (% baseline) in vehicle treated animals ($p=0.0052$) (Figure 5.8b).

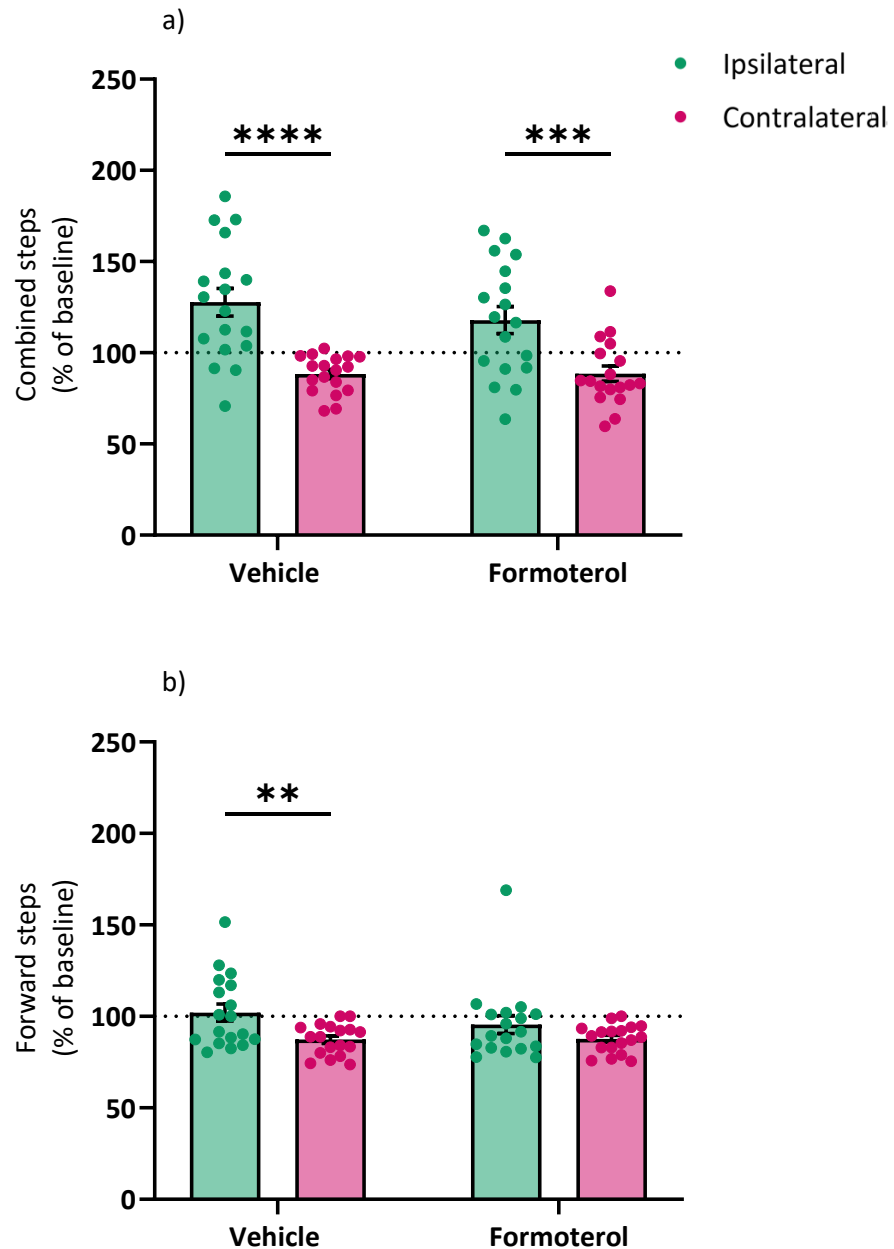


Figure 5.8. Formoterol does not impact stepping test performance five weeks post-intranigral delivery of A53T PFF+AAV. a) Combined steps expressed as a percentage of baseline, b) forward steps expressed as a percentage of baseline. Data are expressed as mean $\pm$ SEM. Two-way ANOVA followed by Fisher's test for multiple comparisons. $n=18$. **= $p\leq 0.01$, ***= $p\leq 0.001$, ****= $p\leq 0.0001$.

5.5.3. Amphetamine-induced rotations test

Students t-tests (for normally distributed data) and Mann-Whitney U tests (for non-parametric data) showed no differences in ipsilateral rotations [$U=154$, $p=0.8086$], contralateral rotations [$U=133$, $p=0.3675$], combined rotations [$U=151.5$, $p=0.7492$], or net ipsilateral rotations [$t(34)=1.343$, $p=0.1881$] between vehicle and formoterol treated animals (Figure 5.9).

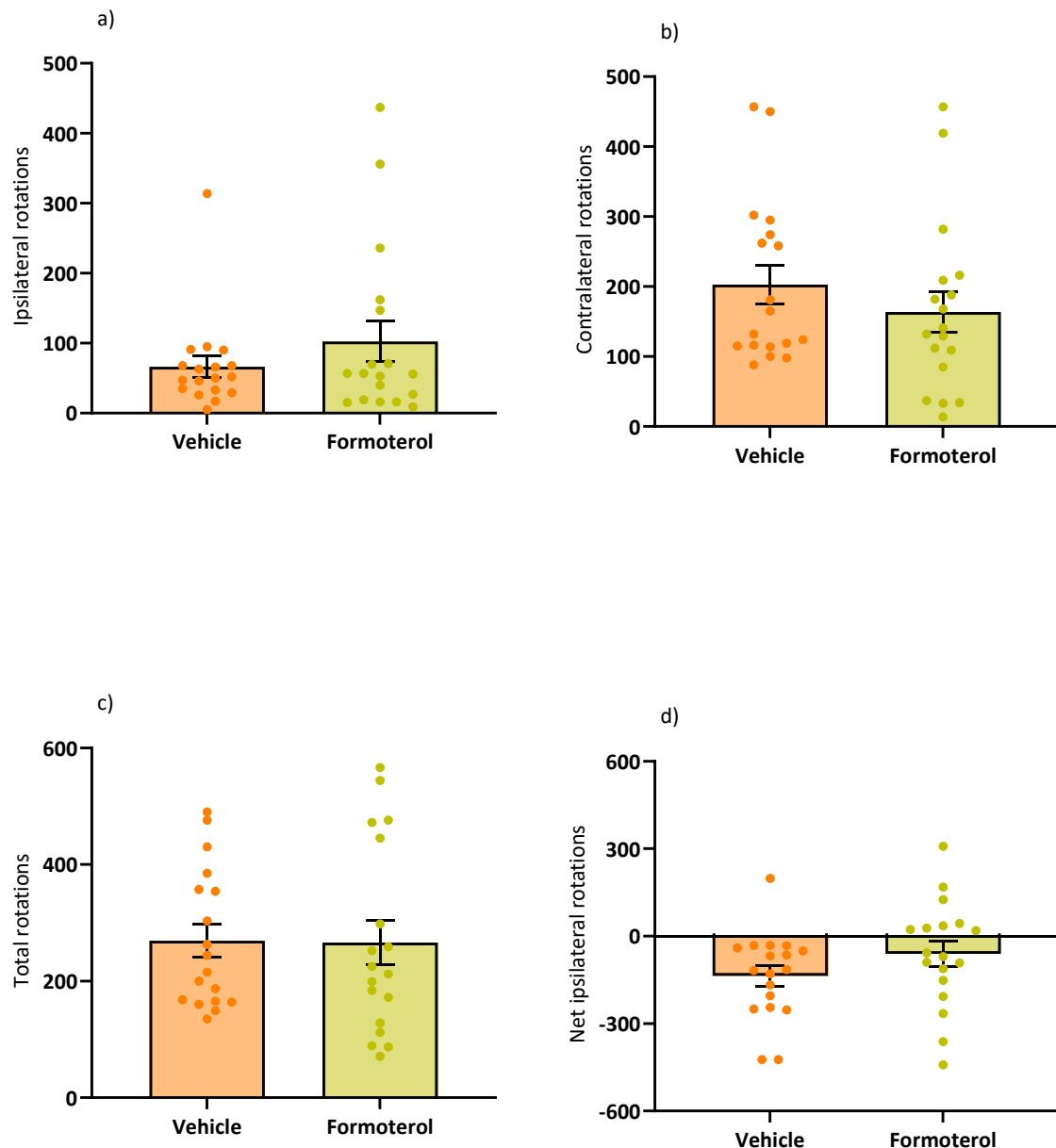


Figure 5.9. Formoterol has no effect on rotational behaviours in the amphetamine-induced rotations test six weeks post-intranigral delivery of A53T PFF+AAV. a) Number of ipsilateral rotations, b) number of contralateral rotations, c) number of total rotations, d) net ipsilateral rotations. Test duration of 90 minutes. Data are expressed as mean $\pm$ SEM. a-c) Mann Whitney U-test, d) students unpaired t-test. $n=18$.

5.5.4. pSer129 immunofluorescence

A two-ANOVA of pSer129 immunofluorescence shows no effect of treatment [$F(1, 48)=0.3380$, $p=0.5637$], an effect of laterality [$F(1, 48)=23.58$, $p<0.0001$], and no interaction between treatment and laterality [$F(1, 48)=0.09048$, $p=0.7649$]. Post hoc comparisons reveal increased pSer129 immunofluorescence in the ipsilateral hemisphere compared to the contralateral hemisphere in vehicle ($p=0.0023$) and formoterol ($p=0.0007$) treated animals (Figure 5.10a). A Mann Whitney U-test shows no effect of formoterol on pSer129 immunofluorescence expressed as a % of the contralateral hemisphere [$U=79$, $p=0.5826$] (Figure 5.10b).

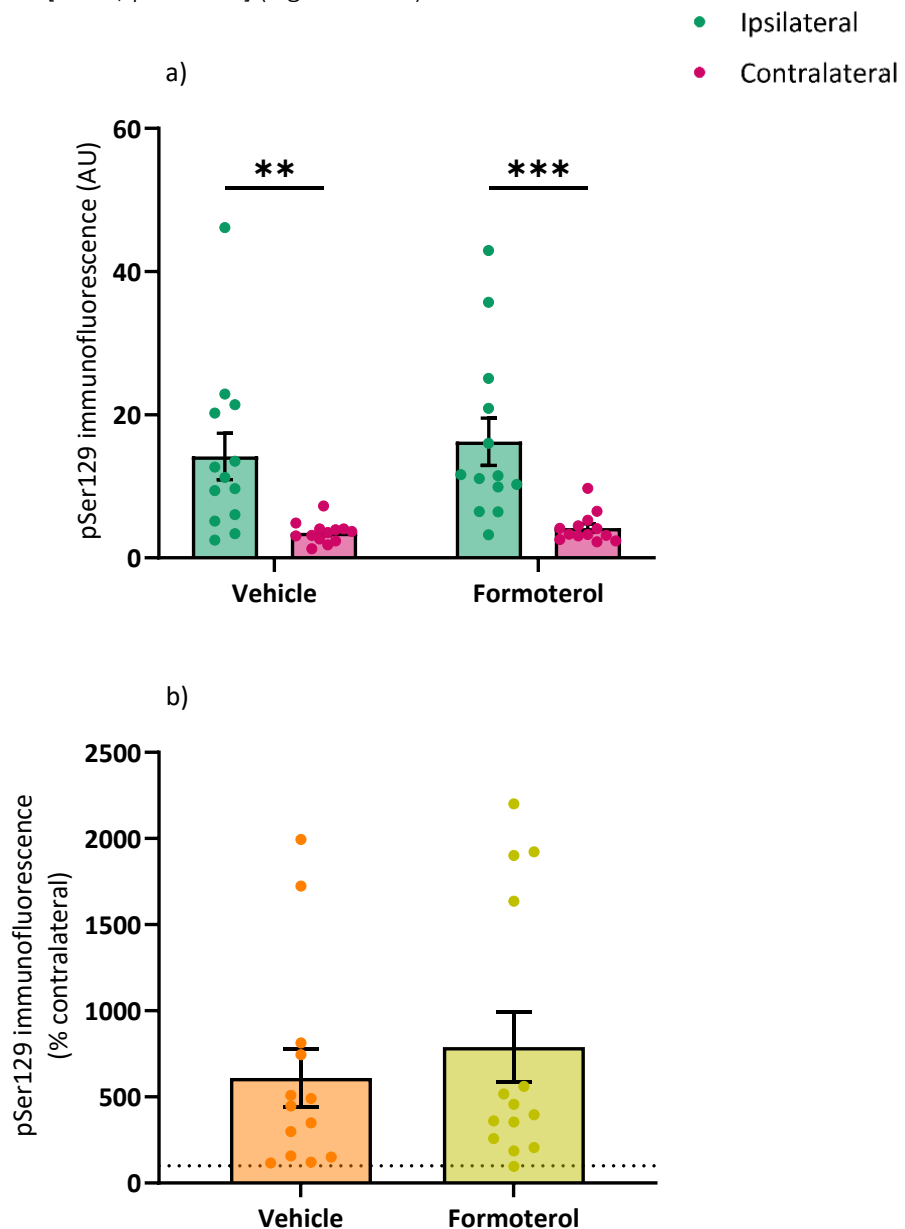


Figure 5.10. Formoterol does not influence pSer129+ α Syn immunofluorescence observed in the ipsilateral hemisphere six weeks post-intraneural delivery of A53T PFF+AAV. a) Mean grey value of pSer129 immunofluorescence in the ipsilateral and contralateral substantia nigra, b) mean grey value of pSer129 immunofluorescence in the ipsilateral hemisphere expressed as a percentage of the contralateral hemisphere. Data is expressed as mean $\pm$ SEM. a) Two-way ANOVA with Fisher's test for multiple comparisons, b) Mann-Whitney U test. $n=18$. **= $p\leq 0.01$, ***= $p\leq 0.001$.

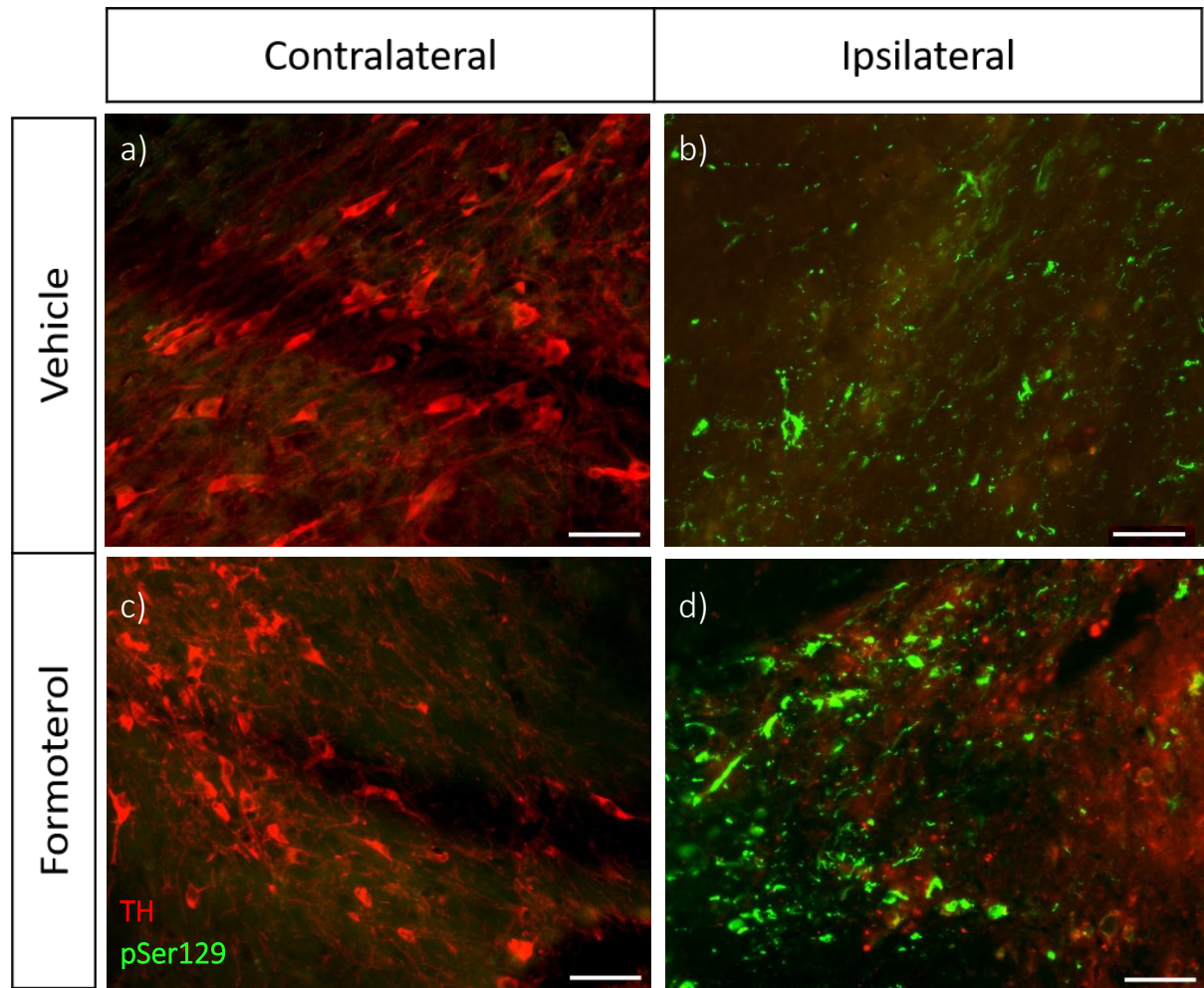


Figure 5.11. pSer129+ α Syn immunofluorescence in the ipsilateral and contralateral substantia nigra six weeks post-intranigral delivery of A53T PFF+AAV. Brain tissue was cryosectioned and fluorescently co-stained for pSer129+ α Syn and TH to confirm location in the substantia nigra. Representative 20x images show pSer129+ α Syn in green and TH+ cell bodies in red. a) Contralateral hemisphere of A53T PFF+AAV + vehicle, b) ipsilateral hemisphere of A53T PFF+AAV + vehicle, c) contralateral hemisphere of A53T PFF+AAV + formoterol, and d) ipsilateral hemisphere of A53T PFF+AAV + formoterol. Scale bar=50 μ m.

5.5.5. Iba1 immunohistochemistry

A two-way ANOVA of Iba1+ cell counts shows no effect of treatment [$F(1, 64)=0.6979$, $p=0.4066$], an effect of laterality [$F(1, 64)=51.96$, $p<0.0001$], and no interaction between treatment and laterality [$F(1, 64)=0.04236$, $p=0.8376$]. Post hoc comparisons reveal increased Iba1+ cell counts in the ipsilateral hemisphere compared to the contralateral hemisphere in both vehicle ($p<0.0001$) and formoterol ($p<0.0001$) treated animals. Mann Whitney U-test shows no effect of formoterol on Iba1+ cell counts expressed as a % of the contralateral hemisphere [$U=131$, $p=0.6584$] (Figure 5.12).

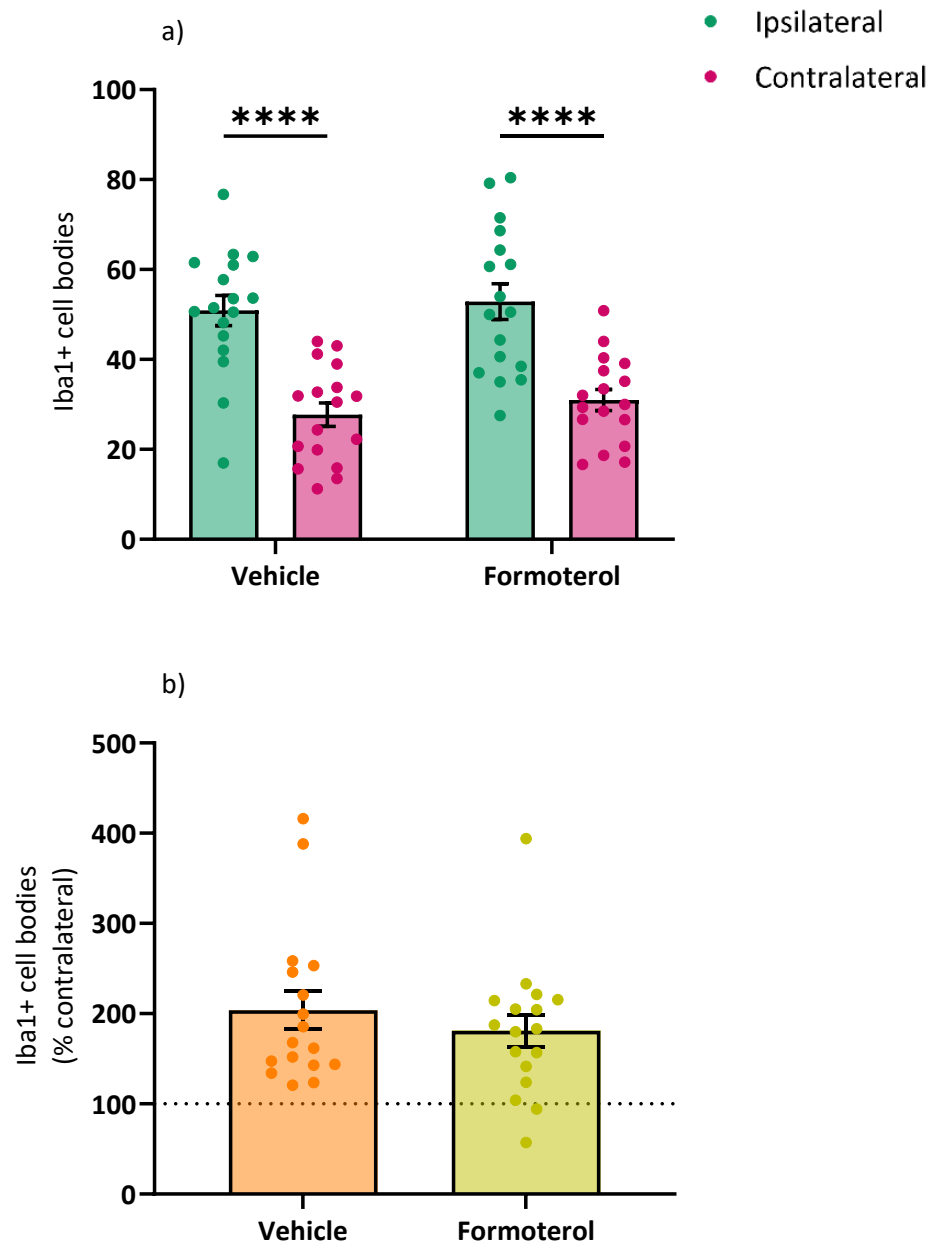


Figure 5.12. Formoterol does not influence the number of Iba1+ microglia in the ipsilateral hemisphere six weeks post-intranigral delivery of A53T PFF+AAV. a) Number of Iba1+ cells in the ipsilateral and contralateral substantia nigra, b) number of Iba1+ cells in the ipsilateral hemisphere expressed as a percentage of the contralateral hemisphere. Data are expressed as mean $\pm$ SEM. a) Two-way ANOVA followed by with Fisher's test for multiple comparisons, b) Mann Whitney U-test. $n=18$. **** $p\leq 0.0001$.

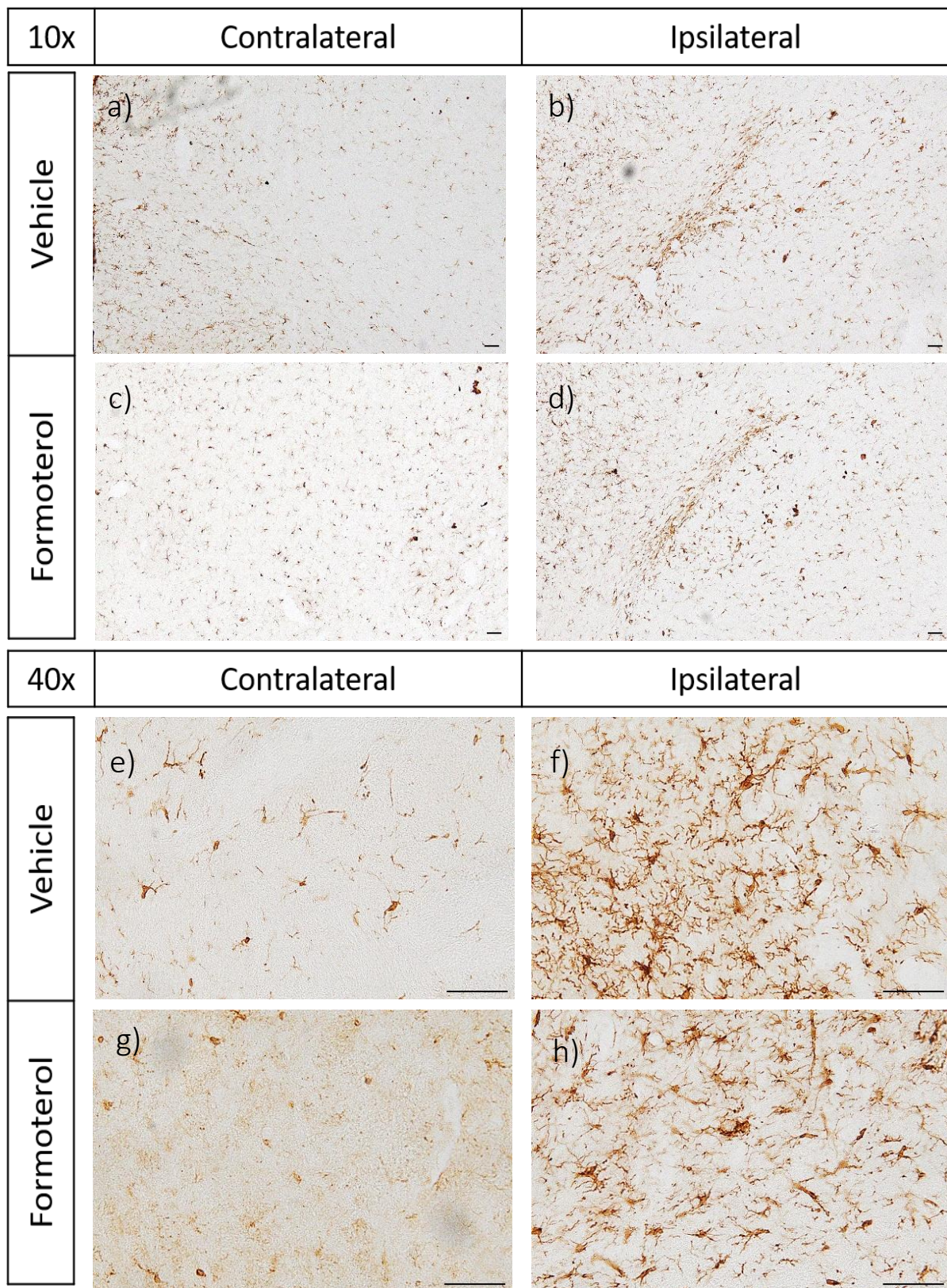


Figure 5.13. Formoterol does not influence the number of Iba1+ microglia in the ipsilateral hemisphere six weeks post-intranigral delivery of A53T PFF+AAV. Brain tissue was cryosectioned and immunohistochemically stained for Iba1 in the substantia nigra. Representative 10x (top panels) and 40x (panels) images show Iba1+ microglia in the a,e) contralateral hemisphere of A53T PFF+AAV + vehicle, b,f) ipsilateral hemisphere of A53T PFF+AAV + vehicle, c,g) contralateral hemisphere of A53T PFF+AAV + formoterol, and d,h) ipsilateral hemisphere of A53T PFF+AAV + formoterol. Scale bar=50µm.

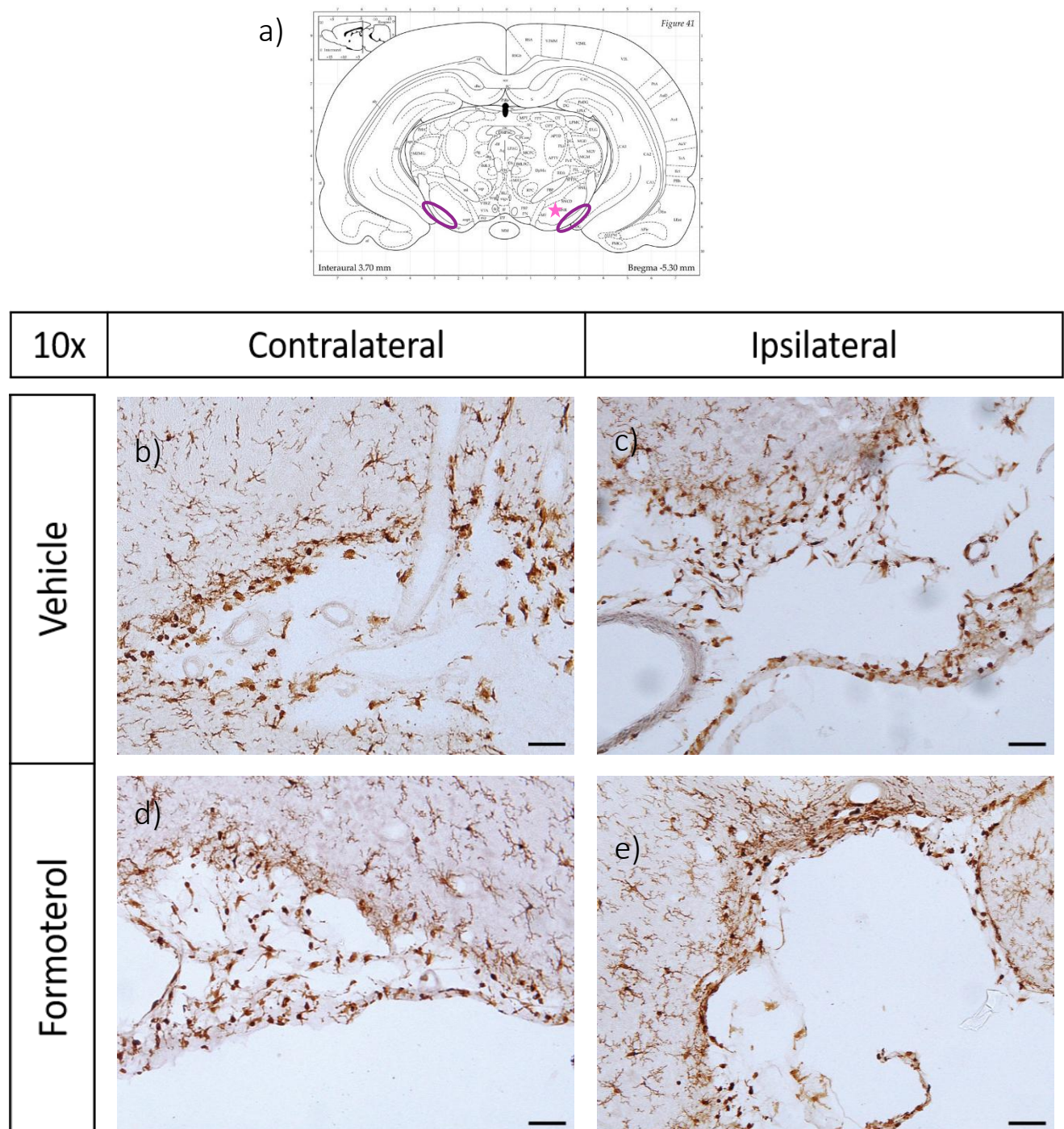


Figure 5.14. Delivery of A53T PFF+AAV into the substantia nigra triggers microglial mobilisation beyond the injection site. Midbrain tissue sections from A53T PFF+AAV-treated animals display Iba1+ microglia in ventricular spaces along outer ventral layers of the cortex and between the cortex and midbrain. a) Annotated atlas map of Wistar rat brain region of interest. Ovals depict region of brain section imaged. Star depicts injection site. Representative 10x images show Iba1+ microglia in the ventricular space between the inner cortex and outer midbrain of b) contralateral hemisphere of vehicle-treated animals, c) ipsilateral hemisphere of vehicle-treated animals, d) contralateral hemisphere of formoterol-treated animals, e) ipsilateral hemisphere of formoterol-treated animals. Scale bar=50µm.

5.5.6. TH Immunohistochemistry

A two-way ANOVA of TH+ cell bodies shows no effect treatment [$F(1, 58)=0.4058$, $p=0.5266$], an effect of laterality [$F(1, 58)=10.19$, $p=0.0023$], and no interaction between treatment and laterality [$F(1, 58)=2.133$, $P=0.1496$]. Post hoc comparisons reveal reduced TH+ cell bodies in the ipsilateral hemisphere compared to the contralateral hemisphere in vehicle treated animals ($p=0.0014$) (Figure 5.15a). A Mann Whitney U-test of TH+ cell bodies expressed as a % of contralateral hemisphere fails to identify a difference between treatment groups [$U=77$, $p=0.0933$], although a trend suggests a reduction in TH+ cell body loss in the ipsilateral hemisphere of formoterol treated animals compared to vehicle treated animals (Figure 5.15b).

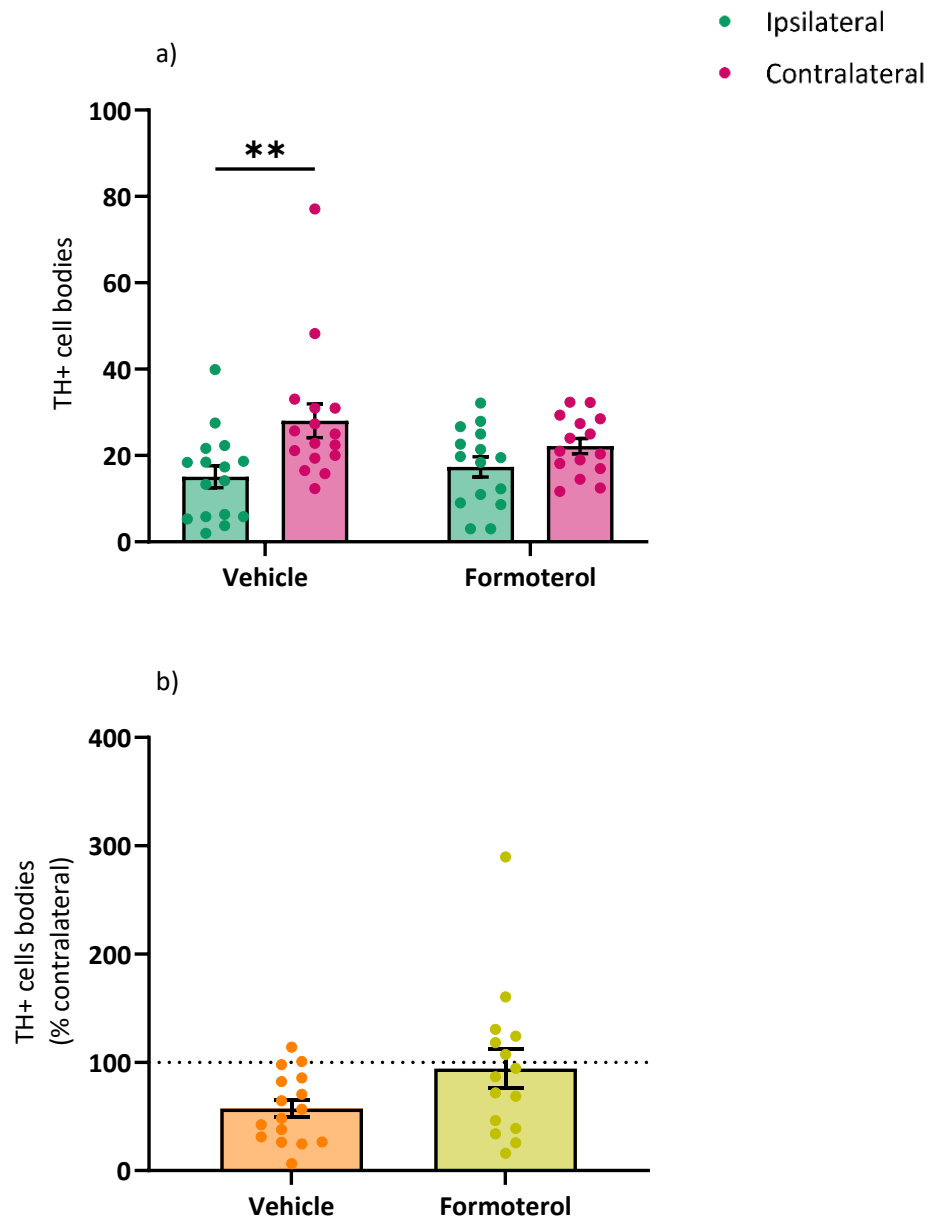


Figure 5.15. Formoterol partially mitigates the reduction in TH+ cell bodies observed in the ipsilateral hemisphere six weeks post-intranasal delivery of A53T PFF+AAV. a) Number of TH+ cell bodies in the ipsilateral and contralateral substantia nigra, b) number of TH+ cell bodies in the ipsilateral hemisphere expressed as a percentage of TH+ cells on the contralateral hemisphere. Data are expressed as mean $\pm$ SEM. a) Two-way ANOVA with Fisher's post hoc test, b) Mann Whitney U-test. $n=18$. **= $p<0.01$.

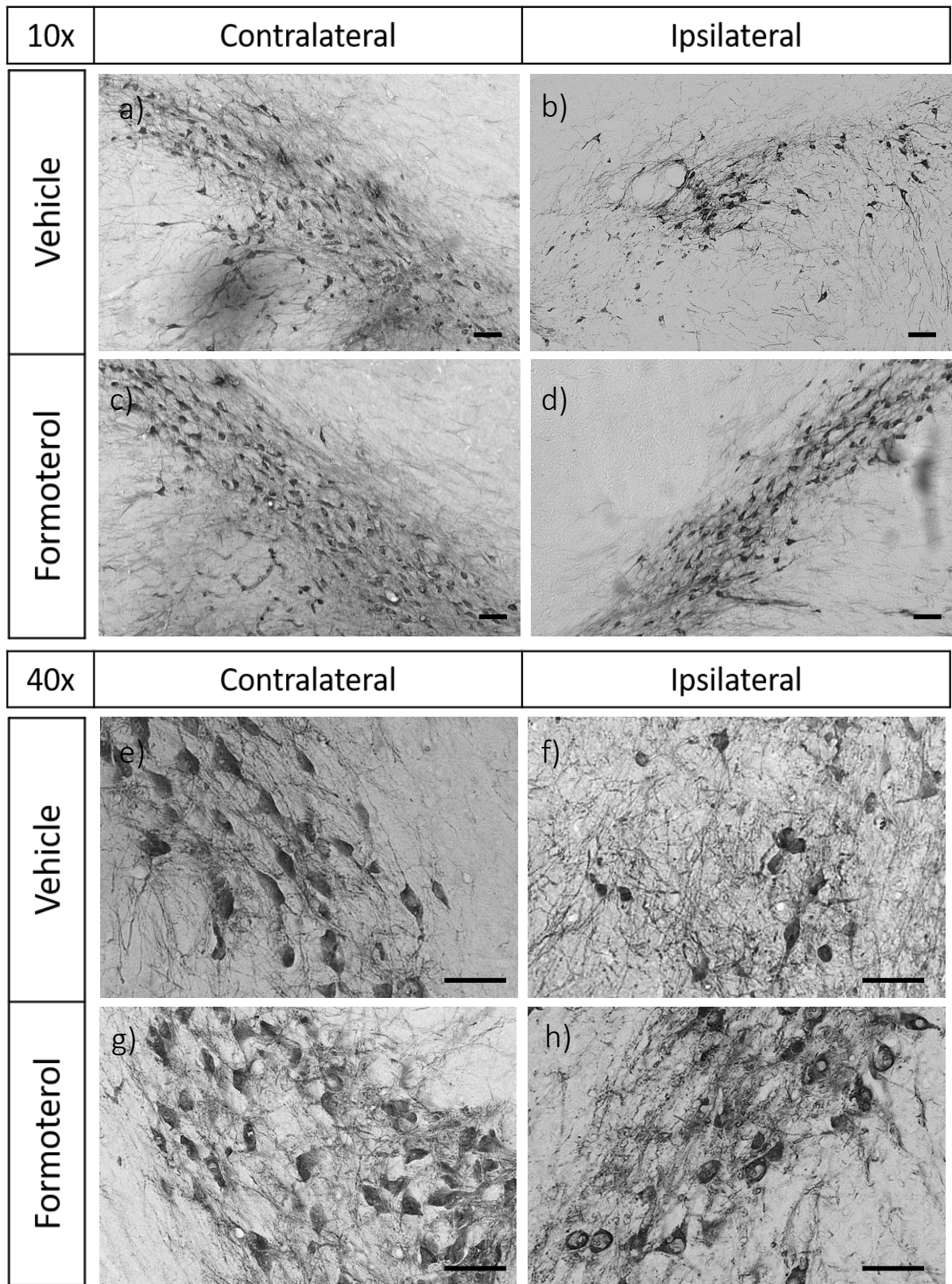


Figure 5.16. TH+ cell bodies in the ipsilateral and contralateral hemisphere six weeks post-intranigral delivery of A53T PFF+AAV. Brain tissue was cryosectioned and immunohistochemically stained for TH in the substantia nigra. Representative 10x (bottom panels) and 40x (top panels) images show TH+ cell bodies in the a,e) contralateral hemisphere of A53T PFF+AAV + vehicle, b,f) ipsilateral hemisphere of A53T PFF+AAV + vehicle, c,g) contralateral hemisphere of A53T PFF+AAV + formoterol, and d,h) ipsilateral hemisphere of A53T PFF+AAV + formoterol. Scale bar=50µm.

5.5.7. Iba1, TH, pSer129 correlation

Correlation analyses show trends of a strong negative correlation between number of Iba1+ cell bodies and number of TH+ cell bodies (expressed as a % of the contralateral hemisphere) in vehicle-treated animals [$r(10) = -0.466$, $p = 0.1747$] (Figure 5.17a), and a weak negative correlation in formoterol-treated animals [$r(12) = -0.239$, $p = 0.4547$] (Figure 5.17b). Correlation analyses show trends of a strong negative correlation between pSer129+ immunofluorescence and number of TH+ cell bodies (expressed as a % of the contralateral hemisphere) in vehicle treated animals [$r(10) = -0.5030$, $p = 0.1384$] (Figure 5.17c), and a weak negative correlation in formoterol-treated animals [$r(12) = -0.1648$, $p = 0.6088$] (Figure 5.17d). Correlation analyses show trends of a weak negative correlation between pSer129+ immunofluorescence and number of Iba1+ cell bodies (expressed as a % of the contralateral hemisphere) in vehicle treated animals [$r(10) = -0.1830$, $p = 0.6128$] (Figure 5.17e), and a weak negative correlation in formoterol-treated animals [$r(12) = -0.1715$, $p = 0.0294$] (Figure 5.17f).

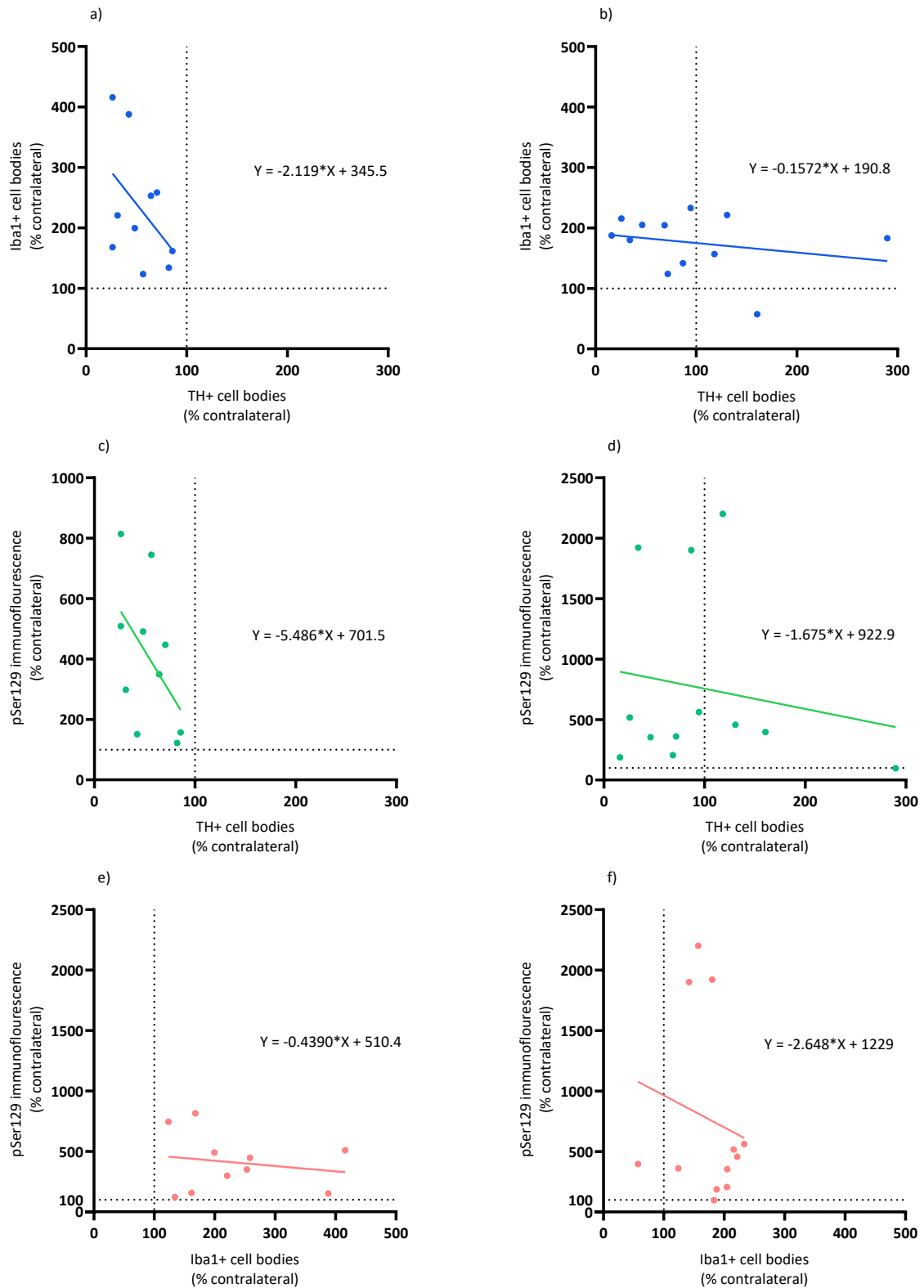


Figure 5.17. Correlation analysis between pSer129+ immunofluorescence, Iba1+ microglial cells counts, and TH+ neuronal cell counts. Correlation plot of Iba1+ cell bodies and TH+ cell bodies expressed as a % of the contralateral hemisphere in a) vehicle and b) formoterol-treated animals. Correlation plot of pSer129+ immunofluorescence and TH+ cell bodies expressed as a % of the contralateral hemisphere in c) vehicle and d) formoterol-treated animals. Correlation plot of pSer129+ immunofluorescence and Iba1+ cell bodies expressed as a % of the contralateral hemisphere in e) vehicle and f) formoterol-treated animals. n=10-12.

5.1. Discussion

In this study, delivery of a single unilateral 4 μ L intranigral injection of human A53T PFFs combined with AAV1/2-human A53T α Syn lead to skilled and spontaneous motor asymmetry observable four weeks post-delivery. Treatment with the β_2 AR agonist formoterol appeared to reduce A53T PFF+AAV-associated motoric asymmetry. Single unilateral injection of A53T PFF+AAV increased pSer129+ α Syn immunoreactivity, increased Iba1+ microglial cell counts, and reduced TH+ dopaminergic cell counts in the ipsilateral substantia nigra, compared to the contralateral substantia nigra, six weeks after delivery. Neither pSer129+ α Syn immunofluorescence nor Iba1+ microglial cell counts were directly affected by treatment with formoterol. Formoterol appeared to reduce the loss of TH+ neuronal cell bodies observed following A53T PFF+AAV delivery, which may indicate a neuroprotective role for formoterol in combating α Syn-induced neurodegeneration. The strong inverse relationship between Iba1+ microglial cells and TH+ neuronal cells (expressed as % contralateral hemisphere) in vehicle-treated animals, was attenuated in the formoterol cohort, as was the strong inverse relationship between pSer129 immunofluorescence and TH+ neuronal cells (expressed as % contralateral hemisphere). Furthermore, formoterol-treated animals appear to show a relationship whereby reduced pSer129 immunofluorescence correlated reduced Iba1+ microglial cells. Together, these findings suggest changes in glial-neuronal interactions associated with A53T PFF+AAV intranigral delivery, the behaviours of which are influenced by formoterol-treatment. Further experimentation will be necessary to confirm this finding.

Combined cylinder scores provide a cumulative score for the use of each limb during spontaneous exploration in the cylinder test. A53T PFF+AAV reduced the number of placements made with the forelimb contralateral to the injected hemisphere. A less apparent reduction in placements made with the limb ipsilateral to the injected hemisphere was also apparent four weeks after intranigral delivery of A53T PFF+AAV. These observations indicate robust reduction in contralateral limb function induced by α Syn administration, accompanied by mild reduction in ipsilateral limb-use. Reduced use of the ipsilateral limb in the cylinder test four weeks after PFF+AAV delivery may be indicative of habituation to the test, rather than a consequence of intranigral α Syn injection. Reduced limb-use in the cylinder test was unaltered by formoterol treatment. These results were further illustrated by expressing combined cylinder performance four weeks after intranigral delivery as a percentage of baseline performance, and as a percentage of the opposite limb. Through both methods of expressing cylinder scores, an A53T PFF+AAV-associated reduction in contralateral forelimb-use was evident, as was the absence of change associated with formoterol treatment.

Detection of treatment-associated improvements in motor dysfunction relies on the specificity of the behaviours assessed in the tests conducted. Vertical exploration (i.e. rearing) is largely controlled at the level of the spinal cord and brain stem. As such, rearing behaviours alone may provide more comprehensive insights into limb-dysfunction associated with an intranigral α Syn burden, and/or treatment-associated benefits. Isolated wall placements made while rats reared in the cylinder test further indicated a reduction in contralateral limb-use four weeks after A53T PFF+AAV delivery to the substantia nigra. While this was evident in both the vehicle and formoterol-treated groups, the broadened range of wall placement scores displayed by formoterol-treated animals four weeks after intranigral delivery, compared to the narrow range of scores of vehicle-treated animals, may indicate early signs of behavioural improvements as a result of drug treatment. When contralateral wall placements were expressed as a percentage of baseline, vehicle-treated animals displayed a 60% reduction in contralateral limb-use overtime. Contralateral limb-use in formoterol-treated animals

reduced to approximately 45% of baseline, which may reflect early evidence of mild, or ensuing improvement afforded by formoterol.

A Mann Whitney U-test comparing the two groups confirms an increase in contralateral limb-use when expressed as a percentage of baseline of formoterol-treated animals compared to vehicle-treated animals. This may indicate a potential partial effect of formoterol in attenuating α Syn-associated rearing asymmetry observed four weeks after A53T PFF+AAV delivery. Together, these results suggest that while formoterol does not impact cumulative forelimb-use in the cylinder test, it appears to mitigate the α Syn-induced reduction of more specific automated motor skills necessary for vertical exploration four weeks after intranigral delivery of A53T PFF+AAV.

In the stepping test, combined contralateral scores of both vehicle-treated and formoterol-treated animals were reduced five weeks after intranigral delivery of A53T PFF+AAV, compared to baseline. Reduced contralateral stepping scores were coupled with increased ipsilateral stepping scores over time. Expressing the number of contralateral steps as a percentage of ipsilateral steps is an approach used to demonstrate a preference towards the use of one limb over the other. When contralateral scores are expressed as a percentage of ipsilateral scores, analysis confirms a reduction in contralateral preference over time. This finding, however, does not reflect a true loss of skilled motor function over time, as a preference towards contralateral limb-use is evident in both groups at baseline. Both vehicle- and formoterol-treated animals displayed balanced use of the right and left limbs in the stepping test five weeks following intranigral A53T PFF+AAV injection. Increased use of the limb ipsilateral to the injection site may indicate a compensatory mechanism to combat a lesion affecting use of the opposite limb, although the imbalance in limb-use identified at baseline suggests this is not the case. Forward stepping scores in isolation provide little additional insight. Reduced contralateral forward stepping is evident in raw scores from both groups, and in the vehicle-treated group when stepping scores are expressed as a percentage of baseline. It appears that formoterol may influence α Syn-associated limb-use asymmetry towards regenerating a more symmetrical pattern of behaviour, although this outcome may be a result of unbalanced baseline scores, and not behaviours modified by treatment five weeks after intranigral delivery of A53T PFF+AAV.

In fact, forward stepping scores expressed as a percentage of baseline suggest a lack of skilled motor asymmetry five weeks after intranigral A53T PFF+AAV delivery. This observation contradicts that reported in the previous chapter, in which the same compounds were delivered to the substantia nigra, albeit via different protocols. The variation in behavioural outcomes raises the question of whether the delivery of the A53T PFF+AAV combination through a single injection produces different outcomes from delivery of the same compounds via two separate injections.

Throughout this body of work, two injection protocols were used to administer A53T PFFs in combination with A53T AAV to the rat substantia nigra. In Chapter 4, 2 μ L of A53T PFFs and 2 μ L of A53T AAV were delivered unilaterally through two independent injections into two different loci within the substantia nigra based on initial recommendations from the manufacturers of the PFF (StressMarq Biosciences) and AAV constructs (Vigene Biosciences). In Chapter 5, 2 μ L of A53T PFF and 2 μ L of A53T AAV were combined to produce a 4 μ L volume which was delivered unilaterally through a single injection into the substantia nigra. Subsequently, more information about the stability of the constructs was shared by the manufacturers, further justifying the combined administration of PFFs with AAV via a single 4 μ L injection to the substantia nigra.

A53T PFFs are stable at room temperature, but degrade down to oligomers or monomers if thawed on ice or exposed to non-ambient temperatures for extended periods of time. AAV1/2-human A53T α Syn must be gradually thawed from storage at -80°C and maintained on ice until ready for use in order to preserve vector integrity. Given the differences between temperatures required to maintain stability of the two reagents, in addition to their sensitivity and vulnerability to denaturation, care was taken to keep them separate throughout the surgical process to ensure optimal construct integrity at the point of stereotactic delivery.

In line with the principles of the 3Rs, which provide a framework for amending protocols to eliminate the unnecessary suffering of animals, we aimed to refine the injection protocol to minimize the length of the surgical procedure. Single unilateral injection of the combined treatment reduced the length of stereotactic surgery and thus exposure to isoflurane anaesthesia by approximately 10 minutes per surgery, which, for rats weighing between 180-250g, is highly beneficial in enhancing the recovery process.

pSer129+ α Syn immunoreactivity was evident within the ipsilateral hemisphere of all A53T PFF+AAV-treated animals. The average fluorescence intensity in the ipsilateral hemisphere was approximately seven times that of the non-injected contralateral hemisphere. pSer129+ α Syn distribution was characterised both by intracellular cytoplasmic expression in the substantia nigra, and ribbon-like structures scattered throughout the midbrain. The cell type in which this expression is observed has yet to be defined. Double immunolabelling of pSer129 with either TH or Iba1 in midbrain tissue sections would verify whether pSer129+ α Syn was localised within nigral dopaminergic neurons and/or microglia, respectively.

In this model, pSer129+ α Syn immunostaining was widespread and ubiquitously distributed across the ipsilateral midbrain of each animal six weeks after intranigral delivery of A53T PFF+AAV (Figure 5.11). Although formoterol treatment did not influence the intensity of pSer129 immunoreactivity, the aforementioned regulation of SNCA expression by β_2 AR remains of interest. An interesting follow-up investigation could assess SNCA gene expression in naïve, PFF, AAV and PFF+AAV-treated rats, with and without formoterol treatment. In addition to immunofluorescence analysis of pSer129+ α Syn, qRT-PCR, and/or Western immunoblotting analysis from brain homogenate would further shed light on a relationship between β_2 AR and SNCA expression, and further position the pharmacological targeting of β_2 ARs as a potential therapeutic strategy for combating α Syn-related pathology.

pSer129+ α Syn immunoreactivity is overtly evident six weeks after intranigral delivery of A53T PFF+AAV into the substantia nigra. As pSer129+ α Syn was not injected into the brain, and is absent in naïve and PBS-injected control animals, α Syn phosphorylation is a likely post-translational modification within the rat brain following interaction between injected materials and endogenous rodent α Syn. pSer129+ α Syn immunoreactivity observed in this model is likely representative of phosphorylation of α Syn protein derived from multiple sources, which have all resulted in hyper-phosphorylated protein. pSer129 expression may derive from PFF-induced phosphorylation of endogenous rat α Syn, phosphorylation of endogenous rat α Syn associated with AAV-induced expression of α Syn, PFF-induced phosphorylation of exogenous human α Syn, and phosphorylation of naïve endogenous rat α Syn generated by newly formed fibrils following delivery of PFF and/or AAV.

Seeding occurs intracellularly or extracellularly in neurons, microglia, and likely other cell types with cell-surface machinery to interact with aggregated proteins. The incorporation of the A53T mutation into the injected materials adds another disease-relevant factor which likely contributes to the development of pathology. Compared to other PD-associated point mutations of the *SNCA* gene, the A53T mutation has shown greater toxicity to dopaminergic neurons than the E46K mutation, while the A30P mutation appears less toxic than both A53T and E46K, when expressed in rodents via AAV-mediated gene transduction (Gaugler et al., 2012; Koprich et al., 2011; Van der Perren et al., 2015a). In line with these reports, thioflavin-T assays of α Syn variants following classic protein purification, show an ability for A30P-mutant α Syn to fibrillise, but at a slower rate than A53T-mutant α Syn (Conway et al., 2000; Winner et al., 2011). An association between the neurotoxic effects and high fibril-forming capacity of A53T-mutant α Syn has been widely reported (Decressac et al., 2012a; von Hovel et al., 2019; Winner et al., 2011). Reports of more severe TH+ cell loss, pSer129+ intraneuronal inclusions, glial activation, and/or motoric asymmetry in rodent models carrying A53T-mutant α Syn, compared to WT or E46K, suggest that A53T-associated animal models more accurately represent late-stages of progressive human synucleinopathic conditions, which constitute neuronal damage and consequential motor impairment, while models associated with other α Syn mutations better recapitulate prodromal disease states.

Supplementary reports from Winner and colleagues show comparative results after implementing an additional streptomycin sulphate precipitation step in the protocol used for the purification of recombinant protein aggregates. Using a lentivirus-mediated approach to express the same α Syn variants in the rat, α Syn variants E35K (glutamic acid to lysine substitution at 35th amino acid) and E57K (glutamic acid to lysine substitution at 57th amino acid) induce a loss of TH+ cells of approximately 50%, variants A30P and E46K induced a loss of approximately 40%, while the A53T variant induced a loss of approximately 17%, despite displaying the highest seeding propensity prior to the change in protocol. Thus, the neurotoxic properties of α Syn relate to its aggregate-forming ability, with E35K and E57K showing a greater ability to fibrillise. Following these experiments, reports from lentivirus PD rat models have ranked α Syn variants from most toxic to least toxic: E57K > E35K $\geq$ A30P $\geq$ E46K $\geq$ WT > A53T (Winner et al., 2011). Complementary *in vitro* analyses of DAT function in cultured fibroblasts and human neuroblastoma cells have shown impaired dopamine uptake by cells expressing WT and A30P mutant α Syn, while cells expressing A53T-mutant α Syn show no such impairment (Wersinger et al., 2003).

A key study to enhance the validity of the present experiments will explore regional and temporal distribution patterns of pSer129+ α Syn. Moreover, distinguishing pSer129+ α Syn derived from exogenous PFF and endogenous rodent α Syn would be an interesting future direction. Genetically, rodent α Syn is highly similar to human α Syn, and primary antibodies which differentiate between human and rat protein have not yet been successfully developed, despite claims of species-specific reactivity made by manufacturers. Although not explicitly reported in this body of work, efforts were made to identify and differentiate rat pSer129+ α Syn from injected human material. This process was difficult given it is not fully understood how PFFs behave after injection into the rat brain.

Delivery of A53T PFF+AAV via a single intranigral injection leads to a robust increase in the presence of Iba1+ microglia (Figure 5.12, 5.13) and a reduction in TH+ cell bodies (Figure 5.15, 5.16) in the ipsilateral hemisphere six weeks after delivery. The number of TH+ cells in the ipsilateral hemisphere of vehicle-treated animals was reduced to approximately 57% of the non-injected contralateral hemisphere. While the increased number of Iba1+ microglia was unchanged by formoterol, α Syn-

associated TH+ cell loss was partially reduced in formoterol-treated animals. In this group, the number of TH+ cells in the injected hemisphere remained within 5% of the non-injected hemisphere. Statistical analysis failed to report a significant difference between formoterol and vehicle-treated groups when TH+ cells were expressed as a percentage of the contralateral hemisphere, but an exploratory interpretation of the data indicates an ability of formoterol to partially attenuate TH+ dopaminergic neuronal loss associated with A53T PFF+AAV delivery.

Although the rotational behaviours measured in the amphetamine-induced rotations test are not characteristic of human conditions of degenerative disease, the motoric asymmetry induced by pharmacological stimulants can be evaluated to indirectly assess the extent of a unilateral lesion in the dopaminergic nigrostriatal tract. In the same vein, extrapolation of behavioural measures in this test may be used to assess the restorative efficacy of neuroprotective interventions which intend to target the dopaminergic system. Interestingly, rotation scores from the amphetamine-induced rotations test do not reflect the changes in TH+ cell counts in the animals treated with formoterol compared to their vehicle-treated counterparts.

Delivery of A53T PFF+AAV into the substantia nigra triggers microglial influx within the injection site, which appears to drive further glial mobilisation beyond the injection region and within the alternate hemisphere. Midbrain tissue sections from A53T PFF+AAV-treated animals display Iba1+ microglia in ventricular spaces and along outer ventral layers of the cortex and between the cortex and midbrain (Figure 5.14). This observation indicates that, in addition to microglial activation in the substantia nigra, injection of A53T PFF+AAV may promote extra-nigral microglial mobilisation, the migration of glial cells through ventricular CSF around and within the brain. Microglial morphology in the substantia was not assessed in the present investigation, how this analysis would be a welcome future direction to further identify the microglial phenotype driven in the CNS in response to intranigral PFF and AAV delivery. Upon recognition of fibrillar α Syn, microglia likely mobilise to the site of delivery and release pro-inflammatory and neurotoxic factors, which drive an inflammatory cellular cascade towards neuronal death in the substantia nigra, and interconnected neuronal networks (Drouin-Ouellet et al., 2014). Intracellular α Syn aggregation also directly interferes with cellular machinery responsible for normal physiological function.

While formoterol did not affect pSer129 or Iba1 immunoreactivity directly, treatment partially reduced the unilateral reduction in TH+ neuronal cell bodies in the substantia nigra, although findings are not statistically significant. Given formoterol treatment was administered two weeks post-delivery of A53T PFF+AAV once daily for seven days, (0.1mg/kg, i.p.), and that neuronal cell death is irreversible, the results suggest that loss of TH+ cells may commence at least 2-3 weeks after intranigral delivery. In keeping with a working hypothesis that targeting β_2 ARs may reduce α Syn-associated neurodegeneration through regulation of microglial activation, the results did not provide evidence in support of formoterol's ability to reduce the α Syn-associated increase in Iba1+ microglia and TH+ cell loss in the substantia nigra.

Microglia maintain a presence in the ipsilateral hemisphere both in vehicle-treated animals, in which TH+ loss is sustained, and in formoterol-treated animals, in which TH+ loss is partially alleviated. Correlation analysis shows a strong negative correlation between Iba1+ positive cells and TH+ cells in vehicle-treated groups, a relationship which dampened by formoterol. Similarly, a strong negative correlation between pSer129+ immunofluorescence and TH+ cell bodies also appears to be dampened by formoterol. These relationships reflect an association between formoterol-mediated

changes in microglial and neuronal interactions within the substantia nigra following delivery of A53T PFF+AAV.

The presence of microglia may be indicative of increased mitochondrial stress and regional production of reactive oxygen species by dying dopaminergic neurons in the surrounding substantia nigra six weeks after intranigral A53T PFF+AAV delivery (Fellner et al., 2013; Rocha et al., 2018; Sarkar et al., 2020). Delivery of A53T AAV+PFF likely triggers the induction of iNOS and NO production while promoting microglial mobilisation. Indeed, the chronic inflammatory component of synucleinopathies may underlie the progressive nature of the disease. In cases of PD, irrespective of the aetiology, cytolysis of dying dopaminergic neurons in the substantia nigra results in the release of immunogenic factors, in addition to otherwise intracellularly deposited α Syn, which activate proximal microglia. Sustained microglial activation subsequently leads to overt production of TNF α , IL-1 β , and IL-6, exacerbating dopaminergic neurotoxicity, which in turn amplifies microglial activation and PD-related neuropathology. Thus, the self-perpetuating cycle of reactive microgliosis and dopamine cell loss in the substantia nigra is likely to accelerate neurodegenerative disease progression (Calabresi et al., 2023; Du et al., 2018; Harms et al., 2017; Sarkar et al., 2020; Tang et al., 2014; Thakur et al., 2017; Zhang et al., 2005). While Iba1+ microglial numbers are unaffected by formoterol, the mechanism of action through which formoterol acts, ultimately leading to neuroprotection, likely still involves the β_2 AR-mediated regulation of microglia.

Further research may be conducted in the future to decipher additional mechanisms mediating the neuroprotective properties of formoterol. To further uncover a role for microglia in formoterol's β_2 AR-mediated neuroprotection, expression of additional cellular and molecular inflammatory markers could be investigated in tissue sections from these animals. In an inflammatory rat model of PD comprising intranigral LPS delivery (10 μ g/2 μ l) and systemic LPS challenge (250 μ g/kg i.p.), treatment with formoterol (0.1mg/kg i.p. once daily for 7 days), commencing 4 hours post systemic LPS challenge, attenuated Iba1+ microgliosis and TH+ cell loss, and alleviated exacerbations in intranigral LPS-driven increases in nigral CD68 and IL-1 β expression (O'Neill et al., 2020). In light of these results, it may prove useful to analyse CD68 and IL-1 β expression in tissue from the current α Syn model. Increased IL-1 β expression in the LPS model also raises the question of the involvement of NLRP3 inflammasome activation in driving inflammation both in the LPS model and in the α Syn model. NLRP3 (and proteins involved in its activation, including caspase 1), IL-1 β , and IL-18 immunolabelling in midbrain tissue from these animals would be a welcome addition to the current dataset. Moreover, histological immunolabelling of TNF α , IL-6, and iNOS expression in this tissue would clarify whether mobilisation of microglia in the ipsilateral substantia nigra was accompanied by upregulation and production of pro-inflammatory cytokines and factors in response to the presence of an α Syn burden.

Furthermore, it remains ill-understood how β_2 AR activation influences other contributors of PD pathology, including gut dysbiosis, oxidative stress, mitochondrial dysfunction, ubiquitin-proteasome dysfunction, and lysosomal damage. It also remains unknown the varying degrees to which treatment with β_2 AR effects symptomology in cases of PD where genetic mutation plays a role in the onset of pathology. Mutations in GBA1, for example, the leading non-Mendelian PD risk gene, results in deficiency of the lysosomal enzyme glucocerebrosidase (GCase), leading to impaired autophagy-lysosomal pathway, ER stress, and subsequent α Syn accumulation and Lewy body formation within lysosomes (Burbulla & Krainc, 2019). Increased levels of α Syn have been reported in human iPSC-derived dopaminergic midbrain neurons from patients with GBA1-associated

parkinsonism (Aflaki et al., 2016), and in human midbrain organoids generated from GBA1^{-/-} embryonic stem cells (Jo et al., 2021). Elevated levels of α Syn were also observed in iPSC-derived neurons following treatment with the GCase inhibitor conduritol β -epoxide (CBE), and in GBA1-mutant iPSC-derived neurons (Menozzi et al., 2023). Reduced GCase activity has been shown to drive oligomerization of α Syn aggregates, thereby impeding GCase migration from the ER to the lysosome, and further reducing GCase activity, thus leading to a pathogenic feedback loop. In human neuroblastoma SH-SY5Y over-expressing exogenous α Syn, GCase activity and protein levels were reduced by 70% and 87% respectively, while protein levels were reduced only by 33% in cells expressing exogenous α Syn at levels 10-fold lower (Gegg et al., 2012). Furthermore, SH-SY5Y and hippocampal and cortical neurons treated with α Syn PFFs showed decreased GCase activity, further highlighting the relationship between GBA1-associated lysosomal damage and α Syn accumulation *in vitro* (Gegg et al., 2020; Henderson et al., 2020b). Although the precise mechanisms behind the effect of α Syn on GCase remain unclear, this bidirectional loop between GCase and α Syn depicts their pathological relationship, likely contributing to PD pathology. As this relationship can be modeled with PFFs *in vitro* and *in vivo*, it therefore remains a research interest and future direction to evaluate the efficacy by which β_2 AR activation can mitigate α Syn PFF-associated lysosomal dysfunction. Noradrenergic interference with these disease-associated pathways may prove an additional therapeutic benefit of drug repurposing. As β_2 AR agonists are not FDA-approved for the treatment of neurodegenerative disease, further evaluation of their anti-inflammatory and neuroprotective nature in experimental models and in clinical trials may determine their potential for translation to therapeutics for synucleinopathic conditions.

To further explore changes in microglia in response to formoterol following intranigral A53T PFF+AAV delivery, a future analysis of microglial morphology would help assess the effects of formoterol on the proportion of amoeboid and ramified microglia in the substantia nigra. Confocal microscopy of fluorescence Iba1⁺ co-immunolabeling with pSer129⁺ α Syn may help to depict phagocytic stage of microglia within the ipsilateral substantia nigra. A time-course analysis of Iba1⁺ microglial mobilisation relative to dopaminergic loss will be critical for identifying the temporal associations between α Syn-driven microglial activation and neuronal cell death.

β_2 AR activation by noradrenaline, or noradrenergic agonists, also alleviate astrocytic mitochondrial oxidative stress and upregulation of neurotrophic factor production, as reported by Mittal and colleagues (Mittal et al., 2017). It is therefore possible that neurotrophic astrocytes played a role in the observed attenuation of TH⁺ cell loss following treatment with formoterol in this study. An analysis of the immunoreactivity of astroglial markers, including GFAP or S β 100, following intranigral A53T PFF+AAV administration could shed light on a potential response of astrocytes in mediating inflammatory homeostasis and conferring neuroprotection to the nigrostriatal bundle. Spatial profiling or single-cell-based techniques could be employed to clarify how microglial and astrocytic functions are differentially regulated in a spatiotemporal context with respect to α Syn inclusions, and contribute to an understanding of the immunopathologic trajectory of synucleinopathies.

In some formoterol-treated animals, unilateral nigral degeneration was still evident, highlighting the need to further optimise this treatment regime in order to achieve consistent, reproducible, and definitive results. The low formoterol dose used in this study is known to be safe in rats, and efficacious in protecting the rat brain against LPS-associated dopaminergic damage and microgliosis (O'Neill et al., 2020). While this formoterol treatment regime exhibits consistent neuroprotective

effects in LPS model, a higher dose or longer treatment regime may be required to fully protect the nigrostriatal bundle from degeneration where there is evidence of an α Syn burden.

In the study presented here in Chapter 5, a seven-day treatment regime of formoterol was employed to mitigate pathology induced by A53T-mutant PFF+AAV in combination, where the combination model was selected based on results presented in Chapters 3 and 4, and the treatment protocol selected based on published literature (O'Neill et al., 2020). In Chapter 3, however, it was reported that intranigral delivery of WT PFFs alone had propensity to drive pSer129+ and Iba1+ immunoreactivity in the nigra within six weeks of delivery to the rat (Figures 3.10-3.14). Following this experiment, an additional investigation was conducted with an aim of assessing an ability for formoterol, administered over an extended 10-day treatment regime, to mitigate these PD-related pathologies, albeit likely indicative only of early-stage disease progression. WT PFFs were administered unilaterally to the rat substantia nigra. After three weeks, rats received a systemic injection of the β_2 AR agonist, formoterol (0.1mg/kg, i.p.) once daily for 10 days, to investigate a potential for pharmacological intervention to influence α Syn accumulation, microglial activation, and mild behavioural deficits following WT PFF delivery. Unexpectedly, pSer129+ immunofluorescence was not detected in brain sections of WT PFF-treated animals from this investigation, and therefore the viability of PFFs used could not be confirmed (Figure A2.8). Downstream behavioural and histological results were deemed unreliable, following various failed attempts to histologically confirm evidence of successful α Syn administration (Appendix IV, Figures A4.1-4.4). While the experiments remain inconclusive at large, it can be confirmed, at the very least, that animals were well tolerant of the extended formoterol treatment regime, an outcome can be implemented in future assessments. Moreover, this experimental design remains one which, should it be repeated, has great promise for assessing the impact of formoterol on both early-stage disease identifiers, like microglial activation and α Syn deposition, and neurodegeneration (Appendix II).

It is reasonable to suggest that chronic β_2 AR activation by noradrenergic agonists would be required in order to effectively modify α Syn-related pathologies. Remarkably, SNCA mRNA expression in the substantia nigra of mice has reportedly been reduced following a single i.p. injection of 10 and 20mg/kg of clenbuterol, but not 40mg/kg. Furthermore, reductions in nigral SNCA mRNA expression were not observed in mice which received 10, 20, or 40mg/kg of clenbuterol every 48 hours for 7 days (Patterson et al., 2022). These findings show a dose dependent effect of β_2 AR agonism on SNCA expression, which becomes desensitized upon repeated treatment and at increased doses. Clenbuterol doses as low as 0.5mg/kg, when administered twice daily for 4–7 days, have been shown to cause functional desensitization of cortical β_2 ARs in rats (Mogilnicka & Nielsen, 1986). β_2 AR desensitization in the rat aorta and adipose tissue have been reported in response to repeated subcutaneous clenbuterol administration at doses as low as 0.3mg/kg twice daily for 14 days, and 0.25mg/kg once daily for 10 days, respectively (Kazanietz & Enero, 1990; Mills & Orcutt, 1989). Other treatment possibilities to consider would include pre-treatment to instigate the effects of administered drugs in advance of introducing an inflammatory stimulus, and to offset tolerance associated with repeated administration. In 1994, a study demonstrated in two MPTP-lesioned non-human primates, the restoration of L-DOPA transport into the brain following pre-treatment with the β_2 AR agonist Isoprenaline (Alexander et al., 1994a).

In future investigations of AAV and/or PFF-mediated α Syn overexpression, care must be taken to employ appropriate controls to differentiate pathology associated with the overexpression of any foreign protein from pathology associated with the overexpression of α Syn specifically. Controls

must not be innately toxic to the dopaminergic neuronal population, which already exhibit enhanced vulnerability to damage and degradation in models of synucleinopathy.

The value of viral vector constructs which overexpress the foreign protein eGFP has been disputed. Some have reported pSer129+ α Syn inclusions and TH+ neuronal cell loss following delivery of AAV2-human WT α Syn, but not AAV2-eGFP, into the rat midbrain, demonstrating an association between pathology and α Syn expression specifically, and not protein expression in general (Taschenberger et al., 2012). Other reports have shown AAV eGFP-induced nigral neurotoxicity and dopaminergic degeneration equal to or greater than those induced by AAV α Syn (Albert et al., 2019; Klein et al., 2006; Koprach et al., 2011; Landeck et al., 2017). Multiple reports show microglial and astrocyte-mediated immune response to eGFP overexpression in the brain (Samaranch et al., 2014). Nigral eGFP expression has also been shown to influence amphetamine-induced rotational patterns in a manner similar to those observed in 6-OHDA models (Albert et al., 2019). Similarly, unilateral delivery of AAV5-eGFP (9.5×10^{12} vg/ml) to the adult Wistar rat substantia nigra has led to more profound limb-use asymmetry, as assessed in the cylinder test, and ipsilateral rotational behaviour in the amphetamine-induced rotations test, 15 weeks post-delivery, compared to PBS, empty vector, and AAV5 WT α Syn delivery. These deficits, however, were not observed within the first six weeks post-delivery, suggesting a slower onset of pathology associated with eGFP overexpression (Burke-Mullaly, 2021). AAV-eGFP may be a useful construct to control for AAV-mediated changes associated with α Syn expression, but its use is likely limited to experiments designed along a timeframe of six weeks or less.

Controls should be chosen considering their relevance to the experimental design, keeping possible adverse interactions in mind. AAV-driven overexpression of glial GDNF, for example, has the propensity to downregulate TH, and thus would be an unsuitable control for models of PD-related pathology (Penttinen et al., 2018). Empty vectors have been widely used as non-toxic controls to account for the effects of AAV-mediated α Syn overexpression in PD models (Burke-Mullaly, 2021; Koprach et al., 2010).

In this unilateral model, the non-injected hemispheres served as internal controls within each animal. Further development of this model will require the inclusion of additional independent controls to confirm study findings. α Syn monomers or filaments could be used as non-toxic recombinant protein controls to account for the effects of recombinant protein inoculation. In combination with recombinant control proteins, empty vectors, or vectors containing a scrambled α Syn sequence co-expressed with eGFP could be delivered to account for the effects of AAV-mediated overexpression of a foreign protein.

Naïve α Syn monomers or misfolded filaments, the starting materials in the generation of PFFs, have generally been accepted as suitable controls for aggregate forms of recombinant α Syn (Corbett et al., 2020; Earls et al., 2019; Fujiwara et al., 2002; Luk et al., 2016). However, caution must be used when implementing these constructs, as α Syn monomers have shown propensity to undergo spontaneous conformational changes in certain conditions, in turn driving the development of pSer129 α Syn+ aggregates in the substantia nigra of rodent models (Paumier et al., 2015). The implementation of meticulously described protocols should minimize these adverse changes (Polinski et al., 2018; Volpicelli-Daley et al., 2014).

5.2. Concluding remarks and next steps

The present investigation demonstrated that delivery of a single unilateral intranigral 4 μ L injection of human A53T PFFs combined with AAV1/2-human A53T α Syn produced comparable levels of pSer129+ α Syn immunofluorescence, Iba1+ microglial activation, and TH+ cell loss in the rat substantia nigra, to two unilateral intranigral injections of 2 μ L A53T PFFs and 2 μ L AAV1/2-human A53T α Syn. Motoric asymmetry, as measured in the cylinder test, also appeared comparable across the two experimental protocols.

The results show pSer129+ α Syn immunofluorescence, increased Iba1+ microglial cell counts, reduced TH+ cell bodies, and associated motor asymmetry in the rat substantia nigra, six weeks following unilateral delivery of A53T PFF+AAV. The presence of pSer129+ α Syn in the rodent brain following delivery of human constructs indicated an ability for exogenous non-rodent α Syn to seed the recruitment and phosphorylation of endogenous rat α Syn upon injection of A53T PFF+AAV into the nigra.

Neither pSer129 expression nor Iba1+ microglial reactivity were directly affected by treatment with formoterol. Formoterol appeared to reduce the loss of TH+ neuronal cell bodies induced by A53T PFF+AAV delivery, which may indicate a neuroprotective role for formoterol in combating α Syn-induced neurodegeneration. Formoterol-treatment appeared to reduce the inverse relationship between Iba1+ microglial cells and TH+ neuronal cells (expressed as % contralateral hemisphere) observed in vehicle-treated animals, and the inverse relationship between pSer129 immunofluorescence and TH+ neuronal cells (expressed as % contralateral hemisphere). Furthermore, formoterol-treated animals appear to show a relationship whereby reduced pSer129 immunofluorescence correlated reduced Iba1+ microglial cells. Together, these findings suggest changes in glial-neuronal interaction associated with A53T PFF+AAV intranigral delivery, the behaviours of which are influenced by formoterol-treatment. Treatment with the β_2 AR agonist formoterol, also appeared to curtail the severity of A53T PFF+AAV-associated motoric asymmetry. Further experimentation will be necessary to confirm this finding.

Although this investigation theorizes a β_2 AR-mediated process through which α Syn-induced neurodegeneration may be alleviated, the mechanisms which underscore this process remain unexplored. The cellular and molecular underpinnings of α Syn-mediated microglial activation and neuronal cell loss can be closely investigated using neurons and microglia in culture. Recapitulating prodromal early stages of pathology is crucial for targeting diseases at early stages of progression. In this model, progressive pathology occurs within a short timeframe of six weeks-post intranigral α Syn delivery, making the model suitable for high throughput testing of treatments targeting prodromal symptoms of synucleopathic disease.

Chapter 6: Utilising alpha-synuclein filaments, oligomers, and preformed fibrils to develop an *in vitro* model of PD-related inflammation and neurodegeneration

6.1. Introduction

In synucleinopathies, α Syn aggregation exists along a trajectory of polymerisation, referred to as the fibril-formation pathway. The conversion of α Syn from inherent soluble monomers into nucleated monomeric filaments, aggregated amyloid-like insoluble oligomers, and β -sheet-rich fibrillar aggregates, impedes normal processing of SNARE-mediated vesicle transport through axonal projections (Burre, 2015). While under physiological conditions α Syn remains localized to the presynaptic terminal, its aggregates can spread throughout neuronal cell bodies and neurites, causing disruption beyond the neuronal cleft. Relative to other conformers of α Syn, fibrillar constructs propagate efficiently through neuronal circuitry, recruiting naïve monomers for polymerisation and post-translational phosphorylation (Lashuel et al., 2013). Formation of pathological forms of α Syn can also materialize through missense mutation (A53T, A30P, and E46K, in the case of familial PD). Genetic multiplication of the WT gene has been observed in sporadic PD (Conway et al., 1998). Mutated α Syn and high levels of its expression have therefore been linked with progressive neurodegeneration observed in synucleinopathies.

Of the conformations in which pathogenic α Syn can exist, oligomers and fibrils have been deemed more immunoreactive and neurotoxic than naïve monomers or pathogenic filaments. However, the relative pathogenicity between fibrillar and oligomeric α Syn is still under debate. Reports from analysis conducted on brain samples from both PD patients and transgenic mice expressing α Syn demonstrate higher levels of soluble oligomers than insoluble fibrils, suggesting oligomeric constructs to be the principal mediators of α Syn-induced neurotoxicity (Sharon et al., 2003). A53T and A30P mutations, which are major genetic pre-determinants of PD, have also been linked to accelerated α Syn oligomerisation, but not fibrillisation (Conway et al., 2000). While striatal administration of oligomers induced dopaminergic depletion in the substantia nigra of WT mice, injection of small-sheet fibril fragments produced significantly greater destruction, not only of nigral dopaminergic neurons, but also of striatal dopamine terminals, resulting in severe motor dysfunction (Froula et al., 2019). Similar findings were reportedly previously by Peelaerts and colleagues, who demonstrated more robust motor dysfunction, dopaminergic cell loss, and synaptic impairment in rats following intranigral PFF delivery, compared to intranigral oligomer delivery (Peelaerts et al., 2015). This discrepancy highlights the need for continued research into the molecular properties of oligomeric α Syn.

While animal models are key for the study of PFF-associated behavioural deficit and tissue damage, the cellular and molecular mechanisms explaining these processes can more easily be elucidated with the use of cellular models. In primary mouse hippocampal neurons, human α Syn PFFs induced the formation of pSer129+ Lewy body-like α Syn inclusions, while monomers did not. Immunocytochemical analysis showed no colocalization between exogenous α Syn and endogenous phosphorylated α Syn, a finding which indicates the ability of PFFs specifically to recruit endogenous mouse α Syn to form p α Syn inclusions in culture (Earls et al., 2019). The structural architecture of PFFs can shift, giving rise to differing seeding capabilities and neurotoxic properties, as observed in primary mouse cortical neurons in culture (Suzuki et al., 2020). The seeding capabilities of fibrils may therefore be explained by interactions between the C-terminal regions of α Syn monomers within

fibrillar compounds, and their neighbouring environments. For example, in the presence of salt, intermolecular repulsion at the acidic and negatively charged C-terminal region of α Syn (residues 96-140) weakens, exposing the C-terminus, and rendering a more tightly packed fibril. The opposite effect is observed in the absence of salt, where intermolecular repulsion at the C-terminus prevails, exposing and elongating the aggregate (Suzuki et al., 2020). Fibrillar fragments have been shown to bind more readily to neuronal NKA pumps than oligomers, thereby contributing more significantly to cellular imbalance through interfering with cellular machinery (Shrivastava et al., 2015). Where fibrils can elongate and recruit endogenous monomers to promote their elongation, oligomers have no propensity to do so, rendering their seeding capability deficient (Froula et al., 2019). Human iPSC-derived neuronal cultures exhibited reduced frequency and amplitude of spontaneous Ca^{2+} oscillations, following 24-hour exposure to exogenous α Syn fibrils, but not monomers (Gribaudo et al., 2019). Taken together, research reported to date supports a hypothesis that fibrillar α Syn, which is capable of neuronal propagation, aggregation, and self-replication through recruitment of naïve monomers, likely contributes more significantly to neuronal damage than other conformers.

In addition to direct inhibition of neuronal function, α Syn aggregates can drive chronic microglial activation, a process known to be toxic to neurons in the vulnerable brain (Gao et al., 2023). In the healthy brain, microglial cells exhibit a quiescent phenotype. As the first line of immune defence in the CNS, microglia serve to protect the brain through microenvironmental surveillance, response to damaged or foreign insults, and interaction with neurons, astrocytes, oligodendrocytes, and infiltrating immune cells. In addition to homeostatic immune maintenance, microglia contribute to synapse pruning, tissue injury repair, phagocytosis, and neurotrophic support of neighbouring cell types. Upon recognition of injury or disease stimuli, microglia shift from their resting state of surveillance and maintenance to an active state of insult clearance. Microglial activation has long been regarded as a consequential hallmark of neurodegenerative disease (Gao et al., 2023). Resting microglia assume a ramified phenotype in the physiological brain, exhibiting cytoskeletal protrusions for assessing and responding to environmental changes more effectively. Upon recognition of a foreign pathogen, cellular debris, or misfolded protein, microglia transfigure into balloon-like amoeboid cells, a morphological transformation which equips them with phagocytic capabilities, and an ability to elicit the appropriate molecular immune response to the pathogen recognized. Historically, morphological changes have been the predominant feature distinguishing microglia in a resting state from those in an activated state. Recently, microglial reactivity has been characterised more dynamically, with reports incorporating the entire complement of omics features and functional consequences associated with their response to pathogenic stimuli. Morphologically, microglial activation is now defined by a variety of morphologies, in addition to simply “resting” or “active” (Kettenmann et al., 2011). Based on the pathogen recognised, activated microglia in the immunocompetent brain alter their transcriptional profile, and produce signalling molecules which enlist additional immune cells to rid the brain of insult, ultimately protecting neuronal populations from damage or death.

In neurodegenerative conditions in which the intrinsic regulatory functions of microglia are impaired, chronic microglial activation can contribute to progression of neuronal damage. Deviation from the normal structure and function of microglia in cortical or hippocampal brain regions are associated with depressive behaviours, cognitive decline, delirium, and impairments in neuroplasticity and neurogenesis. These observations have been corroborated in studies conducted with both human tissue samples and animal models of Alzheimer’s disease (Dantzer et al., 2008; Perry et al., 2007). In

a rodent stroke model, lesion size correlated positively with microglial activation (Lee et al., 2014b). Consequently, pharmacological approaches that regulate microglial activation have neuroprotective effects in reactive microglial neuroinflammatory models of neurodegenerative disease (Culmsee et al., 2004; O'Neill et al., 2020; Yssel et al., 2018; Zheng et al., 2015).

Dopaminergic nigral neurons are particularly vulnerable to intracellular α Syn aggregation in the PD brain. The Braak staging hypothesis, however, suggests α Syn accumulation to occur in noradrenergic neurons of LC prior to the nigrostriatal bundle (Braak et al., 2004). The processes by which α Syn propagates through neuronal networks is relatively unknown, but likely results in the buildup of extra neuronal protein aggregates following secretion by damaged neurons. As the primary scavengers of extracellular stimuli, microglia likely become recruited to sites of secreted α Syn, and engulf the aggregates for clearance. Increased microglial numbers with ameboid morphology have been observed in brain tissue of human PD patients and animal models of synucleinopathy, supporting a likely interaction between α Syn aggregates and microglial activation (Ouchi et al., 2005; Smajic et al., 2022). Recombinant α Syn fibrils can activate microglia *in vitro* and *in vivo* to promote the production of IL-6, IL-1 α , IFN γ , and TNF α through NF κ B-mediated pathways. In addition, α Syn fibrillisation can trigger glial production of NO, superoxide, and other reactive oxygen species, which promote neuronal cytotoxicity (Codolo et al., 2013; Javed et al., 2020; Kettenmann et al., 2011).

Moreover, overactive microglia in the α Syn-burdened brain alter the transcription profiles of cell surface receptor MHC-II, TLRs, inflammasome components, complement factors, and phagocytic receptors (Wolf et al., 2017). Lyophilized full-length α Syn, and α Syn fragments, induced the release of pro-inflammatory cytokines IL-6 and TNF α from enriched primary mouse mesencephalic microglia, an effect which was attenuated by the addition of an α Syn neutralising antibody (Rabenstein et al., 2019). Increased levels of TNF α and IL-6 have been reported by Harms and colleagues, who observed additional surges in glial IL-1 α and IL-17 secretion, and MHC-II and iNOS expression in primary microglial cultures treated with PFFs (Harms et al., 2017). Alvarez-Erviti and colleagues showed an ability for α Syn released by cultured neurons to elicit microglial response in culture. conditioned media from SH-SY5Y neuroblastoma overexpressing A53T-mutant α Syn elicited an increase in IL-1 β mRNA expression and protein release from BV2 microglia, while direct application of WT monomeric α Syn (5 μ M) to BV2 microglia for 48 hours promoted TNF α gene and protein upregulation, and IL-1 α gene upregulation (Alvarez-Erviti et al., 2011). Su and colleagues corroborated these findings in enriched primary mouse microglial cultures, and further suggested the involvement of the CD36 scavenger receptor in mediating a glial response to WT α Syn (Alvarez-Erviti et al., 2011; Su et al., 2008).

Mechanistically, the activation of microglia by aggregated α Syn is thought to be orchestrated directly through glial-expressed transmembrane TLRs. A key finding reported by Stefanova and colleagues highlighted TLR4 as a chaperone for α Syn-mediated microglial activation. In a transgenic mouse model expressing α Syn, the ablation of TLR4 exacerbated motor dysfunction and dopaminergic degeneration along the nigrostriatal tract. Resulting increased levels of α Syn in the brain therefore suggested TLR4 function to be directly related to microglial-mediated α Syn phagocytosis (or lack thereof in the case of TLR4 dysfunction) (Stefanova et al., 2011). Extracellular α Syn is recognized as a DAMP by TLR2, although its ligand activity for α Syn is conformation sensitive. As such, oligomers, but not fibrils, may undergo TLR2-mediated internalization (Kim et al., 2013). Application of A53T and WT α Syn (constructs not specified) to primary mouse astrocytes in culture, lead to the upregulation of TLR2 and TLR3 at mRNA and protein levels, with A53T-mutant constructs driving

more extreme changes than WT (Wang et al., 2018). Interestingly, while blood and brain tissue samples from PD patients displayed increased levels of TLR2 and TLR4, along with α Syn-driven glial over-activation, there is contradictory evidence from PD mouse models, which reveal uncertainty about the involvement of TLR2 and TLR4 in microglia-associated neurodegeneration (Drouin-Ouellet et al., 2014).

Chronic microglial activation and production of pro-inflammatory mediators are a common feature of PD, Alzheimer's disease, and Lewy body dementia. In synucleinopathic diseases, neurodegeneration is likely a consequence of a cyclical loop in which α Syn aggregates trigger inflammation, in turn contributing to neurodegeneration, which further perpetuates existing neuroinflammation (Harms et al., 2013). In primary rat neuron-glial co-cultures comprising 48% astrocytes, 11% microglia, and 40% neurons (1% of which were TH+ neurons), 10-day incubation with WT or A53T α Syn (likely monomers, but construct not defined) resulted in reduced dopamine uptake capability and increased production of superoxide and inducible reactive oxygen species, compared to controls. Reductions in dopaminergic neuronal capacity to reuptake dopamine in culture and production of reactive oxygen species are classical indicators of cellular stress and neuronal damage *in vitro* and *in vivo* (Gao et al., 2008; Guo et al., 2008; Liu et al., 2002; Shukuri et al., 2021). α Syn-induced neuronal dysfunction and superoxide production were ameliorated by depleting the proportion of microglia to 1% (54% astrocytes, 1% microglia, and 45% neurons), suggesting that the α Syn-induced neurotoxicity was driven through a microglial-mediated process. Moreover, direct application of α Syn to neurons had no impact on dopamine uptake (Zhang et al., 2007). These results demonstrate *in vitro* that increased dopaminergic cell death following exposure to α Syn is attributable mainly to its capacity to activate microglia (Barcia et al., 2012; Castano et al., 2002; Cunningham et al., 2009; Flores-Martinez et al., 2018; Nagatsu et al., 2000; Rabenstein et al., 2019; Schroder et al., 2018; Wang et al., 2019).

Under physiological conditions and levels of expression, TNF α is a key regulator of synaptic plasticity, synaptic transmission, astrocytic-mediated glutamate release, and other regulatory processes which support homeostasis within the CNS (Beattie et al., 2002; Santello & Volterra, 2012). Excessive TNF α release, however, drives pro-inflammatory signalling through TNF receptors (TNFR), the activation of which is closely associated with cell death signalling pathways, namely NF κ B (Sriram & O'Callaghan, 2007), cJun NH2-terminal kinase (JNK), and p38MAPK (Sabio & Davis, 2014). In naïve hippocampal neuronal cultures, high concentrations of TNF α lead to increased cell death. On the contrary, cell death was prevented following application of the same TNF α concentration to TNFR1 knockout cultures, suggesting a requirement of the TNFR1 receptor in mediating TNF α -associated cell death. These findings were demonstrated in transgenic mice modelling Alzheimer's disease, in which knockout of the TNFR1 gene prevented amyloid- β -associated neuronal death, memory loss, and learning deficits (He et al., 2007). This observation, along with complementary findings of TNF α -associated neuronal death, has provided rationale for the development of TNF α inhibitors for treating neurodegenerative diseases associated with inflammation (Li et al., 2004).

In a two-hit mouse model comprising both α Syn overexpression and inflammation indicative of PD pathology, Gao and colleagues demonstrated by genetic ablation of α Syn, the dependency of inflammation-mediated dopaminergic degeneration on neuronal α Syn expression. These findings ran parallel with *in vitro* work, which showed that application of primary microglia to primary cortical neuronal cultures generated from α Syn transgenic mice, and not α Syn knockout mice, promoted dopamine-sensitivity to inflammation-mediated neurotoxicity (Gao et al., 2008). Moreover, in

inflammatory models of PD, pharmacological agents which aimed to reduce glial activation and production of pro-inflammatory mediators also attenuated dopaminergic degeneration and motor dysfunction, and promoted the expression of neurotrophic factors (O'Neill et al., 2020; Yssel et al., 2018). Despite growing literature supporting α Syn-associated inflammation, and microglial-mediated neurodegeneration, mechanistic evidence connecting these relationships remains largely unexplored.

In a similar manner to those in the aging-brain, microglia in the α Syn-burdened brain become hyper-responsive to pro-inflammatory stimuli, and may become primed upon repeated exposure to protein aggregates in the CNS (Perry et al., 2007). Microglial priming has been defined as the enhanced and prolonged response of microglia to a relatively small homeostatic disturbance following prior activation by disease-salient stimuli. The persistent reaction drives a neurotoxic glial response. Microglia have been shown to become primed in synucleinopathies in response to the prion-like propagation and aggregation of α Syn. While primed, microglia exhibit enlarged cell somas and short dendritic arbores, and follow the characteristic upregulation of pro-inflammatory TNF α , IL-1 β , and IL-6, but also IL-10 and transformation growth factor (TGF) β .

Roodveldt, Labrador-Garrido, and colleagues investigated in primary cultures, the innate immune response of α Syn-primed microglia following stimulation with TLR-targeting ligands. They concluded that, while it largely depended on the nature of the TLR agonist challenge applied, microglia primed with WT α Syn can indeed alter the glial response, either by increasing the secretion of the pro-inflammatory cytokines IL-6 and TNF α , or by reducing the expression of the anti-inflammatory cytokine IL-13. No changes in cytokine secretion profile were observed following an LPS challenge to primed microglia. This finding suggests a differential involvement between TLR4 ligand interaction in primed and non-primed microglial states (Roodveldt et al., 2013). Similarly, systemic LPS administration generated exaggerated IL-1 β expression in mice exposed previously to α Syn via intranigral injection, compared to naïve mice (Couch et al., 2011).

Some suggest that ensuing neurotoxicity in CNS environments where microglia have become primed may be through concomitant downregulation of neuronal ligands which normally inhibit microglial activation, such as fractalkine, CD47, and CD200 (Wolf et al., 2017). In LPS-primed primary murine microglia subsequently treated with fibrillar α Syn, which has previously been shown to drive α Syn pathology *in vivo* (Luk et al., 2012a), a time-dependent increase in IL-1 β secretion was detectable after 18 hours (Gordon et al., 2018). In humans, α Syn-primed microglia in the CNS may render the brain ill-equipped to react and respond to peripheral stimuli, such as inflammation, injury, or stress. As such, synucleinopathic disease progression is likely accelerated by inflammatory insults, which, prior to α Syn accumulation, may not have posed such a threat to the immunocompetent brain. Interestingly, in the aged brain, LPS-induced proinflammatory cytokine secretion is not suppressed by IL-10, demonstrating that microglial priming associated with aging may be less sensitive to combative anti-inflammatory regulation (Wolf et al., 2017).

The generation of an effective immune response to an infection or disease-salient protein aggregate, requires a delicate balance between pro- and anti-inflammatory responses. IL-10 has potent anti-inflammatory effects in the CNS, and is essential for regulation of immune responses. However, the immunosuppressive properties of IL-10 can also be exploited by pathogens to facilitate their own survival (Iyer & Cheng, 2012). In contrast to expected outcomes, intraspinal injection of AAV-IL-10 to transgenic mice expressing human A53T-mutant α Syn upregulated microgliosis and led to early

death. IL-10 preconditioning also reduced the lifespan of transgenic mice injected with mouse α Syn PFFs in hindlimb muscles (intramuscular injection of 5 μ g PFF into each hindlimb). Moreover, sustained expression of a naturally occurring IL-10 variant (which has widely been characterised by its predominant immunosuppressive properties), led to further upregulation of microgliosis, accumulation of pathological α Syn aggregates in the midbrain and spinal cord, and autophagic impairment in midbrain and spinal cord neurons of PFF-seeded mice. Interestingly, neither IL-10 nor its variant triggered astrogliosis in the spinal cord, relative to eGFP-expressing control mice (Cockey et al., 2021). This investigation first, highlights a disconnect between microglial and astrocytic response to immune stimuli throughout various regions of the CNS axis, while also demonstrating that immunosuppressive conditioning, in this case by IL-10, may in fact exacerbate the pathological phenotype instigated by α Syn aggregates.

A cyclic relationship exists between microglial response to inflammatory stimuli and astrocytic response to microglial activation, which drives a continuous loop of inflammation in the neurodegenerating brain. In their ability to endocytose α Syn via dynamin-related pathways, astrocytes likely contribute to α Syn-induced microglial activation, inflammation, and subsequent neuronal dysfunction (Lee et al., 2008a; Lee et al., 2010). When exposed to α Syn fibrils, human embryonic stem cell-derived astrocytes exhibit increased cell surface expression of costimulatory molecules CD80, CD86 & CD40, which are necessary for T-cell activation. Colocalization of both astrocytic MHC-I and MHC-II with α Syn aggregates after astrocytic ingestion suggests a role for astrocytes as antigen presenting-cells in α Syn-associated pathologies (Rostami et al., 2020). Intracellular endocytosis of α Syn by astrocytes in primary cultures has led to the upregulation of the chemokine ligand 10 (CXCL10), chemokine ligand 2 (CCL2), and IL-6, the expression levels of which correlated with the extent of intracellular α Syn accumulation (Wang et al., 2018). In primary astrocytes, A53T-mutant α Syn triggered the upregulation of GFAP, cyclooxygenase (COX) 2, and iNOS expression, in addition to increasing secretion of TNF α and IL-1 β (Yu et al., 2018).

IFN γ is a pro-inflammatory cytokine produced by type 1 T-helper cells that plays a critical role in early immunological responses to viral and tumourigenic insult (Mamane et al., 1999). In healthy brains with robust innate immune systems, IFN γ contributes to microglial management and clearance of cellular waste and debris. In age-related neurodegenerative disease, IFN γ -producing T-cell infiltration can become enhanced as a likely result of blood-brain barrier permeability. IFN γ activation of microglia drives the JAK/STAT signalling pathway, ultimately leading to iNOS production, and activation of a pro-inflammatory immune response in the CNS (Mount et al., 2007). IFN γ -mediated iNOS production, increased microglial activation, and glial production of pro-inflammatory signalling molecules have been shown both *in vitro* and *in vivo* to be contributing factors in chronic neuropathology (Barcia et al., 2012; Mount et al., 2007; Papageorgiou et al., 2016). In male 8-week-old mice, intracerebroventricular delivery of IFN γ , over a range of concentrations (0.5, 1, 2, 4, or 8 μ g in 1 μ L) increased the number of MHC-II⁺Iba1⁺ microglia in the prefrontal cortex and hippocampus, compared to vehicle-treated mice, 1-, 3-, and 7-days post injection. Of note, both vehicle-treated animals and IFN γ -treated animals displayed increased Iba1⁺ microglia compared to non-injected animals, indicating immunogenic effects of the injection. IFN γ injection (2 μ g/ μ L), but not sham injection, stably induced the continued expression of iNOS, IL-6, IL-1 β , and TNF α mRNA in the prefrontal cortex and hippocampus, which was accompanied by reductions in proliferation and survival of immature hippocampal neurons. *In vitro*, microglia extracted from IFN γ -treated mice halted proliferation and induced apoptosis of co-cultured primary mouse neural precursor cells. In

line with the theory that the observed neuronal damage resulted from glial-mediated inflammation, microglial inhibition with the tetracyclic antibiotic minocycline, ameliorated pro-inflammatory cytokine production, and prevented neuronal damage both in *in vivo* and *in vitro*. Similarly, as IFN γ activates microglia through the JAK/STAT pathway, the JAK inhibitor ruxolitinib suppressed upregulation of MHC-II⁺Iba1⁺ microglia and mRNA expression of iNOS, IL-6, IL-1 β , and TNF α , 1-, 3-, and 7-days post IFN γ injection. In these cultures, pre-treatment with minocycline or ruxolitinib promoted neuronal cell proliferation and decreased apoptosis of immature neurons (Zhang et al., 2020).

While IFN γ alone can promote microglial proliferation and activation in culture, it can also serve as a priming agent, to evoke an exaggerated immune response from microglia subsequently stimulated with LPS or amyloid- β . The enhanced immune response ultimately leads to neuronal dysfunction, inhibition of neurogenesis, or neurodegeneration (Mount et al., 2007). While the exact significance is unknown, there exists a positive spatial and temporal correlation between expression profiles of α Syn and IFN γ in the PD brain (Liscovitch & French, 2014). Moreover, plasma and CSF levels of IFN γ are elevated in PD patients, which may reflect increased central IFN γ afforded by lymphocytes and other CNS infiltrating cells (Nakamura et al., 1992; Widner et al., 2002). Evidence for a relationship between IFN γ and microglial activation in PD has been suggested by allelic differences in the IFN γ gene between early- and late- onset PD patients (Hakansson et al., 2005; Mizuta et al., 2001). In monkeys with MPTP-induced parkinsonism, inflammation-associated dopaminergic neurodegeneration was dependent on the expression of IFN γ , without which, glial activation was not maintained (Barcia et al., 2012). As a potent inducer of inflammation, IFN γ may play a key role in exacerbating microglial activation in the α Syn-primed brain. As such, its use *in vitro* may prove useful for exploring glial and neuronal mechanisms underpinning α Syn-mediated inflammation and associated neurodegeneration.

6.2. Aims and objectives

The collective aim of the experiments described in this chapter was to assess a role for glial cells as mediators of α Syn-induced neuronal atrophy, and explore variant forms of α Syn in producing these effects *in vitro*.

Specifically, human recombinant α Syn filaments, oligomers, WT PFFs, or A53T PFFs were applied to primary cortical mixed glial or enriched microglial cell cultures to explore microglial activation state and pro-inflammatory transcription profile associated with α Syn-mediated microglial activation *in vitro*. Moreover, human recombinant primary cortical mixed glial or enriched microglial cultures were pre-treated with α Syn A53T PFFs and subsequently stimulated with IFN γ , to assess microglial activation state and resulting cytokine transcription profile. An LDH assay was used to assess cytotoxic response of glial cultures to α Syn treatment. Microglial activation state was assessed through Iba1+ immunocytochemistry and cell morphology analysis. qRT-PCR was conducted on glial cell lysate to assess changes in mRNA transcription of key pro-inflammatory cytokines IL-1 β , IL-1 α , IL-6, and TNF α in response to α Syn stimulation.

The subsequent aim was to assess the impact of the conditioned media from α Syn and/or IFN γ -treated mixed glial or enriched microglial cultures on the complexity and integrity of primary cortical neuronal cells *in vitro*. Conditioned media from glial cell cultures treated with human recombinant α Syn filaments, oligomers, or PFFs, and/or IFN γ , was applied to primary cortical neurons in culture. Neuronal integrity following conditioned media transfer was measured with an LDH assay. Neuronal complexity was assessed with MAP2 immunocytochemistry and Sholl analysis.

6.3. Experimental design

Primary cortical mixed glia were isolated from P2-3 Wistar rat neonates and cultured to maturity for 14 days in a humidified incubation chamber at 37°C with 5% carbon dioxide. Mature mixed glia and enriched glia were maintained in 24-well plates with 300µL DMEM per well. DMEM was changed every 3-4 days throughout maturation. On DIV14, growth media was removed and replaced with treatment media. For single treatment experiments, 300µL treatment was applied per well. αSyn filaments, oligomers, or PFFs were prepared in NBM at 4µg/ml and applied for 24-96 hours. IFNγ was prepared in NBM at a range of 0-500ng/ml and applied for 24 hours. For priming experiments, A53T PFFs (4µg/ml) were applied for 24 hours, followed by IFNγ (10ng/ml) for 24 hours, and in the reverse order for the reverse-priming protocol. Treatment application was randomized across culture plates to control for growth variability. After treatment, LDH assay was conducted. Mixed and enriched glia were fixed with 4% paraformaldehyde solution in preparation for Iba1+ immunocytochemical staining. Fluorescence microscopy was conducted, and morphology assessed with ImageJ. Glial cell lysate was collected for analysis of cytokine mRNA expression through qRT-PCR. Conditioned media was collected and filtered for the transfer to primary neurons.

Primary cortical neurons were isolated from P1-2 Wistar rat neonates and cultured to maturity for 14 days in a 37°C humidified incubation chamber with 5% carbon dioxide. Neurons were maintained in NBM. Media was changed every 7 days throughout maturation. On DIV14-21, neuronal media was removed and replaced with conditioned media from mixed glial or enriched glial cultures. Primary neurons were stimulated with 300µL treatment media for 24 hours in all experiments. Treatment application was randomized across culture plates to control for growth variability. 24 hours after treatment, LDH assay was conducted. Neurons were fixed with 4% paraformaldehyde solution for MAP2+ immunocytochemical staining. Fluorescence microscopy was conducted and Sholl analysis performed on MAP2+ neurons to assess neurite outgrowth and branching morphology.

For analysis of MAP2 and Iba1 immunocytochemistry, 7-10 cells were included in the analysis of each technical replicate, with each experiment comprising 3-4 technical replicates per treatment group. A minimum of 3 experiments were conducted and included in statistical analyses.

6.4. Methods

All methods were conducted as described in chapter 2.

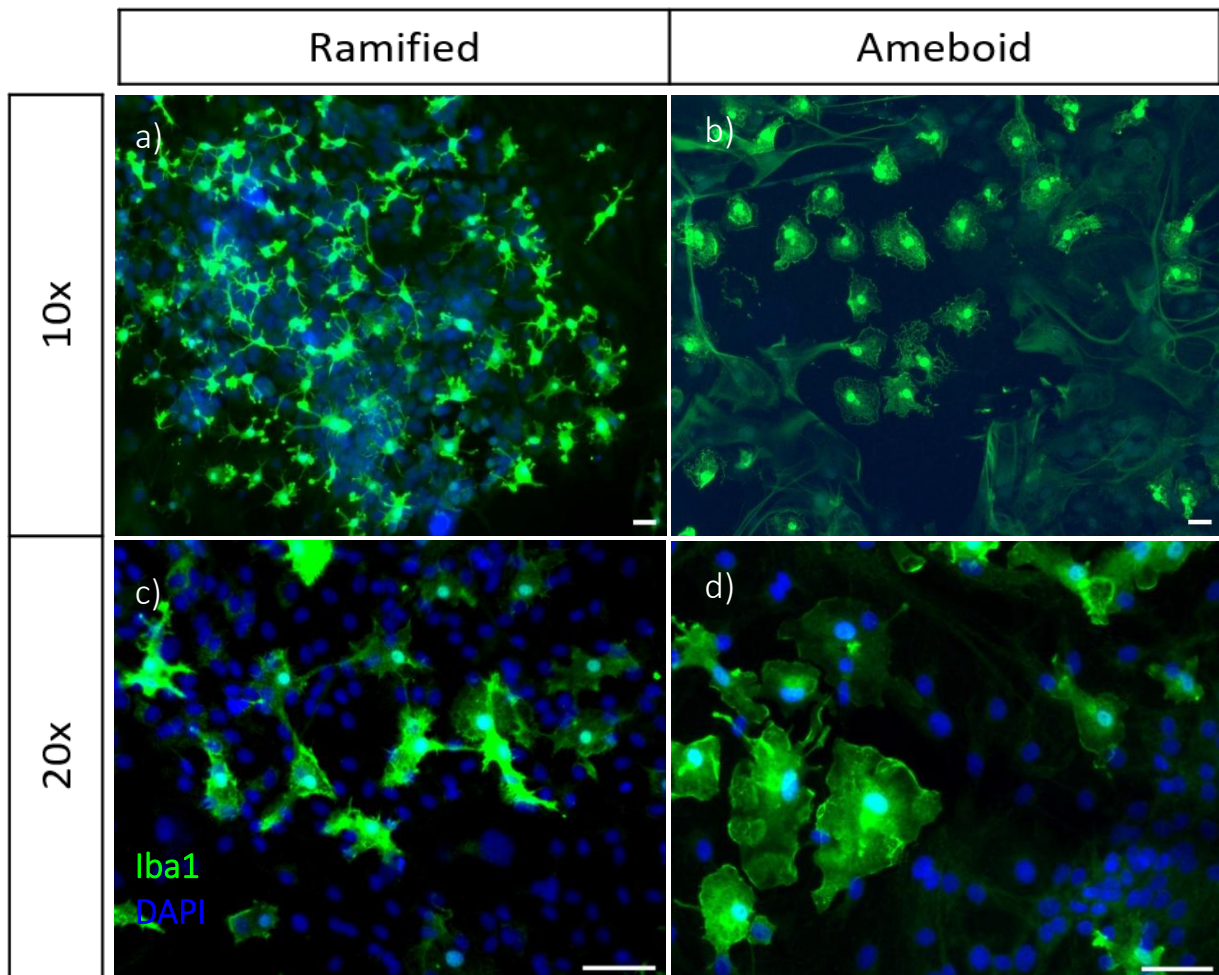


Figure 6.1. Iba1+ primary microglia in primary cortical mixed glial cultures. Representative 10x (top panel) and 20x (bottom panel) images show primary cortical microglial cells stained with Iba1 in green and DAPI in blue. a,c) ramified Iba1+ microglia in mixed glial cultures, and b,d) ameboid Iba1+ microglia in mixed glial cultures. Scale bar=50µm.

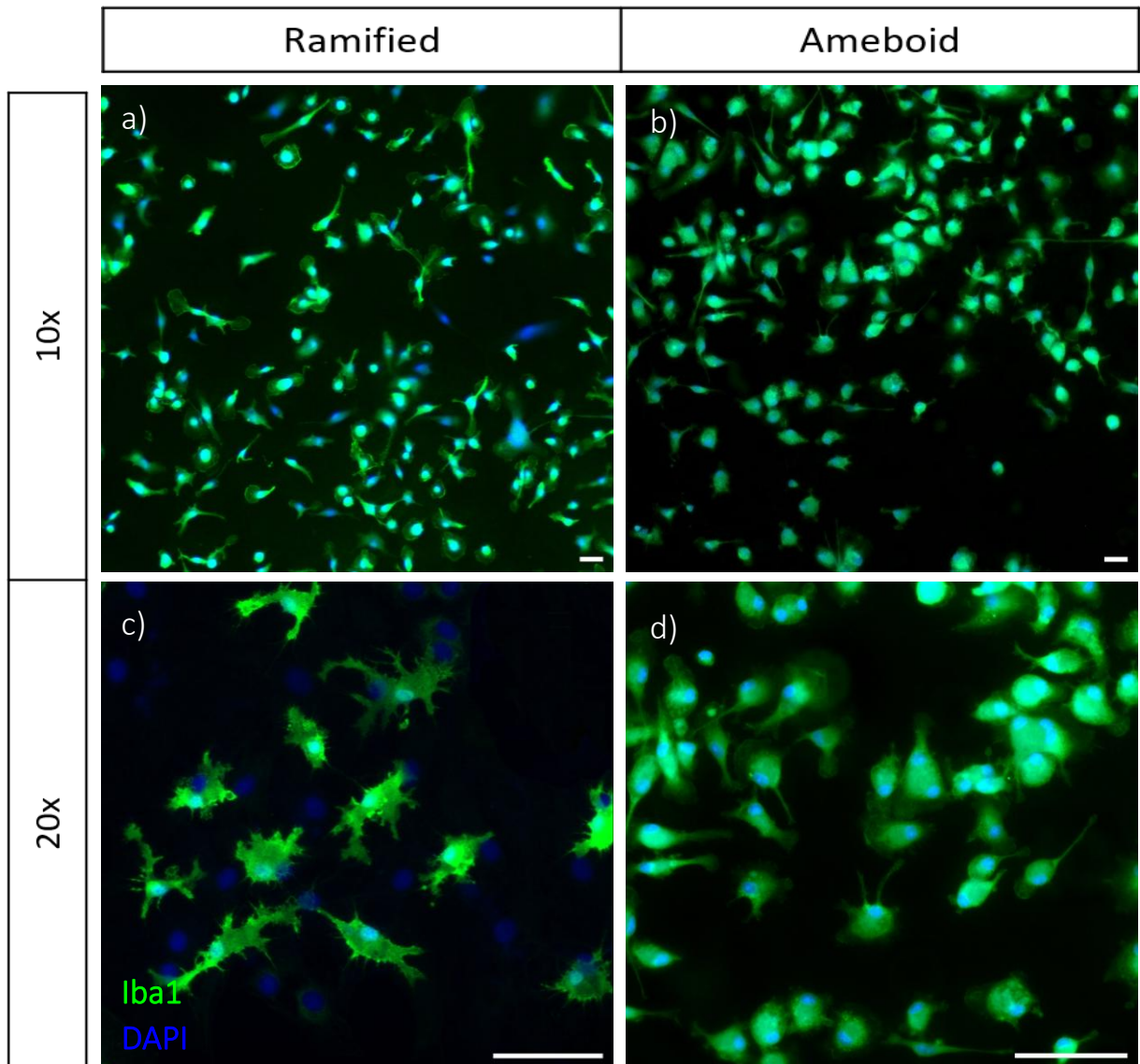


Figure 6.2. Iba1+ primary microglia in primary cortical enriched microglial cultures. Representative 10x (top panel) and 20x (bottom panel) images show primary cortical microglial cells stained with Iba1 in green and DAPI in blue. a,c) ramified Iba1+ microglia in enriched microglial cultures, and b,d) ameboid Iba1+ microglia in enriched microglial cultures. Scale bar=50µm.

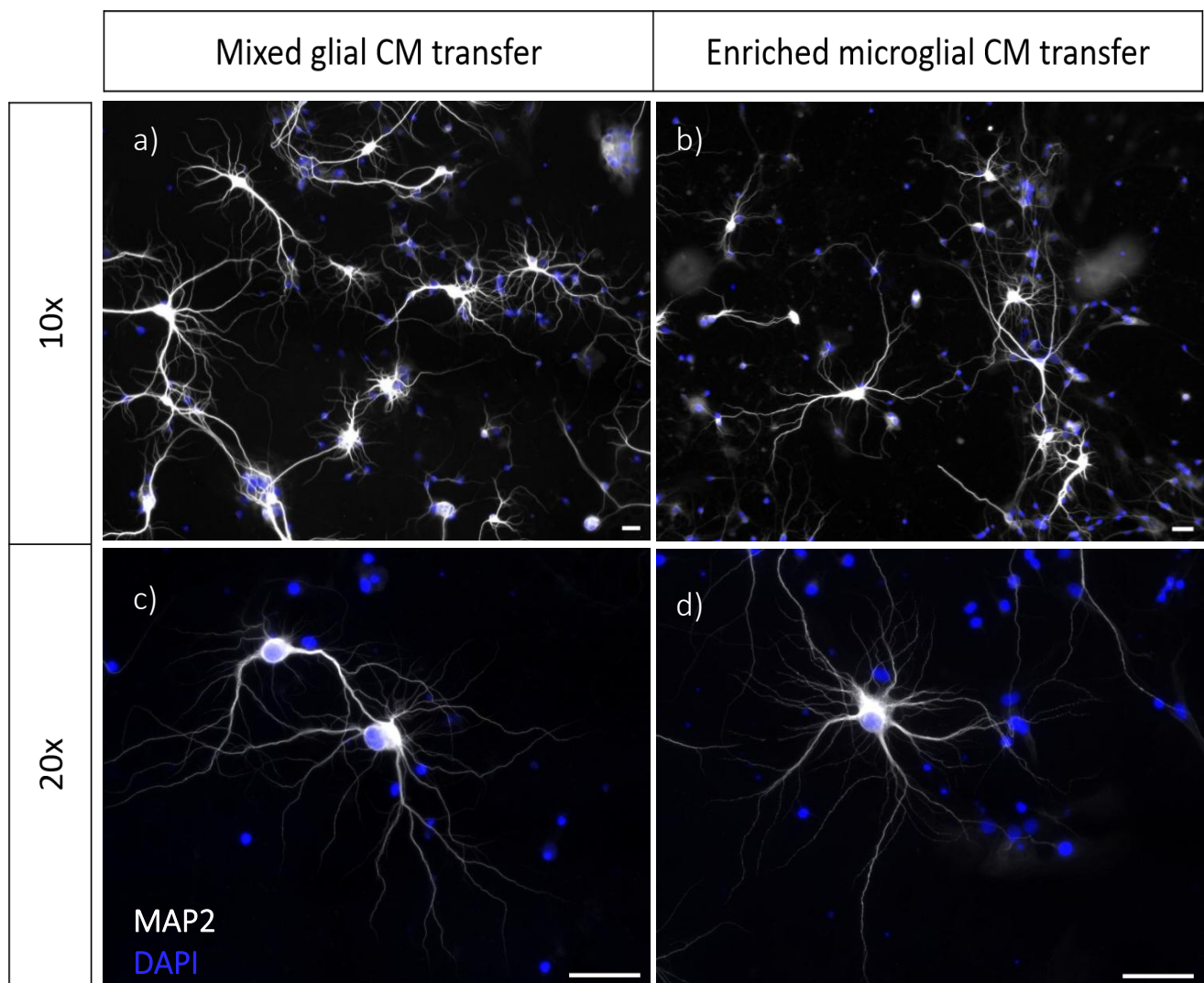


Figure 6.3. MAP2+ primary neurons treated with conditioned media transferred from primary mixed glial and enriched microglial cultures. Representative 10x (top panel) and 20x (bottom panel) images show primary cortical neuronal cells stained with MAP2 in white and DAPI in blue. Primary neurons treated with a,c) conditioned media transferred from mixed glial cultures, and b,d) conditioned media transferred from enriched microglial cultures. Scale bar=50µm.

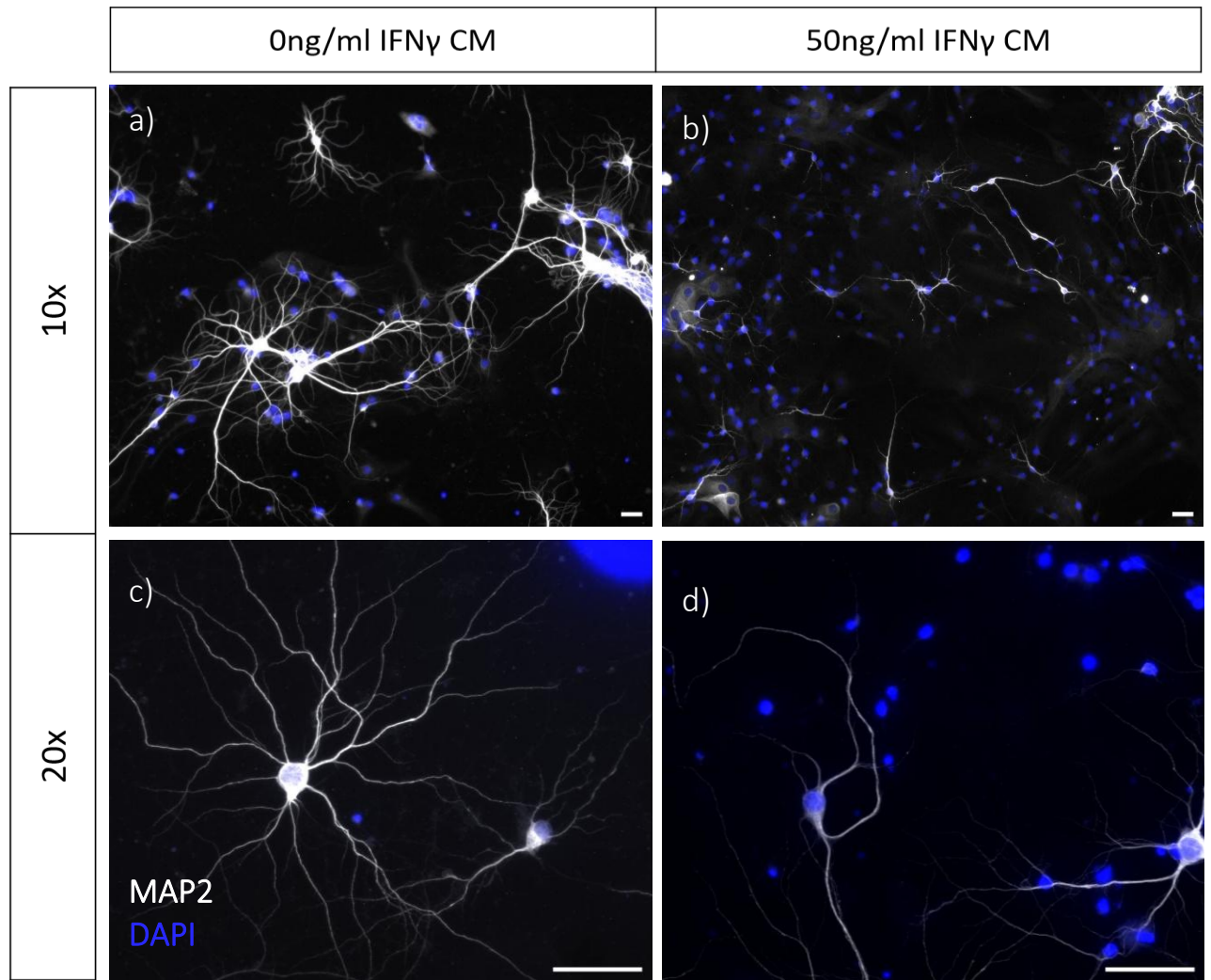


Figure 6.4. MAP2+ primary neurons treated with conditioned media transferred from enriched microglia treated with IFN γ in DMEM. Representative 10x (left panel) and 20x (right panel) images show primary cortical neuronal cells stained with MAP2 in white and DAPI in blue. Primary neurons treated with a,c) conditioned media transferred from enriched microglial glial cultures treated with 0ng/ml IFN γ , and b,d) conditioned media transferred from enriched microglial glial cultures treated with 50ng/ml IFN γ . Scale bar=50 μ m.

6.5. Results

6.5.1. Mixed glial response to 24-hour treatment with α Syn filaments, oligomers, or PFFs (4 μ g/ml).

In mixed glial cell cultures included in the following results, Iba1+ microglia comprised approximately of 25% of all DAPI+ cells (Figure 6.5a). One-way ANOVA of glial LDH activity expressed as a % of controls shows no effect of treatment with α Syn filaments, oligomers, or PFFs [$F(5, 12)=0.8422$, $p=0.5451$] (Figure 6.5b).

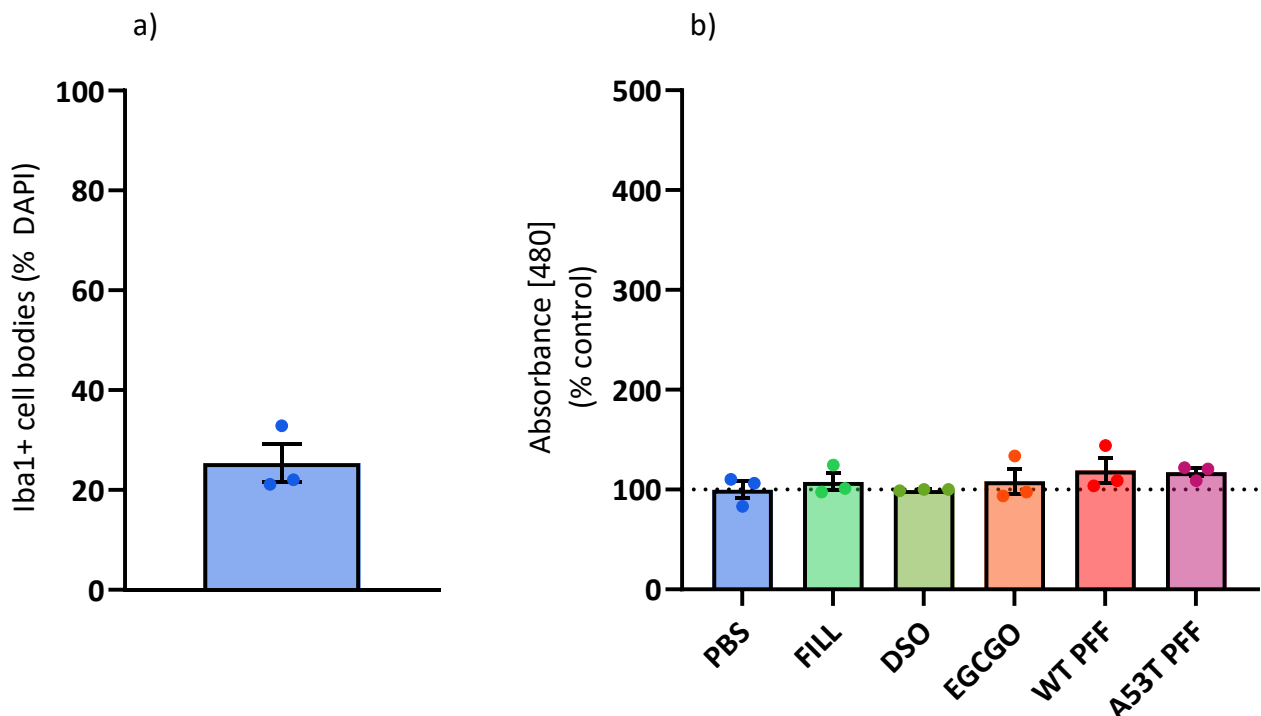


Figure 6.5. Proportion of Iba1+ cells and LDH activity in primary mixed glial cultures treated with α Syn filaments, oligomers, or PFFs for 24 hours. Mature primary cortical mixed glia (DIV14) were treated with 4 μ g/ml soluble filaments (FILL), dopamine-stabilised oligomers (DSO), epigallocatechin gallate-stabilised oligomers (EGCGO), WT PFFs, A53T-mutant PFFs, or PBS for 24 hours. a) % of Iba1+ cells in mixed glial cultures used in experiments 6.5.1-6.5.2, b) mixed glial LDH activity expressed as a percentage of PBS-treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H_2O_2). Data are expressed as mean $\pm$ SEM. One-way ANOVA. $n = 3$.

Independent one-way ANOVAs show no effect of treatment with α Syn filaments, oligomers, or PFFs on cell area [$F(5, 14)=1.692$, $p=0.2011$] perimeter [$F(5, 14)=1.136$, $p=0.3867$], Feret's diameter [$F(5, 14)=1.841$, $p=0.1693$], circularity [$F(5, 14)=0.8359$, $p=0.5457$], or solidity [$F(5, 14)=0.8728$, $p=0.5236$] (Figure 6.6a-e). One-way ANOVA shows an effect of α Syn treatment on immunofluorescence of Iba1+ microglial cells [$F(5,14)=3.432$, $p=0.0312$]. Post hoc comparisons reveal increased Iba1+ immunofluorescence in Iba1+ DSO-treated microglia compared to Iba1+ PBS-treated microglia ($p=0.02277$) (Figure 6.6f).

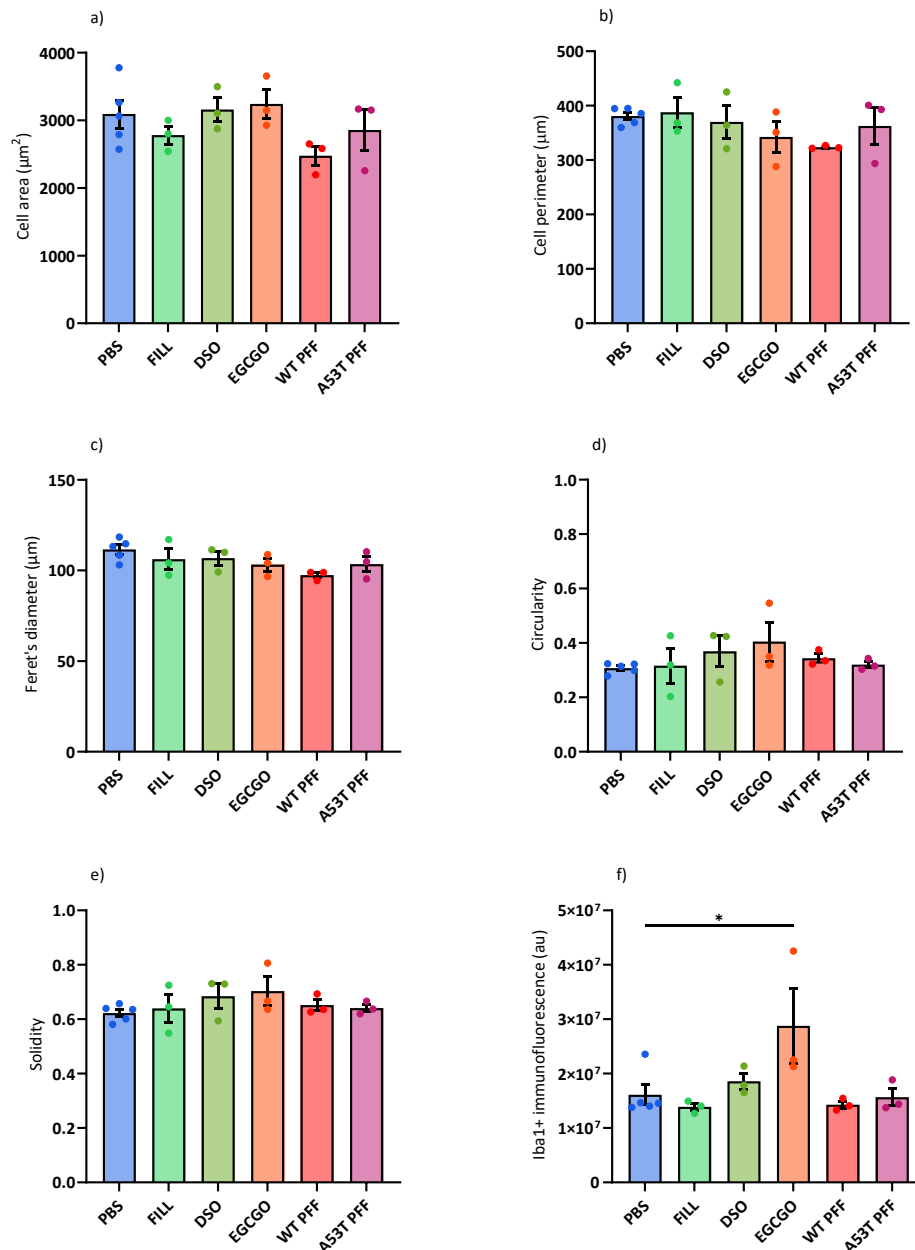


Figure 6.6. Morphology of Iba1+ microglia in primary mixed glial cultures treated with α Syn filaments, oligomers, or PFFs (4 $\mu\text{g}/\text{ml}$) for 24 hours. Mature primary cortical mixed glia (DIV14) were treated with 4 $\mu\text{g}/\text{ml}$ soluble filaments (FILL), dopamine-stabilised oligomers (DSO), epigallocatechin gallate-stabilised oligomers (EGCGO), WT PFFs, A53T-mutant PFFs, or PBS for 24 hours. Cells were fixed for Iba1 immunofluorescence. Image analysis was performed on ImageJ to quantify a) area, b) perimeter, c) Feret's diameter, d) circularity, e) solidity, and Iba1+ immunofluorescence of Iba1+ microglia. Data are expressed as mean $\pm$ SEM. One-way ANOVA followed by Dunnett's test for multiple comparisons. $n = 3-5$.

One-way ANOVA of IL-1 β mRNA expression shows an effect of treatment with α Syn [F(5, 12)=4.722=0.7901]. Post hoc comparisons reveal increased IL-1 β mRNA expression in mixed glial cells stimulated with DSO compared to PBS control (p=0.0268) (Figure 6.7a). One-way ANOVA of IL-1 α mRNA expression shows an effect of treatment with α Syn [F(5, 12)=5.783, p=0.0061]. Post hoc comparisons reveal increased IL-1 α mRNA expression in mixed glial cells treated with DSO compared to PBS control (p=0.0027) (Figure 6.7b). Independent one-way ANOVAs show no effect of α Syn treatment on IL-6 [F(5, 12)=0.1567, p=0.9738], TNF α [F(5, 12)=0.9157, p=0.5031], or IL-10 [F(5, 12)=1.558, p=0.2449] mRNA expression (Figure 6.7d-e).

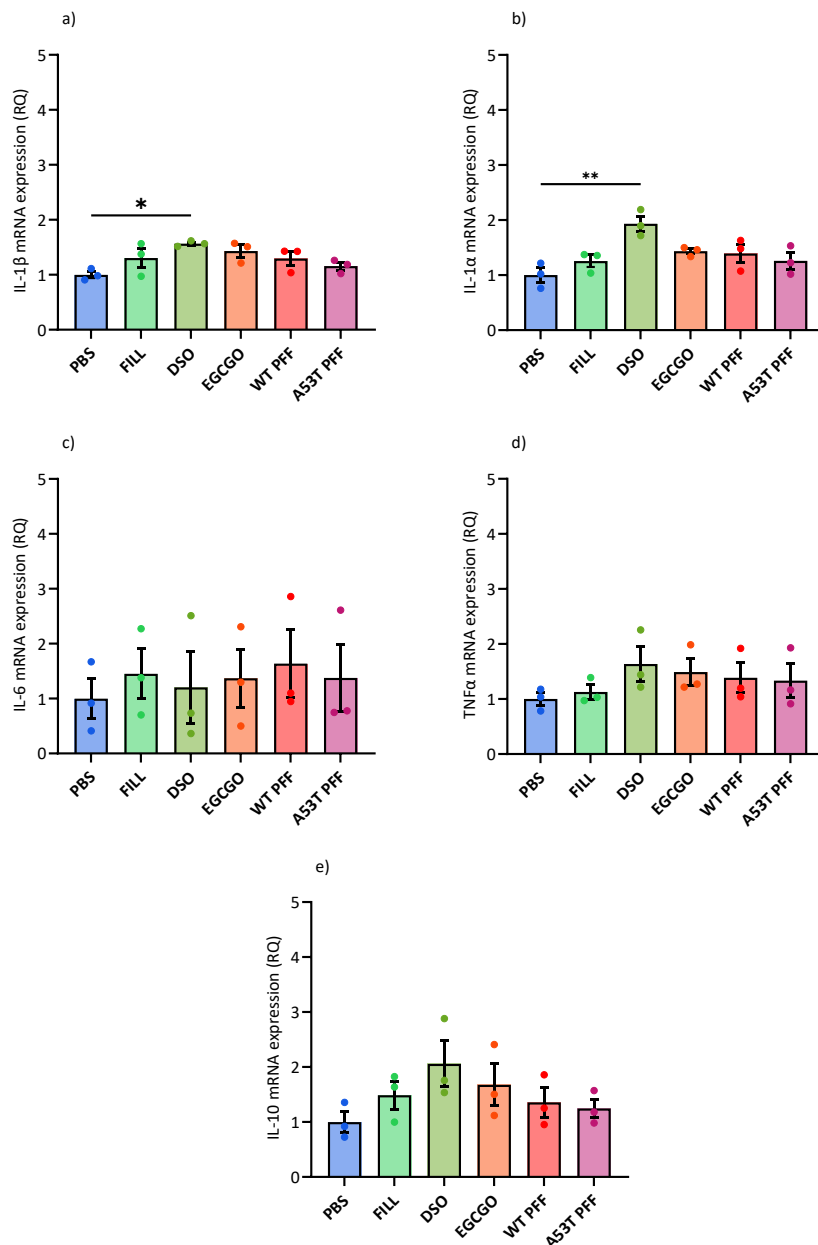


Figure 6.7. mRNA expression of target cytokines in mixed glial cells treated with α Syn filaments, oligomers, or PFF (4 μ g/ml) for 24 hours. Mature primary cortical mixed glia (DIV14) were treated with 4 μ g/ml soluble filaments (FILL), dopamine-stabilised oligomers (DSO), epigallocatechin gallate-stabilised oligomers (EGCGO), WT PFF, A53T-mutant PFF, or PBS for 24 hours. Mixed glial mRNA expression of a) IL-1 β , b) IL-1 α , c) IL-6, d) TNF α , and e) IL-10 expressed as relative quantity compared to PBS-treated controls. GAPDH was used as an internal control. Data are expressed as mean $\pm$ SEM. One-way ANOVA followed by Tukey's test for multiple comparisons. n = 3. * = p $\leq$ 0.05, ** = p $\leq$ 0.01.

6.5.2. Neuronal response following transfer of conditioned media from mixed glial cells treated with α Syn filaments, oligomers, or PFFs (4 μ g/ml) for 24 hours.

One-way ANOVA of neuronal LDH activity expressed as a % of controls shows no effect of conditioned media treatment [$F(5, 12) = 0.5601$, $p = 0.7288$] (Figure 6.8).

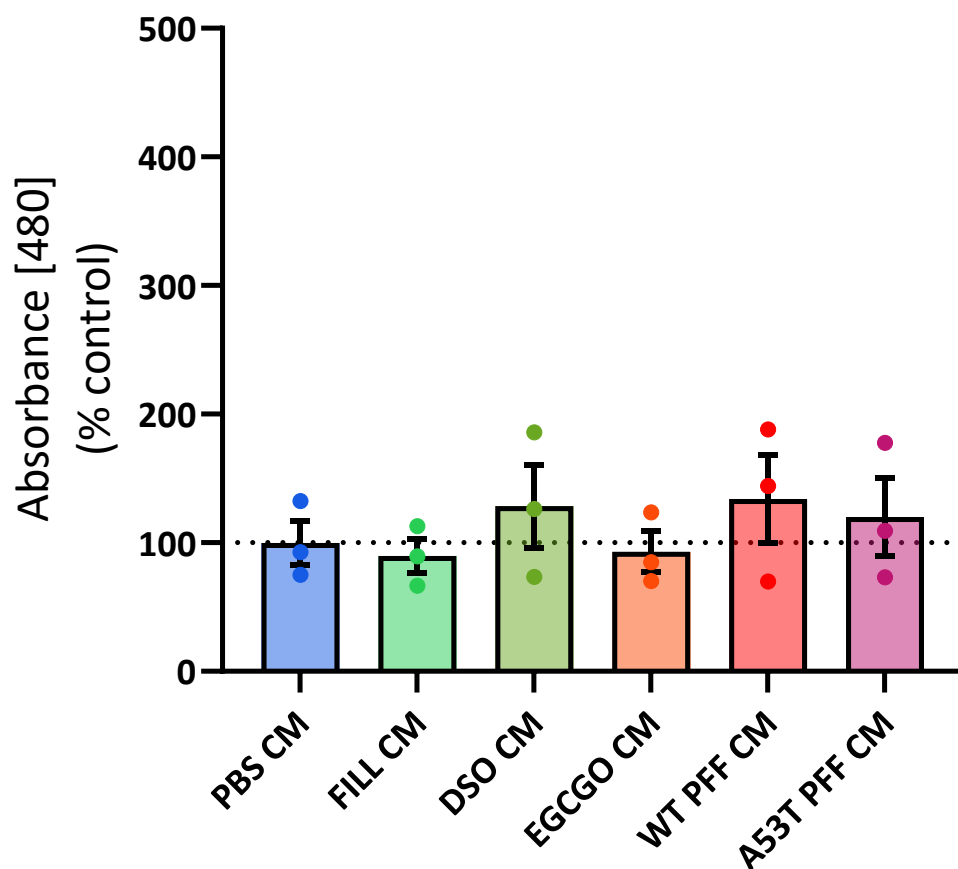


Figure 6.8. LDH activity in primary neuronal cultures treated for 24 hours with conditioned media from mixed glial cells treated with α Syn filaments, oligomers, or PFF (4 μ g/ml) for 24 hours. Mature primary neurons (DIV14) treated with conditioned media from mixed glial cultures treated with soluble filaments (FILL), dopamine-stabilised oligomers (DSO), epigallocatechin gallate-stabilised oligomers (EGCGO), WT PFFs, A53T-mutant PFFs, or PBS for 24 hours. LDH activity expressed as a percentage of PBS conditioned media-treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H_2O_2). Data are expressed as mean $\pm$ SEM. One-way ANOVA. $n = 3$.

One-way ANOVAs show no effects of conditioned media treatment on number of primary neurites [$F(5, 17)=0.5362$, $p=0.7462$], length of primary neurites [$F(5, 17)=0.2178$, $p=0.9500$], or number of branches [$F(5, 17)=1.158$, $p=0.3695$] (Figure 6.9a-c). Two-way ANOVA of Sholl profile shows an effect of conditioned media treatment [$F(5, 312)=6.3$, $p<0.0001$], and an effect of radial distance [$F(25, 312)=78$, $p<0.0001$], and no interaction between conditioned media treatment and radial distance [$F(125, 312)=0.35$, $p>0.9999$]. Post hoc comparisons reveal no differences in Sholl profiles between treatment groups (Figure 6.9d).

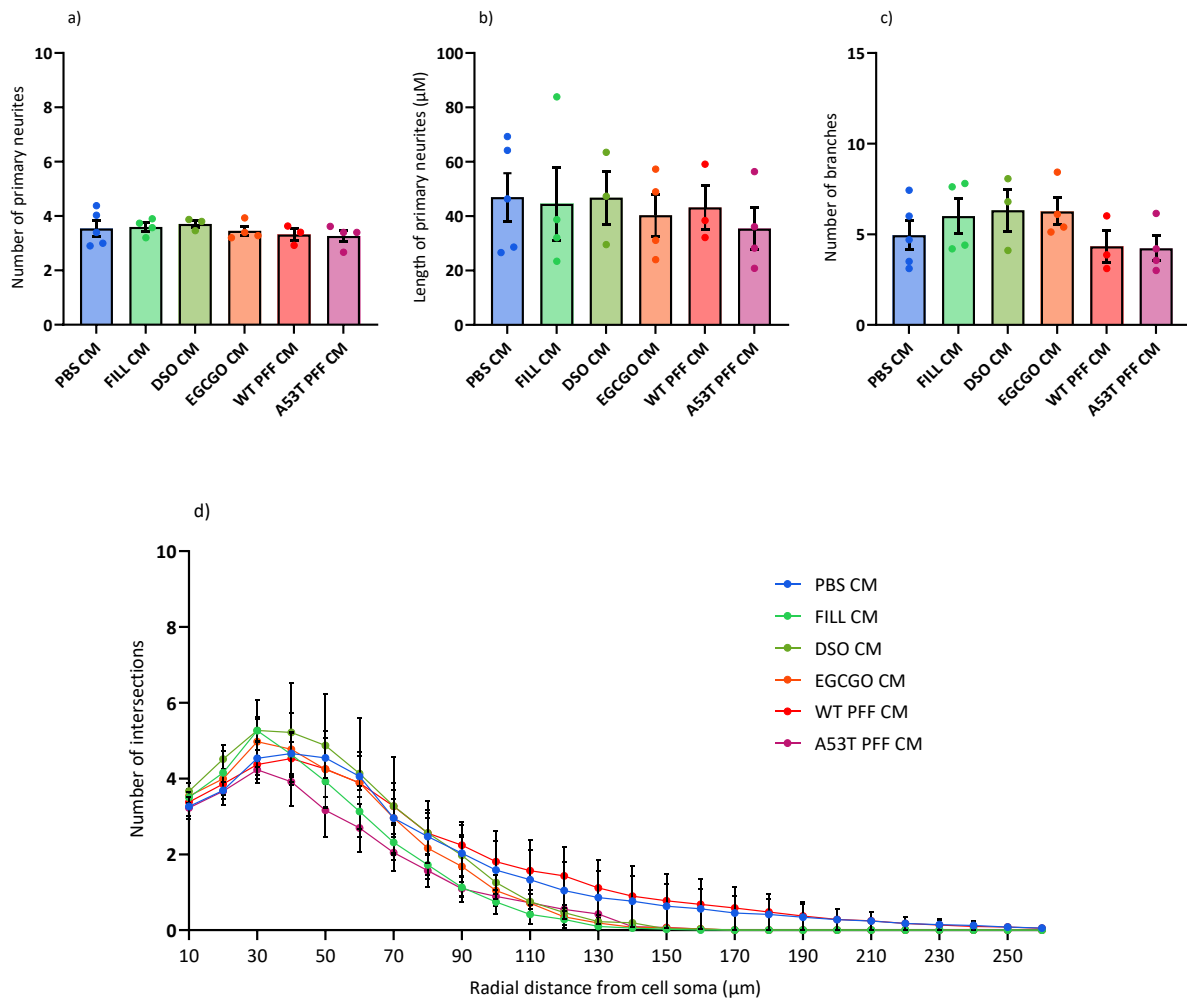


Figure 6.9. Sholl profile of primary neurons treated for 24 hours with conditioned media from mixed glial cells treated with α Syn filaments, oligomers, or PFFs (4μg/ml), for 24 hours. Mature primary neurons (DIV14) treated with conditioned media from mixed glial cultures treated with soluble filaments (FILL), dopamine-stabilised oligomers (DSO), epigallocatechin gallate-stabilised oligomers (EGCGO), WT PFFs, A53T-mutant PFFs, or PBS for 24 hours. a) Number of primary neurites, b) length of primary neurites, c) number of branches, d) Sholl profile. Data are expressed as mean $\pm$ SEM. (a-c) One-way ANOVA d) Two-way ANOVA followed by Tukey's test for multiple comparisons. $n = 5$.

6.5.3. Mixed glial response to treatment with WT PFFs (4µg/ml) for 24-96 hours.

In glial cell cultures included in the following results, Iba1+ microglia comprised approximately of 30% of all DAPI+ cells (Figure 6.10a). A two-way ANOVA of glial LDH activity expressed as percentage of 24-hour PBS-treated controls shows no effect of treatment [$F(1, 16)=0.0037$, $p=0.9525$], an effect of time [$F(3, 16)=16$, $p<0.0001$], and no interaction between treatment and time [$F(3, 16)=1.0$, $p=0.4115$] (Figure 6.6b). Post hoc comparisons reveal increased % absorbance in after 96 hours of both PBS-treatment ($p=0.0015$) and WT PFF-treatment ($p=0.0370$) (Figure 6.10b).

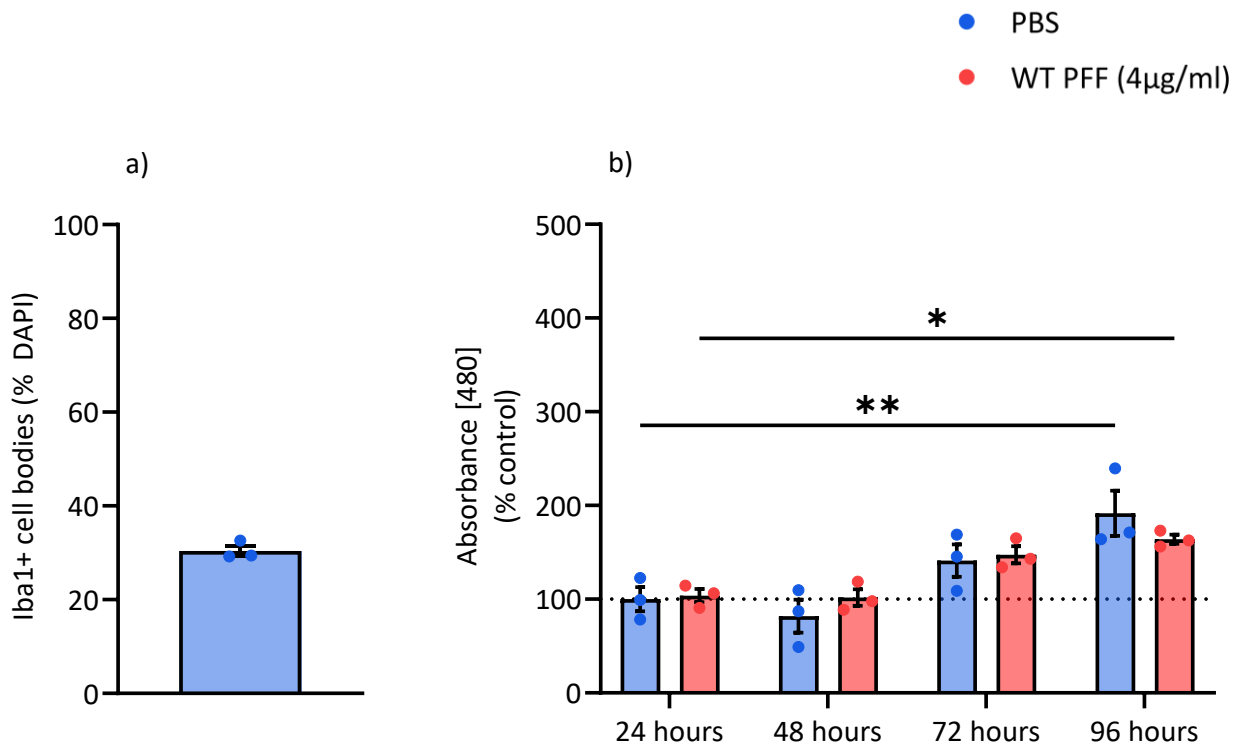


Figure 6.10. Proportion of Iba1+ cells and LDH activity in primary mixed glial cultures treated with WT PFFs for 24-96 hours. a) Proportion of Iba1+ cells in mixed glial cultures used in experiments 6.5.3-6.5.4, b) mixed glial LDH activity expressed as a percentage of 24-hour PBS-treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H_2O_2). Data are expressed as mean $\pm$ SEM. Two-way ANOVA followed by Tukey's test for multiple comparisons. $n = 3$.

A two-way ANOVA of cell area shows no effect of treatment [$F(1,17)=0.01284$, $p=0.9111$], time [$F(3,17)=0.2253$, $p=0.8775$], or interaction between treatment and time [$F(3,17)=0.1710$, $p=0.9145$] (Figure 6.11a). A two-way ANOVA of cell perimeter shows no effect of treatment [$F(1,17)=1.838$, $p=0.1929$], time [$F(3,17)=0.2011$, $p=0.8942$], or interaction between treatment and time [$F(3,17)=0.1067$, $p=0.9550$] (Figure 6.11b). A two-way ANOVA of Feret's diameter shows no effect of treatment [$F(1,17)=0.7184$, $p=0.4084$], time [$F(3,17)=0.4122$, $p=0.7463$], or interaction between treatment and time [$F(3,17)=0.001523$, $p>0.9999$] (Figure 6.11c). A two-way ANOVA of circularity shows no effect of treatment [$F(1,17)=2.609$, $p=0.1247$], time [$F(3,17)=0.1485$, $p=0.9293$], or interaction between treatment and time [$F(3,17)=0.3079$, $p=0.8193$] (Figure 6.11d). A two-way ANOVA of solidity shows no effect of treatment [$F(1,17)=2.720$, $p=0.1175$], time [$F(3,17)=0.09412$, $p=0.9623$], or interaction between treatment and time [$F(3,17)=0.2466$, $p=0.8626$] (Figure 6.11e). A two-way ANOVA of cell integrated density shows no effect of treatment [$F(1,17)=0.03419$, $p=0.8555$], time [$F(3,17)=0.1048$, $p=0.9561$], or interaction between treatment and time [$F(3,17)=0.2619$, $p=0.8519$] (Figure 6.11f).

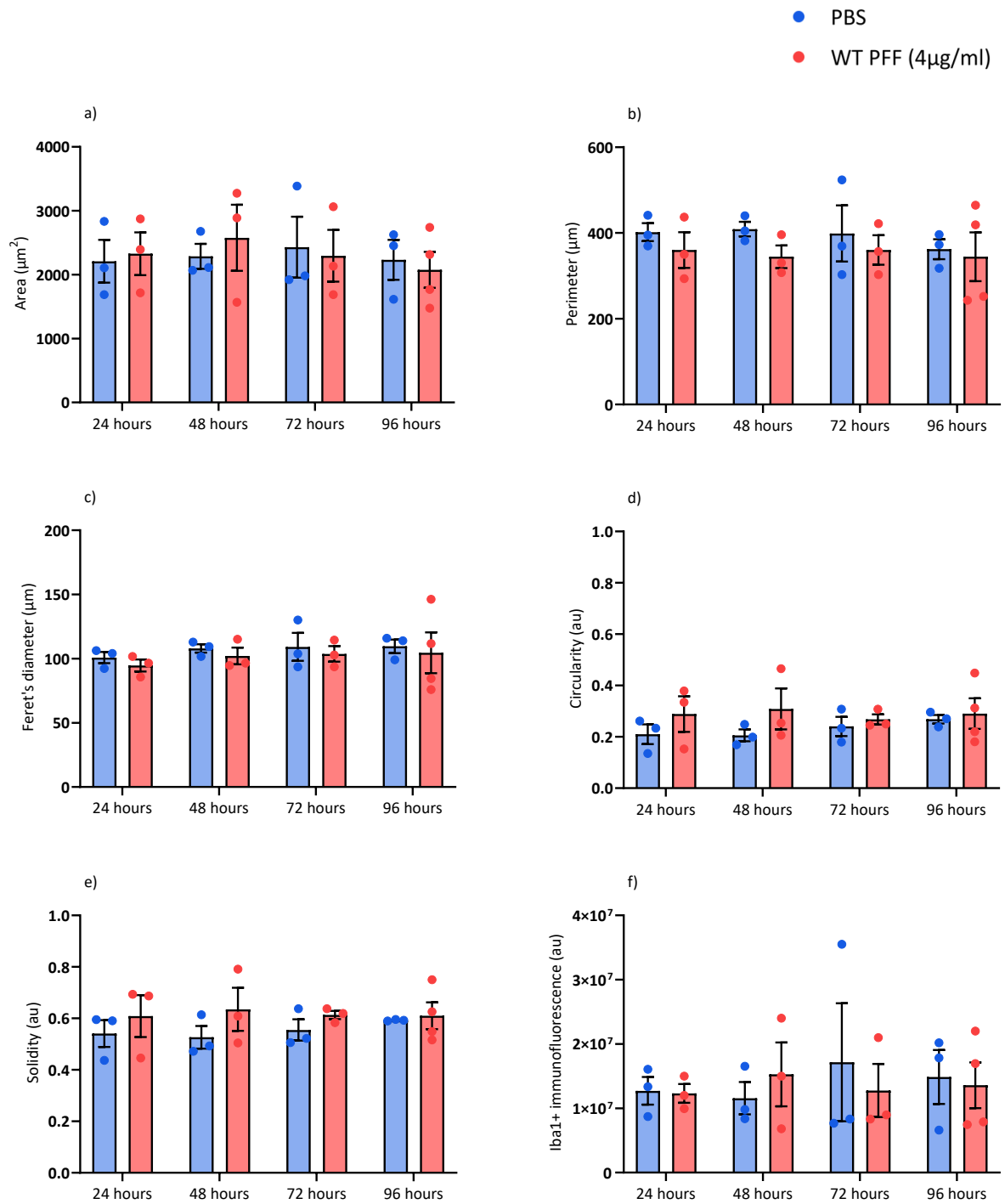


Figure 6.11 Morphology of Iba1+ microglia in primary mixed glial cultures treated with WT PFF for 24-96 hours. Cells were fixed for Iba1 immunofluorescence. Image analysis was performed on ImageJ to quantify cell a) area, b) perimeter, c) Feret's diameter, d) circularity, e) solidity, and f) Iba1+ immunofluorescence of Iba1+ cells in mixed glial cultures. Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's test for multiple comparisons. n = 3-4.

A two-way ANOVA of IL-1 β mRNA expression shows no effect of treatment [$F(1, 26)=0.72$, $p=0.4033$], an effect of time [$F(4, 26)=18$, $p<0.0001$], and no interaction between treatment and time [$F(4, 26)=0.20$, $p=0.9350$]. Post hoc comparisons reveal reductions in IL-1 β mRNA expression in mixed glia treated with PBS for 72 hours ($p=0.0043$), 96 hours ($p=0.0027$), and 144 hours ($p=0.0006$) compared to mixed glia stimulated with PBS for 24 hours. IL-1 β mRNA expression is reduced in mixed glia treated with WT PFF for 48 hours ($p=0.0082$), 72 hours ($p = 0.0007$), 96 hours ($p=0.0004$), and 144 hours ($p<0.0001$) compared to mixed glia stimulated with WT PFF for 24 hours (Figure 6.12a)

A two-way ANOVA of IL-1 α mRNA expression shows no effect of treatment [$F(1, 26)=3.3$, $p=0.0792$], an effect of time [$F(4, 26)=43$, $p<0.0001$], and no interaction between treatment and time [$F(4, 26)=0.70$, $p=0.5997$]. Post hoc comparisons reveal reductions in IL-1 α mRNA expression in mixed glia treated with PBS for 48 hours ($p=0.0003$), 72 hours ($p<0.0001$), 96 hours ($p<0.0001$), and 144 hours ($p<0.0001$), compared to mixed glia treated with PBS for 24 hours. IL-1 α mRNA expression is reduced in mixed glia treated with WT PFF for 48 hours ($p<0.0001$), 72 hours ($p<0.0001$), 96 hours ($p<0.0001$), and 144 hours ($p<0.0001$), compared to mixed glia treated with WT PFF for 24 hours (Figure 6.12b)

A two-way ANOVA of IL-6 mRNA expression shows no effect of treatment [$F(1, 26)=4.1$, $p=0.0543$], an effect of time [$F(4, 26)=9.8$, $p<0.0001$], and no interaction between treatment and time [$F(4, 26)=0.55$, $p=0.6990$]. Post hoc comparisons reveal reductions in IL-1 α mRNA expression in mixed glia treated with WT PFF for 48 hours ($p=0.0060$), 72 hours ($p=0.0077$), 96 hours ($p<0.0010$), and 144 hours ($p=0.0019$) compared to mixed glia treated with WT PFF for 24 hours. No changes in IL-6 mRNA expression are revealed following PBS-treatments over time (Figure 6.12c)

A two-way ANOVA of TNF α mRNA expression shows no effect of treatment [$F(1, 26)=1.6$, $p=0.2185$], an effect of time [$F(4, 26)=6.7$, $P=0.0007$], and no interaction between treatment and time [$F(4, 26)=0.58$, $p=0.6826$]. Post hoc comparisons reveal increased TNF α expression in mixed glia treated with WT PFF for 72 hours compared to 24 hours ($p=0.0160$), and a reduction in TNF α expression in mixed glia treated with WT PFF for 144 hours compared to 72 hours ($p = 0.0025$). No changes in TNF α mRNA expression are revealed in PBS-treated cells over time (Figure 6.12d).

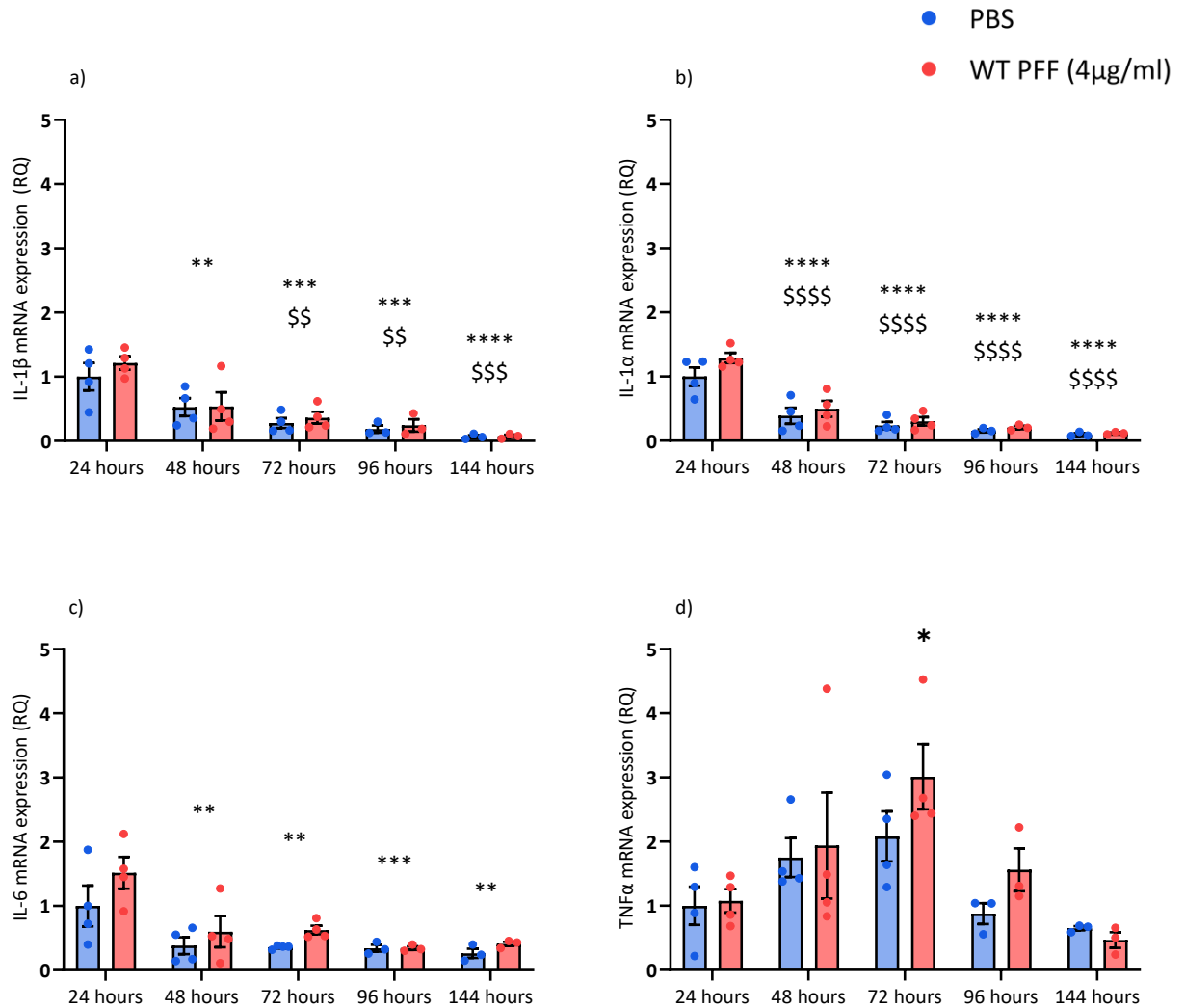


Figure 6.12. mRNA expression of target cytokines in mixed glial cells treated with WT PFF for 24-144 hours. Mixed glial mRNA expression of a) IL-1β, b) IL-1α, c) IL-6, and d) TNFα expressed as relative quantity compared to 24-hour PBS-treated controls. GAPDH was used as an internal control. Data are expressed as mean ± SEM. Two-Way ANOVA followed by Tukey's test for multiple comparisons. n = 3-4. \$'s represent significant differences between the annotated PBS-group and 24-hour PBS treatment only. \$\$\$=p≤0.001, \$\$\$\$=p≤0.001. *'s represent significant differences between the annotated WT PFF-group and 24-hour WT PFF treatment only. *=p≤0.05, **=p≤0.01, ***=p≤0.001, ****=p≤0.001.

6.5.4. Neuronal response to treatment with conditioned media transferred from mixed glial cultures treated with WT PFFs (4µg/ml) for 24-96 hours.

Two-way ANOVA of neuronal LDH activity expressed as a % of controls shows no effect of treatment [$F(1,16)=0.16$, $p=0.6936$], an effect of time [$F(3,16)=4.4$, $p=0.0198$], and no interaction between treatment and time [$F(3,16)=0.62$, $p=0.6142$]. Post hoc comparisons reveal an increase in LDH activity in neuronal cultures treated with PBS for 72 hours compared to 24 hours ($p=0.0218$) (Figure 6.13).

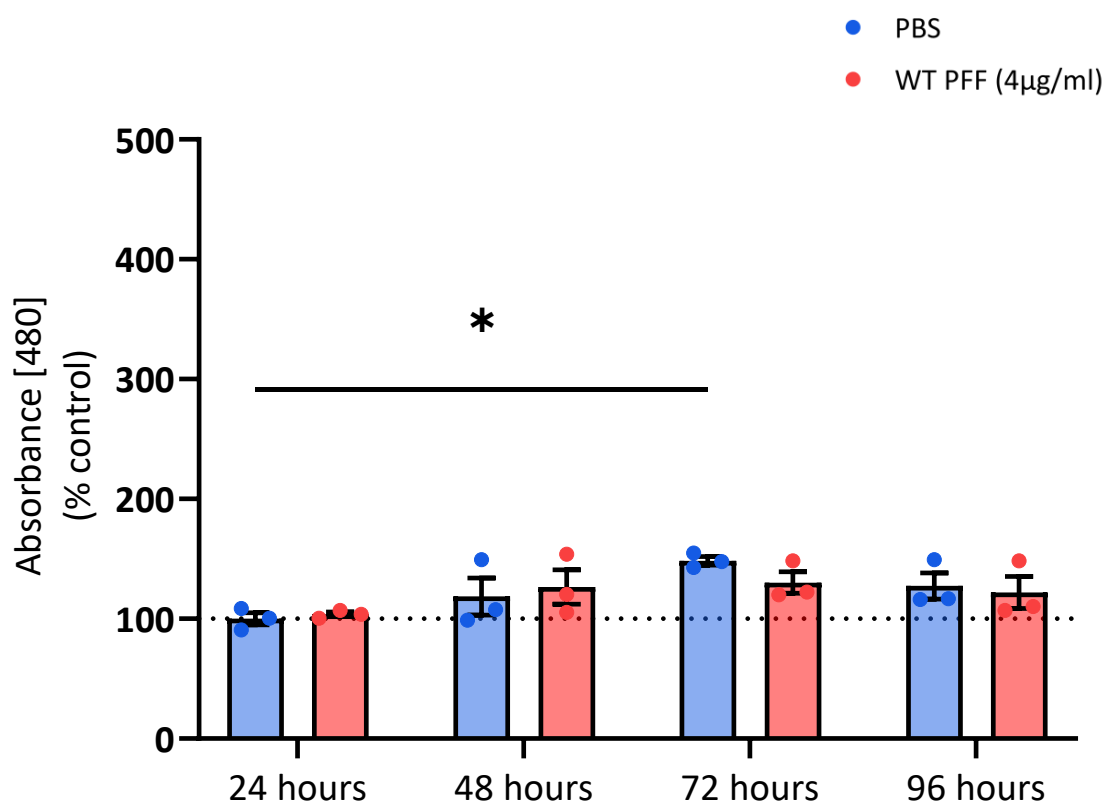


Figure 6.13. LDH activity in primary neuronal cultures treated for 24 hours with conditioned media transferred from mixed glial cells treated with WT PFFs for 24-96 hours. a) LDH activity expressed as percentage of 24-hour PBS-treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H_2O_2). Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's test for multiple comparisons. $n=3$. *= $p \leq 0.05$.

A one-way ANOVA shows no effect of conditioned media treatment on number of primary neurites [$F(7,23)=0.6934$, $p=0.6769$], length of primary neurites [$F(7,23)=1.163$, $p=0.3611$], or number of branches [$F(7,23)=0.2129$, $p=0.9788$] (Figure 6.14a-c). A two-way ANOVA of Sholl profile shows an effect of treatment [$F(7,624)=7.0$, $p<0.0001$], an effect of radial distance [$F(25,624)=184$, $p<0.0001$], and no interaction between treatment and radial distance [$F(175,624)=0.35$, $p>0.9999$]. Post hoc comparisons reveal no differences in Sholl profile between treatment groups (Figure 6.14d).

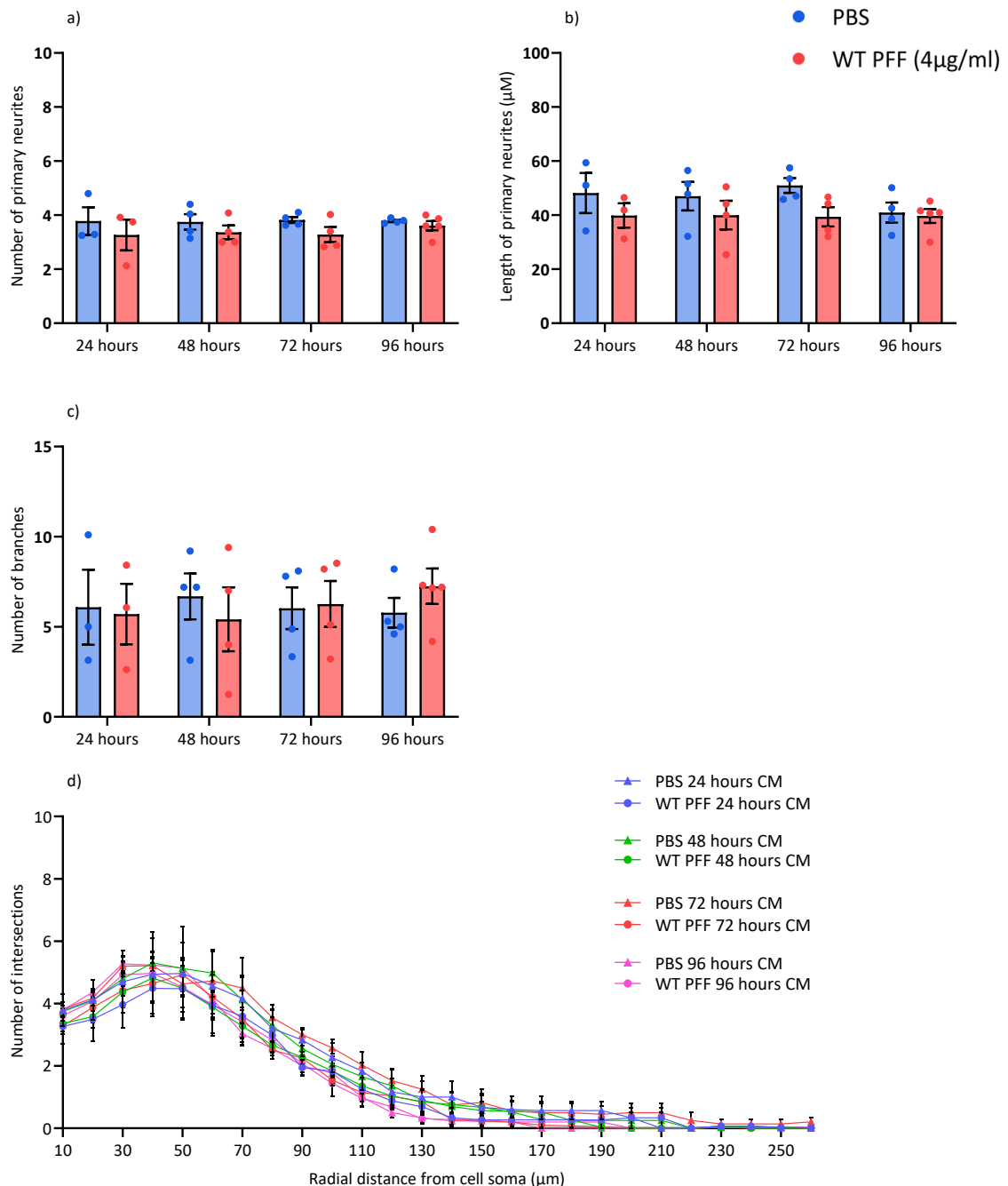


Figure 6.14. Sholl profile of primary neuronal neurons treated for 24 hours with conditioned media transferred from mixed glial cells treated with WT PFF for 24-96 hours. a) Number of primary neurites, b) length of primary neurites, c) number of branches, d) Sholl profile. Data are expressed as mean ± SEM. (a-c) One-way ANOVA d) Two-way ANOVA followed by Tukey's test for multiple comparisons. n = 3-4.

6.5.5. Mixed glial response to 24-hour treatment with IFN γ .

In the following mixed glial cell cultures, Iba1+ microglia comprised approximately 30% of all DAPI+ cells (Figure 6.15a). One-way ANOVA of glial LDH activity expressed as a % of controls shows an effect of treatment with IFN γ [$F(5, 42)=9.151$, $p<0.0001$]. Post hoc comparisons reveal increased LDH activity in mixed glia treated with 500ng/ml IFN γ compared to vehicle-treated controls ($p<0.0001$) (Figure 6.15b).

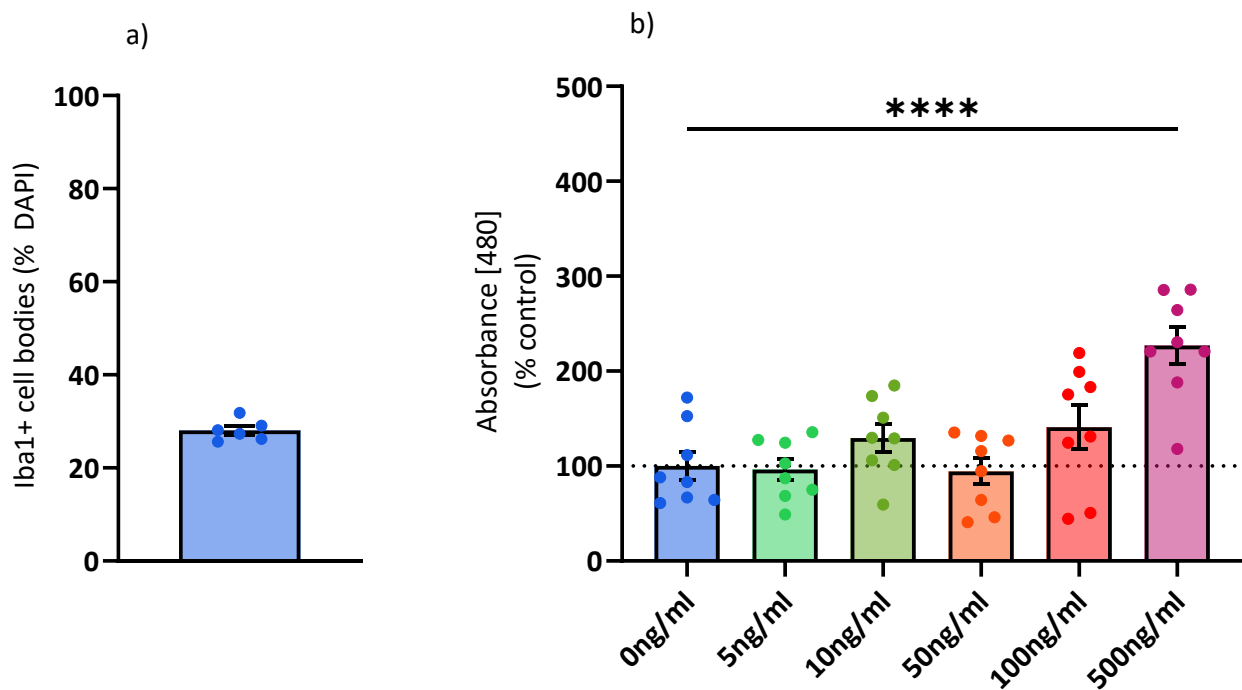


Figure 6.15. Proportion of Iba1+ cells and LDH activity in primary mixed glial cultures treated with 0-500ng/ml IFN γ for 24 hours. a) Proportion of Iba1+ cells in mixed glial cultures used in experiments 6.5.5-6.5.6, b) mixed glial LDH activity expressed as a percentage of 0ng/ml IFN γ -treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H $_2$ O $_2$). Data are expressed as mean $\pm$ SEM. One-Way ANOVA with Dunnett's post hoc. $n = 8$. **** = $p \leq 0.0001$.

One-way ANOVA shows no effect of IFN γ treatment on area [$F(5,21)=1.434$, $p=0.2533$], perimeter [$F(5, 21)= 2.480$, $p=0.0648$], Feret's diameter [$F(5, 21)= 1.410$, $p=0.2612$], circularity [$F(5,21)=1.346$, $p=0.2840$], or solidity [$F(5,21)=1.050$, $p=0.4151$] (Figure 6.16a-e). One-way ANOVA shows an effect of IFN γ treatment on Iba1+ immunofluorescence [$F(5,21)=5.247$, $p=0.0028$]. Post hoc comparisons reveal increased Iba1+ immunofluorescence in glial cells treated with 10ng/ml IFN γ ($p=0.0006$), 50ng/ml IFN γ ($p=0.0108$), 100ng/ml IFN γ ($p=0.0043$), and 500ng/ml IFN γ ($p=0.0225$), compared to those treated with vehicle-treated controls (Figure 6.16f).

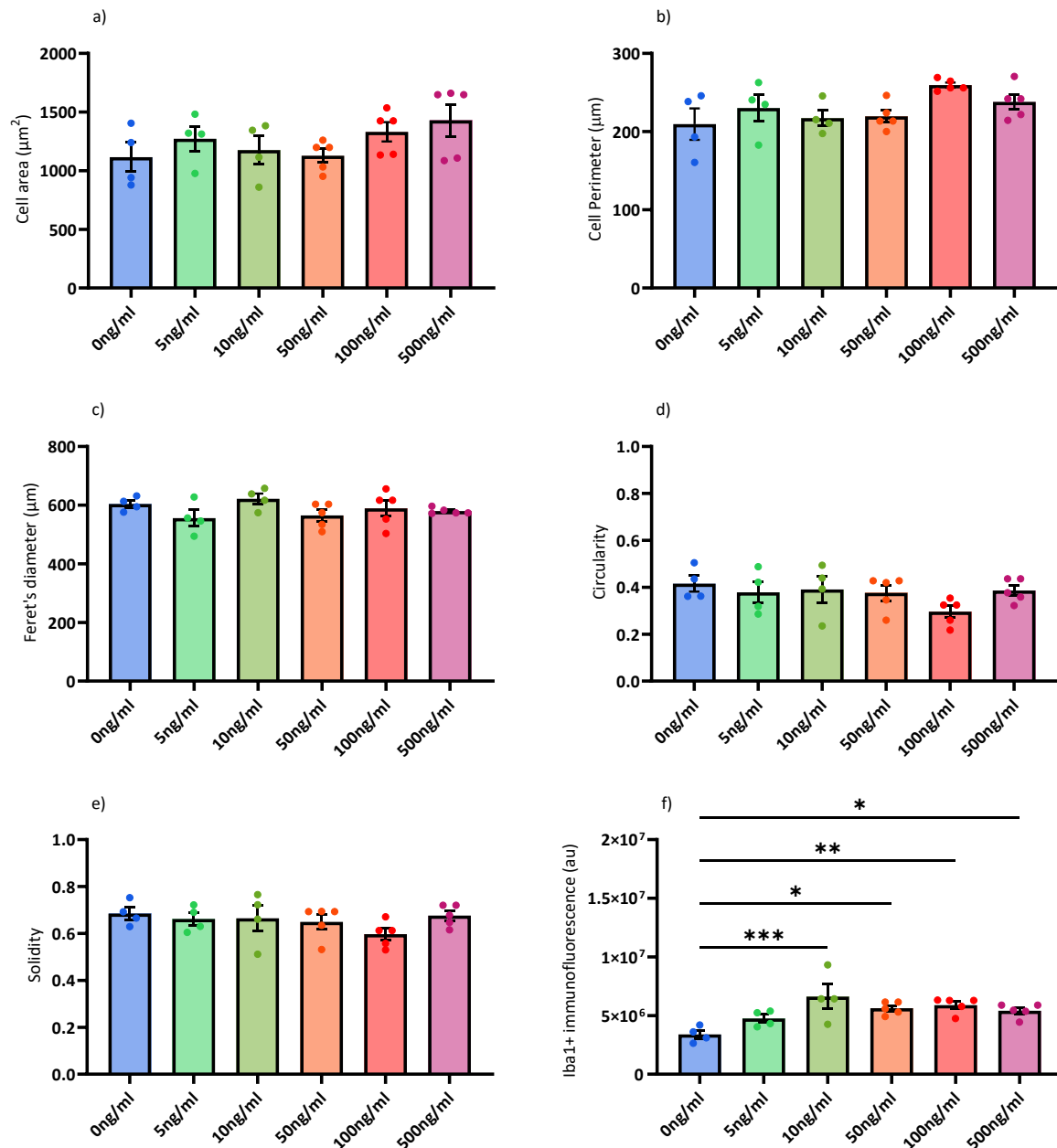


Figure 6.16. Morphology of Iba1+ microglia in primary mixed glial cultures treated with 0-500ng/ml IFN γ for 24 hours. Cells were fixed for Iba1 immunofluorescence. Image analysis was performed on ImageJ to quantify cell a) area, b) perimeter, c) Feret's diameter, d) circularity, e) solidity, f) Iba1+ immunofluorescence. Data are expressed as mean $\pm$ SEM. One-way ANOVA followed by Dunnett's test for multiple comparisons. n = 4-5. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

One-way ANOVA of glial mRNA expression shows no effect of IFN γ treatment on IL-1 β [F(5,24)=1.822, p=0.1466], IL-1 α [F(5,24)=1.703, p=0.1724], IL-6 [F(5,18)=1.261, p=0.3230], TNF α [F(5,18)=0.8489, p=0.5332], or IL-10 [F(5,12)=0.4969, p=0.7729] mRNA expression (Figure 6.17).

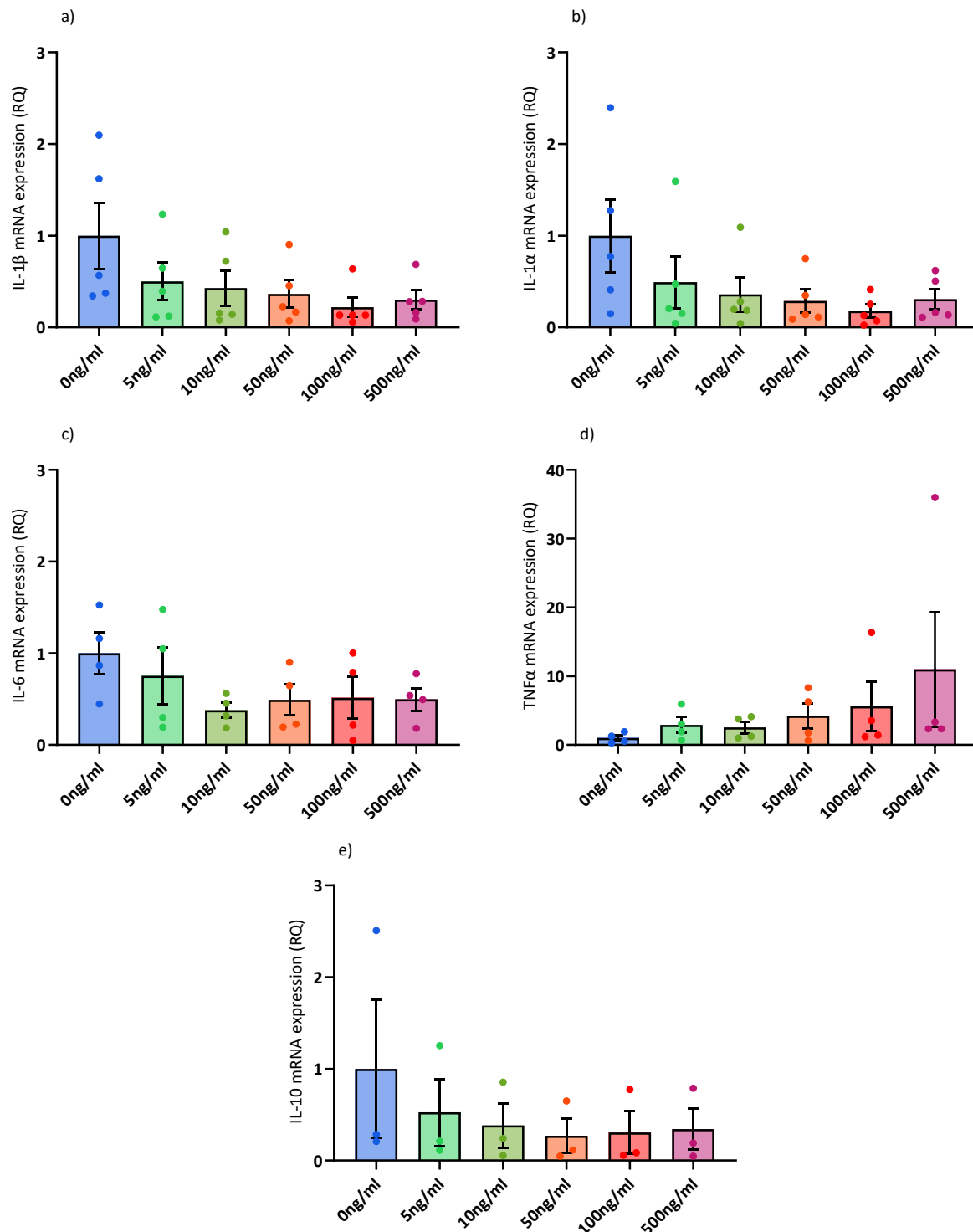


Figure 6.17. mRNA expression of target cytokines in mixed glial cells treated with 0-500ng/ml IFN γ for 24 hours. Mixed glial mRNA expression of a) IL-1 β , b) IL-1 α , c) IL-6, d) TNF α , and e) IL-10 expressed as relative quantity compared to 0ng/ml IFN γ -treated controls. GAPDH was used as an internal control. Data are expressed as mean $\pm$ SEM. One-Way ANOVA. n=3-5.

6.5.6. Neuronal response to treatment with conditioned media from mixed glia treated for 24 hours with IFN γ .

One-way ANOVA of neuronal LDH activity expressed as a % of controls shows an effect of conditioned media treatment [$F(5,18)=22.07$, $p<0.0001$]. Post hoc comparisons reveal reduced LDH activity in neuronal cultures treated with 5ng/ml IFN γ conditioned media ($p<0.0001$), 10ng/ml IFN γ conditioned media ($p<0.0001$), 50ng/ml IFN γ conditioned media ($p<0.0001$), 100ng/ml IFN γ conditioned media ($p<0.0001$), and 500ng/ml IFN γ conditioned media ($p<0.0001$) compared to vehicle conditioned media-treated controls (Figure 6.18).

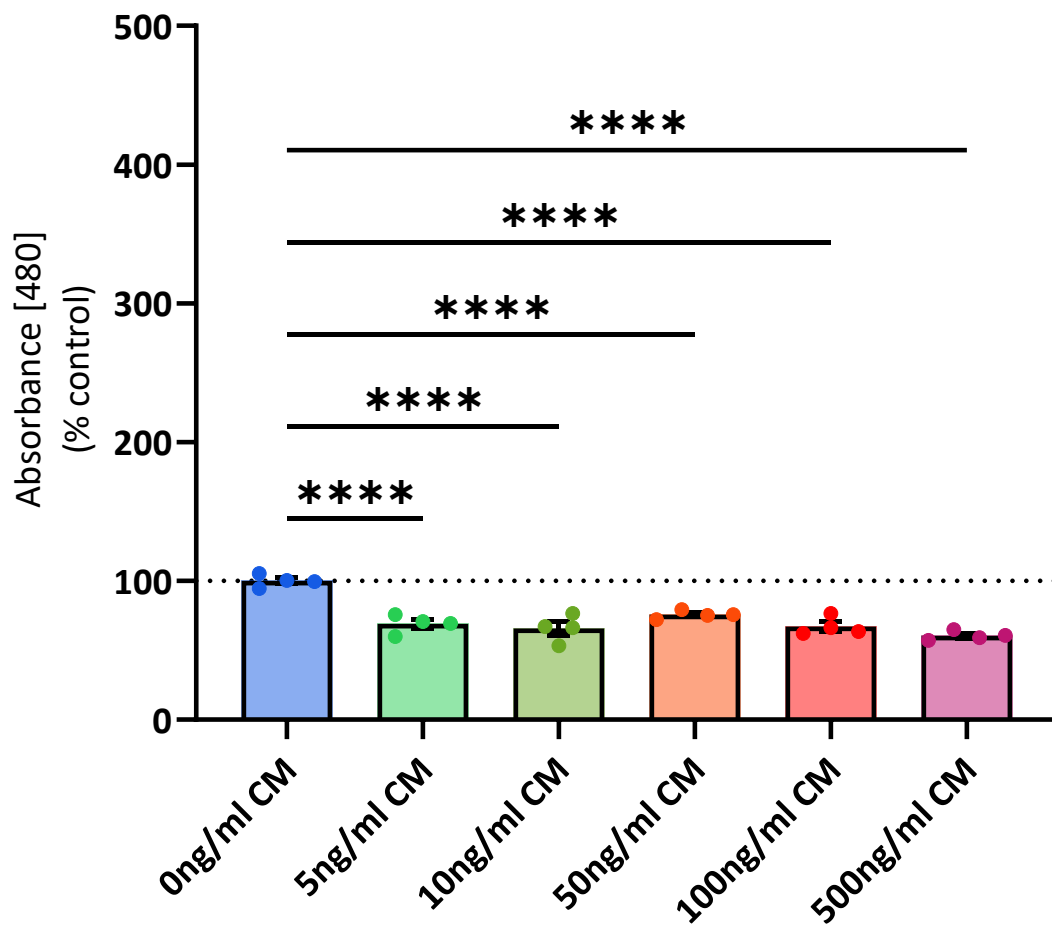


Figure 6.18. LDH activity in primary neuronal cultures treated for 24 hours with conditioned media transferred from mixed glial cells treated with 0-500ng/ml IFN γ for 24 hours. LDH reactivity expressed as percentage of 0ng/ml IFN γ -treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H $_2$ O $_2$). Data are expressed as mean $\pm$ SEM. One-Way ANOVA with Dunnett's post hoc. $n=4$. ****= $p\leq 0.001$.

One-way ANOVAs of neuronal complexity measures show no effect of IFN γ treatment on number of primary neurites [F(5,22)=0.3618, p=0.8690], length of primary neurites [F(5,22)=0.6972, p=0.6312], or number of branches [F(5,22)=0.4772, p=0.7893] (Figure 6.19a-c). Two-way ANOVA of Sholl profile shows an effect of treatment [F(5,572)=5.478, p<0.0001], an effect of radial distance [F(25,572)=292.2, p<0.0001], and no interaction between treatment and radial distance [F(125,572)=0.5038, p>0.9999]. Post hoc comparisons reveal no differences in Sholl profile between treatment groups (Figure 6.19b).

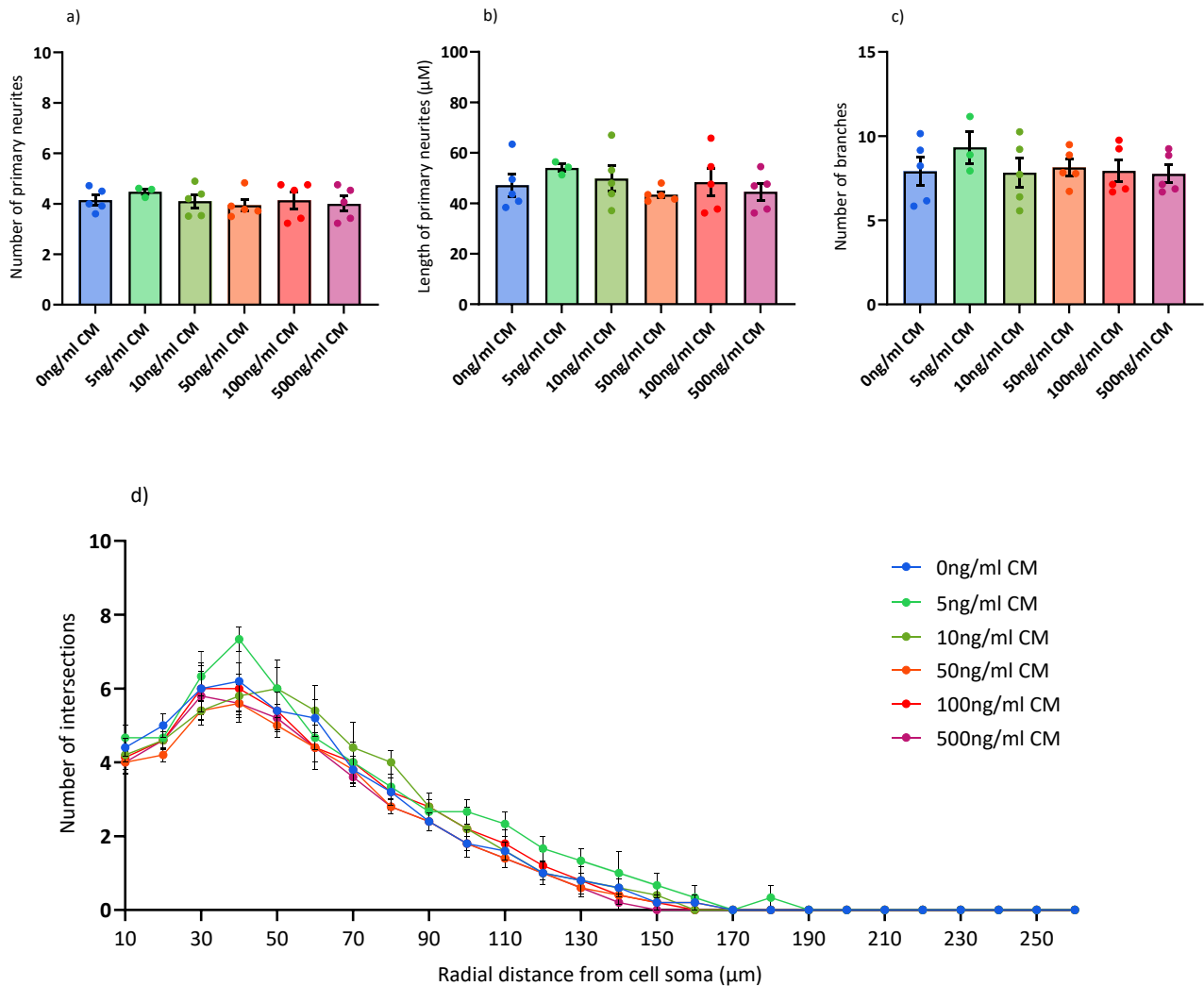


Figure 6.19. Sholl profile of MAP2+ primary neurons treated for 24 hours with conditioned media transferred from mixed glial cells treated with 0-500ng/ml IFN γ for 24 hours. a) Number of primary neurites, b) length of primary neurites, c) number of secondary branches. Data are expressed as mean $\pm$ SEM. a-c) One-way ANOVA, d) Two-Way ANOVA followed by Dunnett's post hoc. n=3-5.

6.5.7. Mixed glial response to pre-treatment with A53T PFFs for 24 hours prior to treatment with IFN γ .

In the following mixed glial cell cultures, Iba1+ microglia comprised approximately of 32% of all DAPI+ cells (Figure 6.20a). A two-way ANOVA of glial LDH activity expressed as a % of controls shows no effect of PFF [$F(1,8)=2.729$, $p=0.1272$], IFN γ [$F(1,8)=2.729$, $p=0.1372$], or interaction between PFF and IFN γ [$F(1,8)=0.1773$, $p=0.6848$] (Figure 6.20b).

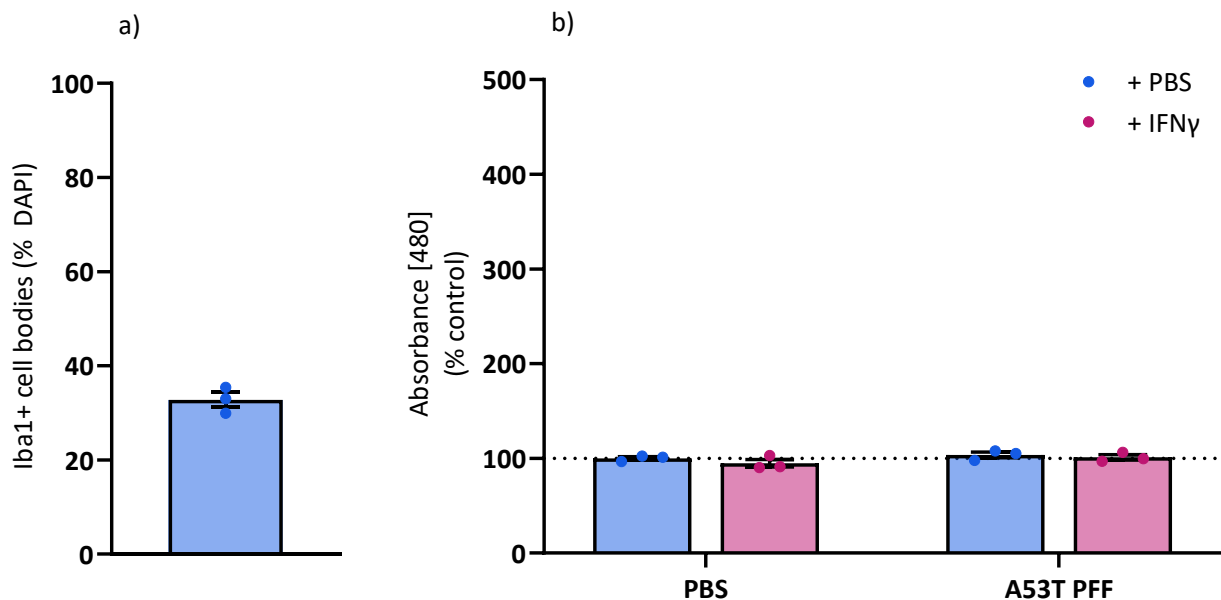


Figure 6.20. Proportion of Iba1+ cells and LDH activity in primary mixed glial cultures pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ . a) Proportion of Iba1+ cells in mixed glial cultures used in experiments 6.5.7-6.5.8, b) LDH activity expressed as percentage of PBS+PBS-treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H $_2$ O $_2$). Data are expressed as mean $\pm$ SEM. Two-Way ANOVA. $n=3$.

A two-way ANOVA of IL-1 β mRNA expression shows no effect of PFF [$F(1,8)=3.036$, $p=0.1196$], an effect of IFN γ [$F(1,8)=21.58$, $p=0.1196$], and no interaction between PFF and IFN γ [$F(1,8)=0.7584$, $p=0.4092$]. Post hoc comparisons reveal a decrease in IL-1 β mRNA expression in cells treated with PBS+IFN γ compared to PBS+PBS ($p=0.0190$) (Figure 6.21a).

A two-way ANOVA of IL-1 α expression shows no effect of PFF [$F(1,8)=0.1444$, $p=0.7137$], an effect of IFN γ [$F(1,8)=23.90$, $p=0.0012$], and no interaction between PFF and IFN γ [$F(1,8)=0.07290$, $p=0.7940$]. Post hoc comparisons reveal reduced IL-1 α mRNA expression in cell treated with PBS+IFN γ compared to PBS+PBS ($p=0.0457$), and cells treated with A53T PFF+IFN γ compared to A53T PFF+PBS ($p=0.0269$) (Figure 6.21b).

A two-way ANOVA of IL-6 mRNA expression shows no effect of PFF [$F(1,8)=0.08163$, $p=0.7824$], no effect of IFN γ [$F(1,8)=0.8597$, $p=0.3809$], and no interaction between PFF and IFN γ [$F(1,8)=0.08936$, $p=0.7726$] (Figure 6.21c).

A two-way ANOVA of TNF α mRNA expression shows no effect of PFF [$F(1,8)=2.846$, $p=0.1301$], no effect of IFN γ [$F(1,8)=2.313$, $p=0.1668$], and no interaction between PFF and IFN γ [$F(1,8)=0.03645$, $p=0.8533$] (Figure 6.21d).

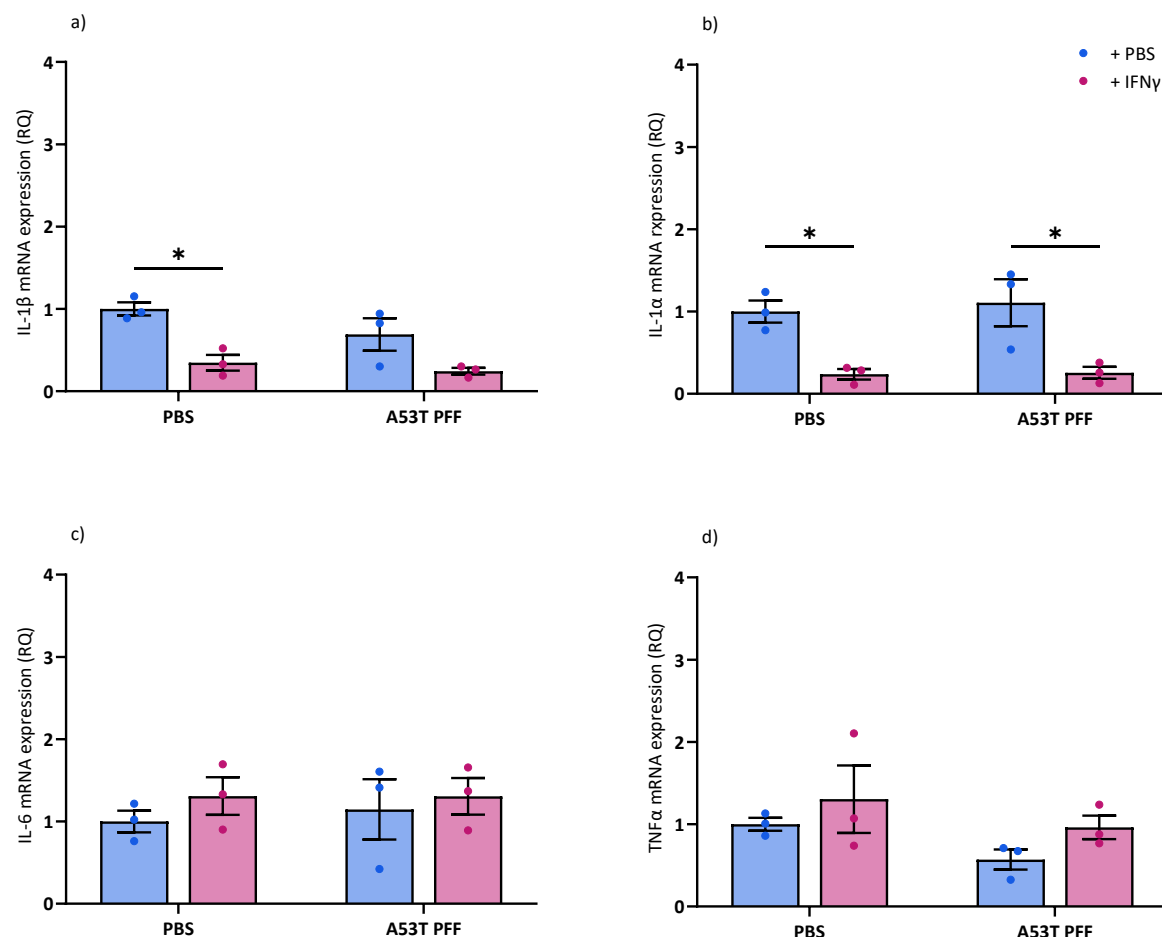


Figure 6.21. mRNA expression of target cytokines in mixed glial cells pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ . Mixed glial mRNA expression of a) IL-1 β , b) IL-1 α , c) IL-6, and d) TNF α expressed as relative quantity compared to PBS+PBS-treated controls. GAPDH was used as an internal control. Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's post hoc. $n=3$.

6.5.8. Neuronal response to treatment with conditioned media transferred from mixed glia pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ .

One-way ANOVA of neuronal LDH activity expressed as a % of controls shows no effect of conditioned media treatment [$F(3,8)=0.3969$, $p=0.7590$] (Figure 6.22).

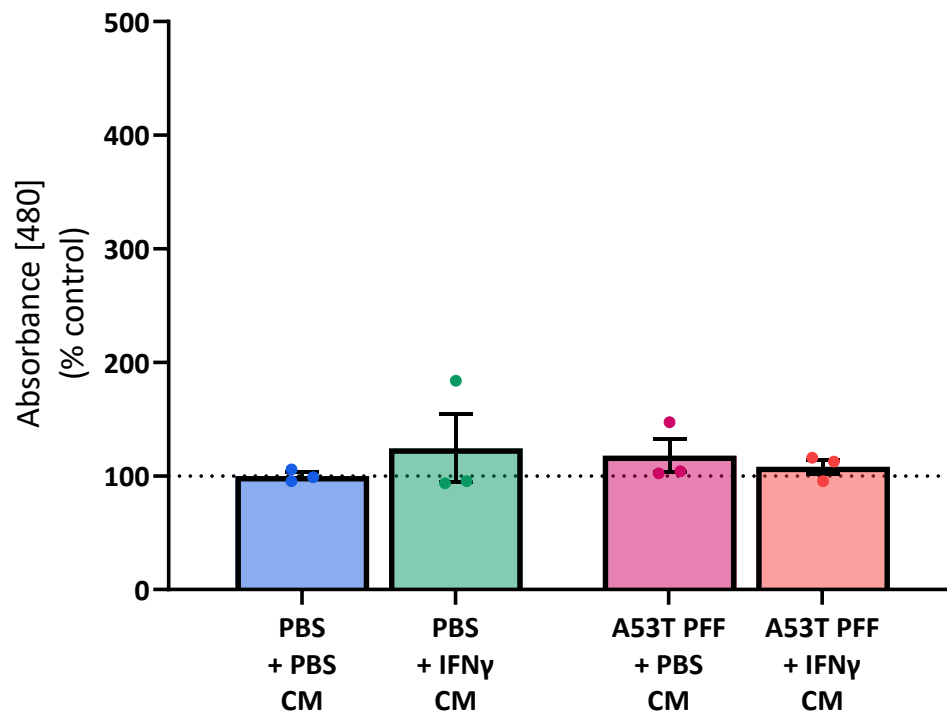


Figure 6.22. LDH activity in primary neuronal cultures treated with conditioned media from mixed glial cells pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ . LDH activity expressed as percentage of PBS+PBS-treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H₂O₂). Data are expressed as mean $\pm$ SEM. One-way ANOVA. $n=3$.

One-way ANOVAs of neuronal complexity measures show no effect of conditioned media treatment on number of primary neurites [$F(3,14)=0.6103$, $p=0.6194$], length of primary neurites [$F(3,14)=0.4228$, $p=0.7396$], or number of branches [$F(3,14)=0.3892$, $p=0.7626$] (Figure 6.23a-c). Two-way ANOVA of number of intersections shows an effect of conditioned media treatment [$F(3,338)=4.6$, $p=0.0034$], an effect of radial distance [$F(25,338)=46$, $p<0.0001$], and no interaction between conditioned media treatment and radial distance [$F(75, 338)=0.26$, $p>0.9999$]. Post hoc comparisons reveal no differences in Sholl profile between treatment groups (Figure 6.23d).

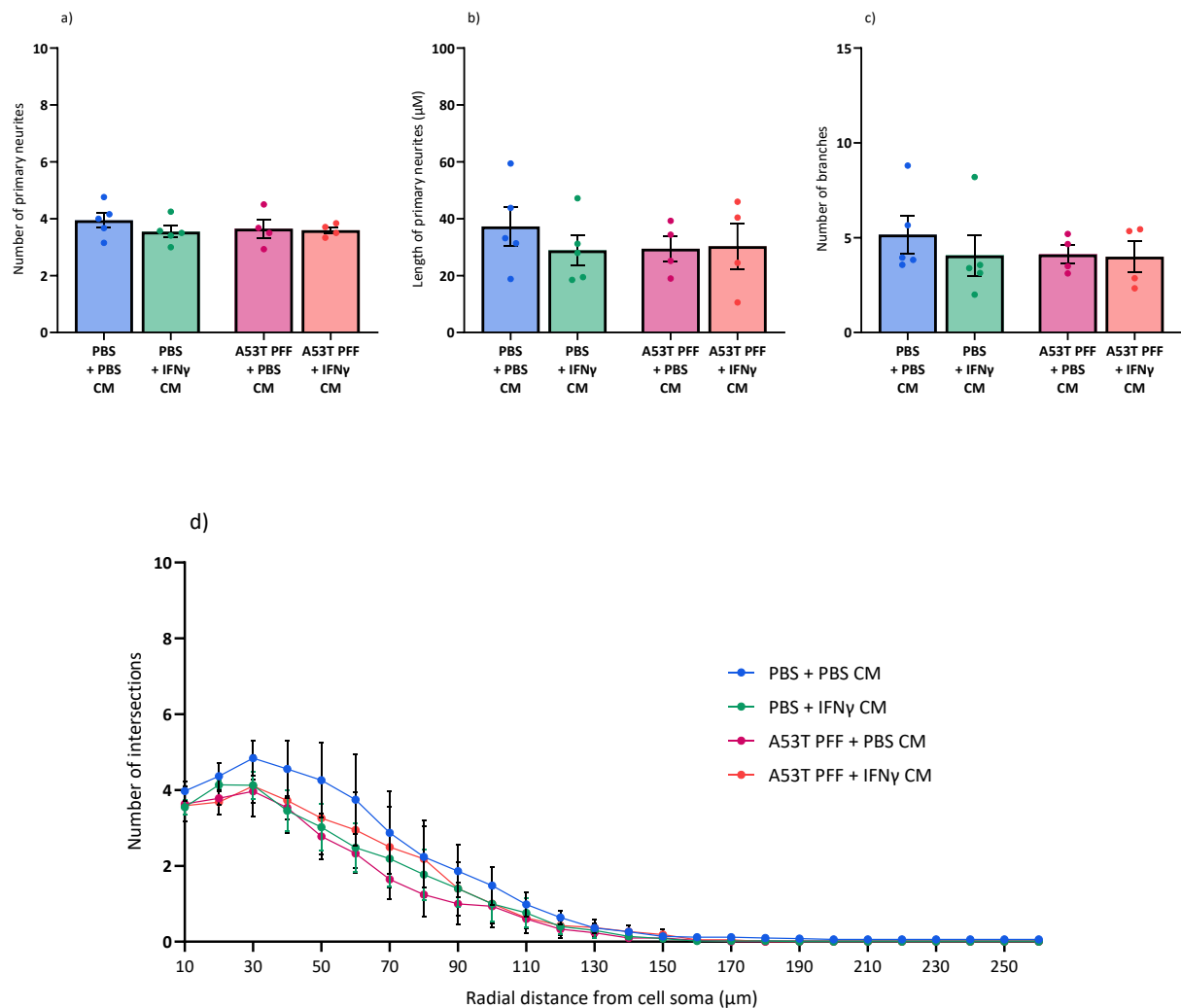


Figure 6.23. Sholl profile of MAP2+ primary neurons treated with conditioned media transferred from mixed glial cells pre-treated with A53T PFFs for 24 for hours prior to treatment with IFN γ . a) Number of primary neurites, b) length of primary neurites, c) number of secondary branches. Data are expressed as mean $\pm$ SEM. a-c) On-way ANOVA, d) two-way ANOVA followed by Dunnett's post hoc. n=4-5.

6.5.9. Mixed glial response to pre-treatment with IFN γ for 24 hours prior to treatment with A53T PFF.

In the following mixed glial cell cultures, Iba1+ microglia comprised approximately of 32% of all DAPI+ cells (Figure 6.24a). A two-way ANOVA of LDH activity in mixed glial cultures expressed as a % of controls shows an effect of IFN γ [$F(1,8)=66.12$, $p<0.0001$], no effect of PFF [$F(1,8)=3.614$, $p=0.0938$], and no interaction between IFN γ and PFF [$F(1,8)=0.006832$, $p=0.9362$]. Post hoc comparisons reveal an increase in % LDH activity in cells treated with IFN γ +PBS compared to PBS+PBS ($p=0.0018$), and from cells treated with IFN γ +PFF compared to PBS+PFF ($p=0.0021$) (Figure 6.24b).

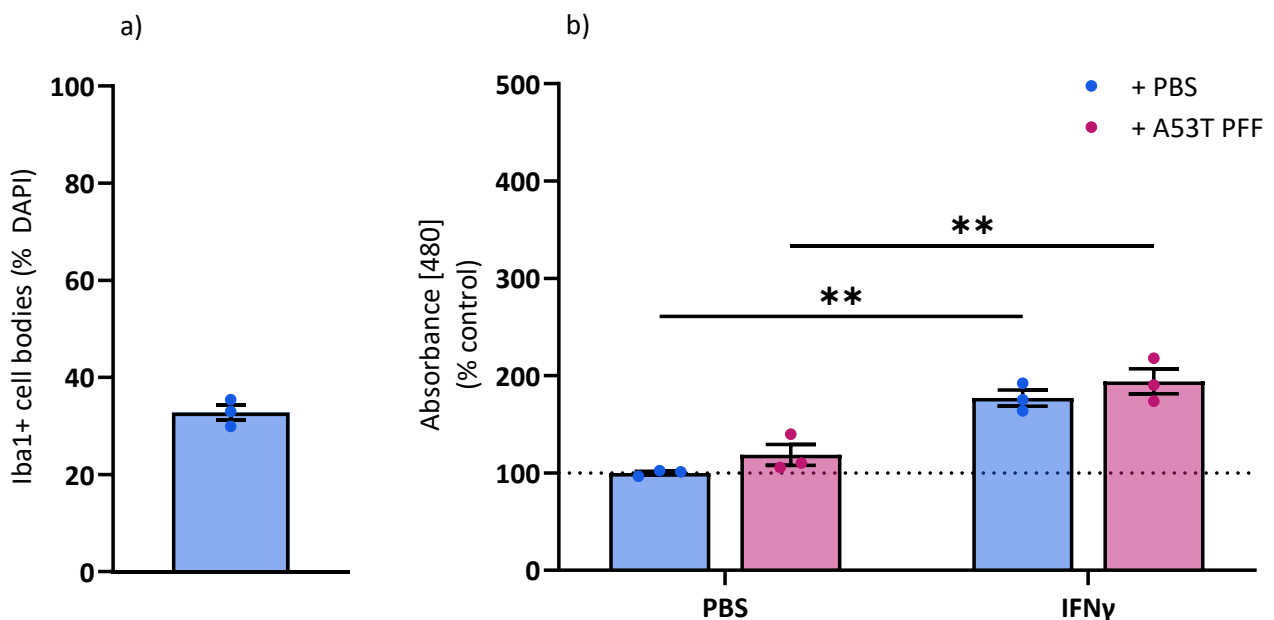


Figure 6.24. Proportion of Iba1+ cells and LDH activity in mixed glial cultures pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF. a) Proportion of Iba1+ cells in mixed glial cultures used in experiments 6.5.9-6.5.10, b) LDH activity expressed as percentage of PBS+PBS-treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H $_2$ O $_2$). Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's test for multiple comparisons. $n=3$. **= $p\leq 0.01$.

A two-way ANOVA of IL-1 β mRNA expression shows an effect of IFN γ [F(1,8)=24.24, p=0.0012], an effect of PFF [F(1,8)=14.613, p=0.0056], and an interaction between IFN γ and PFF [F(1,8)=15.86, p=0.0040]. Post hoc comparisons reveal a reduction in IL-1 β mRNA expression in cells treated with PBS+PFF (p=0.0026), IFN γ +PBS (p=0.0011), and IFN γ +PFF (p=0.0012) compared to PBS+PBS (Figure 6.25a).

A two-way ANOVA of IL-1 α mRNA expression shows no effect of IFN γ [F(1,8)=3.798, p=0.0872], no effect of PFF [F(1,8)=0.4083, p=0.5407], and an interaction between IFN γ and PFF [F(1,8)=9.194, p=0.0163]. Post hoc comparisons reveal a reduction in IL-1 α mRNA expression cells treated with IFN γ +PBS (p=0.0320) compared to PBS+PBS (Figure 6.25b).

A two-way ANOVA of IL-6 mRNA expression shows an effect of IFN γ [F(1,8)=12.68, p=0.0074], no effect of PFF [F(1,8)=1.003, p=0.3459], and no interaction between IFN γ and PFF [F(1,8)=3.037, p=0.1195]. Post hoc comparisons reveal a reduction in IL-6 mRNA expression in cells treated with IFN γ +PBS (p=0.0233) and IFN γ +PFF (p=0.0482) compared to PBS+PBS (Figure 6.25c).

A two-way ANOVA of TNF α mRNA expression shows no effect of IFN γ [F(1,8)=0.01642, p=0.9012], no effect of PFF [F(1,8)=3.009, p=0.1210], and an interaction between IFN γ and PFF [F(1,8)=11.01, p=0.0106]. Post hoc comparisons reveal reduction in TNF α mRNA expression in cells treated with

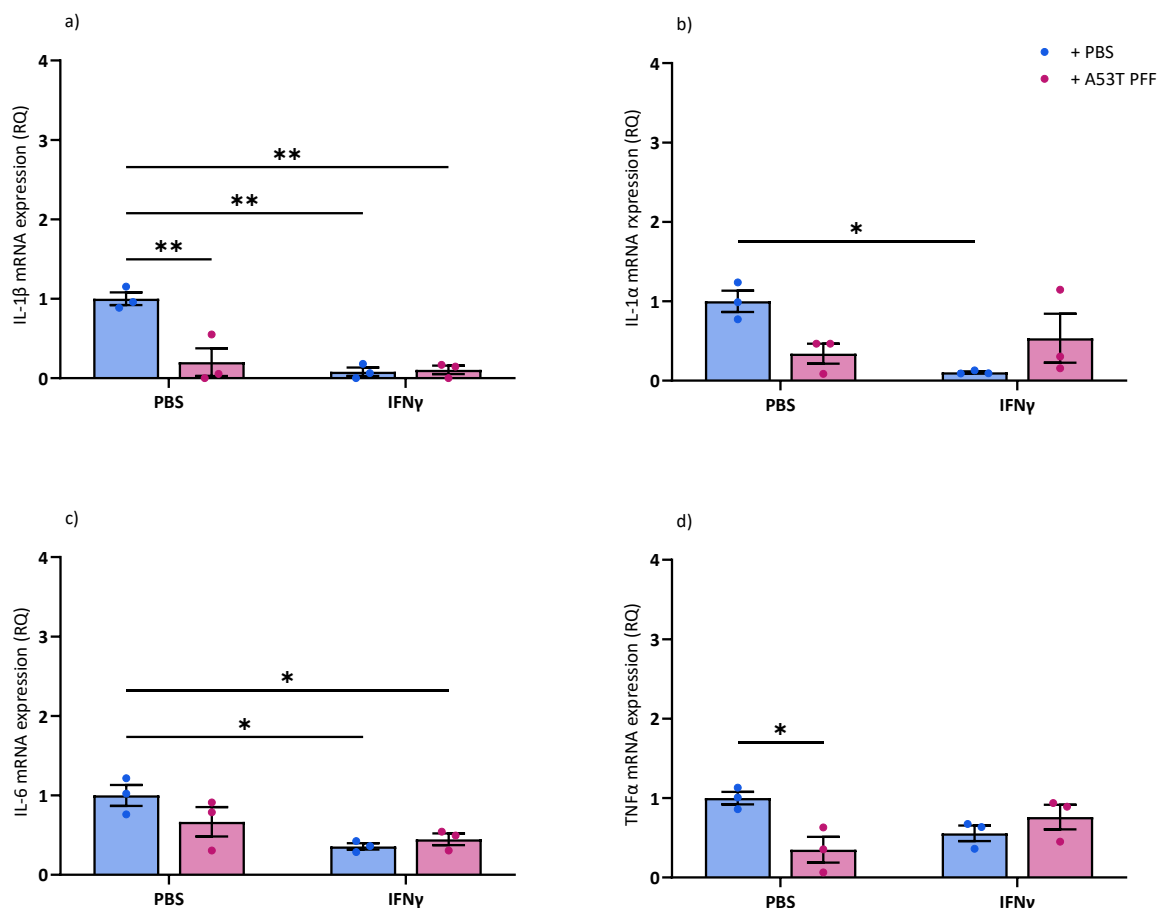


Figure 6.25. mRNA expression of target cytokines in mixed glial cells pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF. Mixed glial mRNA expression of a) IL-1 β , b) IL-1 α , c) IL-6, and d) TNF α expressed as relative quantity compared to PBS+PBS-treated controls. GAPDH was used as an internal control. Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's test for multiple comparisons. n=3. * = p<0.05, ** = p<0.01.

6.5.10. Neuronal response to treatment with conditioned media transferred from mixed glia pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF.

One-way ANOVA of neuronal LDH activity expressed as a % of control shows no effect of conditioned media treatment [$F(3,8)=1.531$, $p=0.2796$] (Figure 6.26).

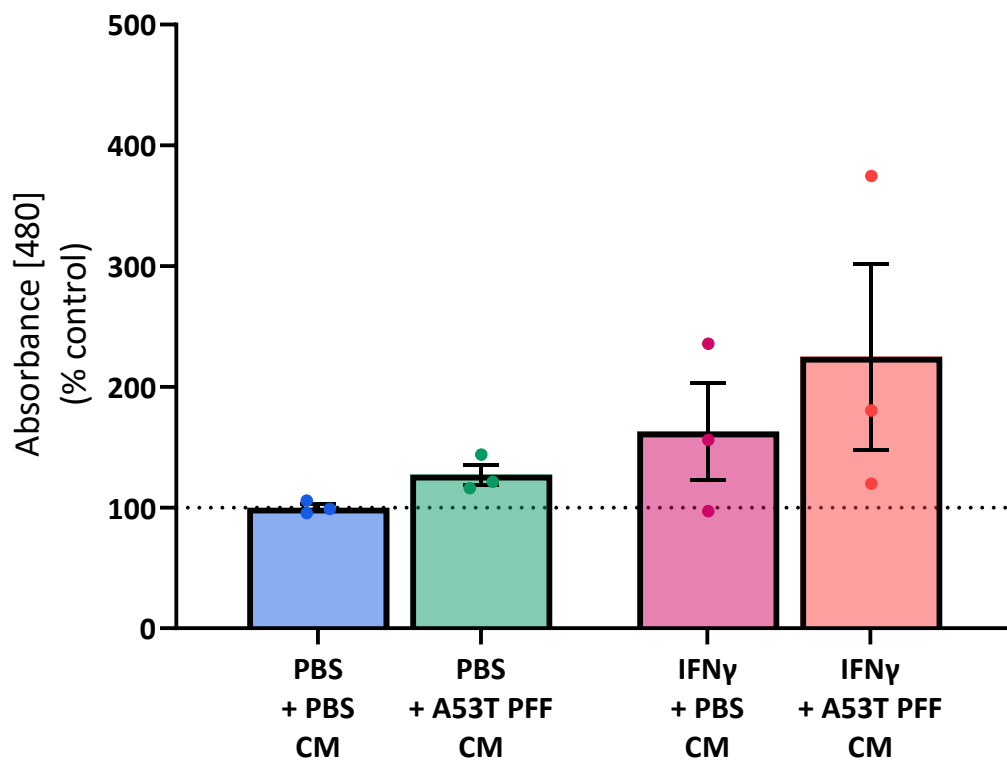


Figure 6.26. LDH activity in primary neuronal cultures treated with conditioned media from mixed glial cultures pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF. LDH activity expressed as percentage of PBS+PBS-treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H₂O₂). Data are expressed as mean $\pm$ SEM. One-way ANOVA. $n=3$.

One-way ANOVAs of neuronal complexity measures show no effect of conditioned media treatment on number of neurites [$F(3,14)=0.6356$, $p=0.6043$], length of neurites [$F(3,14)=0.2081$, $p=0.8891$], or number of branches [$F(3,14)=0.3119$, $p=0.8165$] (Figure 6.27a-c). Two-way ANOVA of number of intersections shows no effect of conditioned media treatment [$F(3,364)=2.4$, $p=0.0672$], an effect of radial distance [$F(25,364)=51$, $p<0.0001$], and no interaction between conditioned media treatment and radial distance [$F(75,364)=0.15$, $p>0.9999$]. Post hoc comparisons reveal no differences in Sholl profiles between treatment groups (Figure 6.27d).

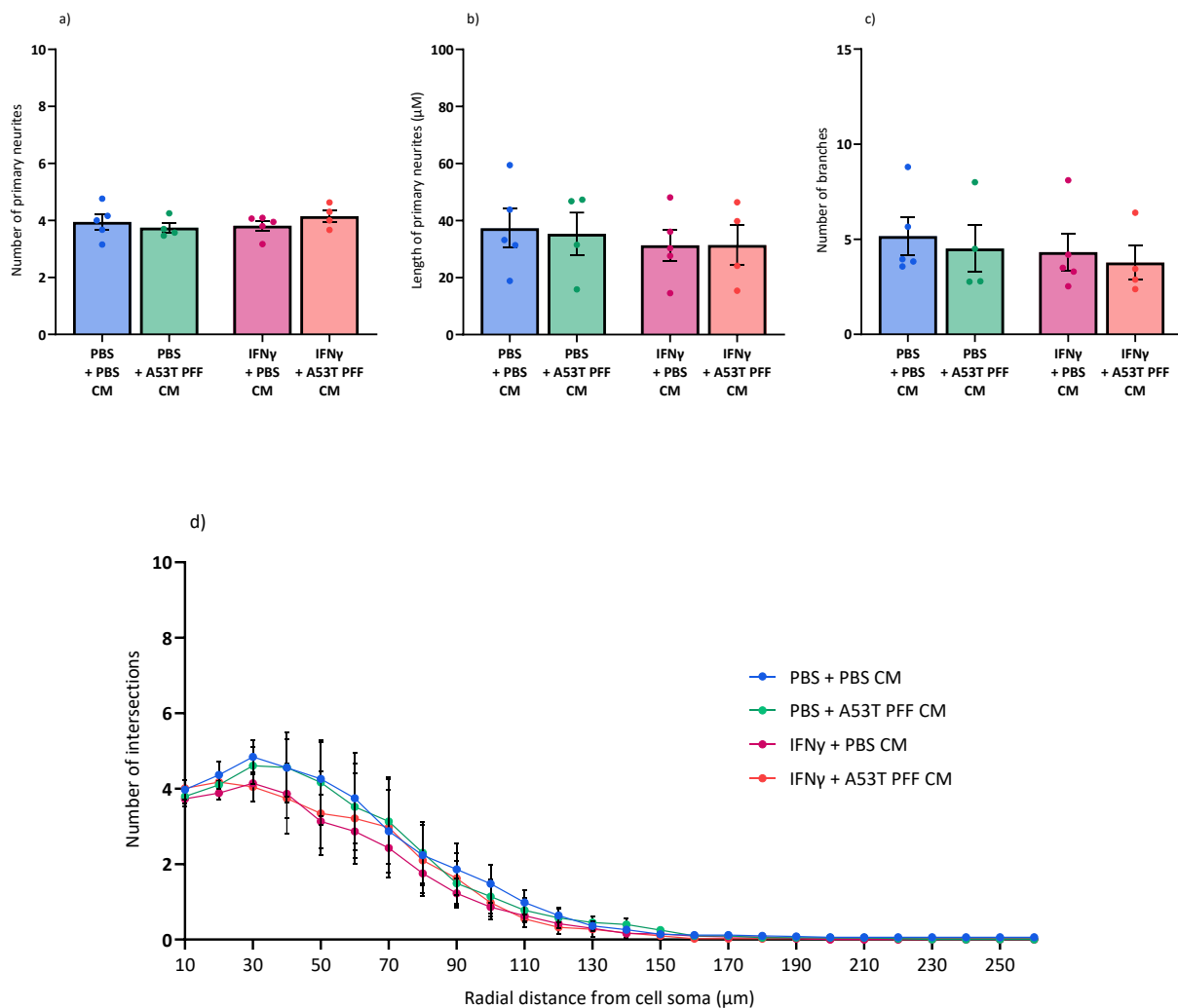


Figure 6.27. Sholl profile of MAP2+ primary neurons treated with conditioned media from mixed glial cultures pre-treated with IFN γ for 24 hours prior to treatment with A53T PFF. a) Number of primary neurites, b) length of primary neurites, c) number of secondary branches. Data are expressed as mean $\pm$ SEM. a-c) One-way ANOVA, d) two-way ANOVA. $n=4-5$.

6.5.11. Enriched microglial response to treatment with IFN γ for 24 hours.

In the following microglial enriched cell cultures, Iba1+ microglia comprised an average of 70% of all DAPI+ cells (Figure 6.28a). A one-way ANOVA of enriched microglial LDH activity expressed as a % of controls shows no effect of IFN γ treatment [$F(2,9)=0.5909$, $p=0.5740$] (Figure 6.28b).

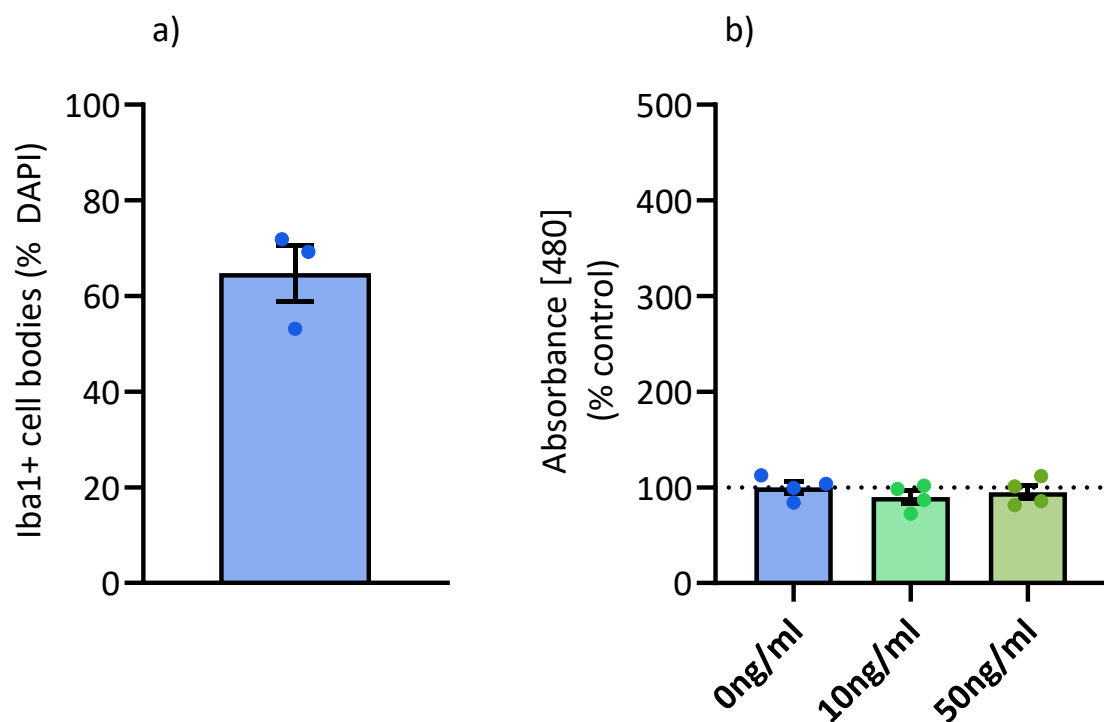


Figure 6.28. Proportions of Iba1+ cells and LDH activity in primary enriched microglial cultures treated with 0-50ng/ml IFN γ for 24 hours. a) Proportion of Iba1+ cells in enriched glial cultures used in experiments 6.5.11-6.5.12, b) enriched microglial LDH activity expressed as a percentage of 0ng/ml IFN γ -treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H $_2$ O $_2$). Data are expressed as mean $\pm$ SEM. One-Way ANOVA. $n=3-4$.

One-way ANOVAs show no effect of IFN γ treatment on cell area [$F(2,8)=0.4239$, $p=0.6684$], perimeter [$F(2,8)=0.06320$, $p=0.9392$], Feret's diameter [$F(2,8)=0.01780$, $p=0.9824$], or circularity [$F(2,8)=0.4845$, $p=0.6330$] (Figure 6.29a-d). One-way ANOVA of cell solidity shows an effect of IFN γ treatment [$F(2,8)=8.832$, $p=0.0094$]. Post hoc comparisons reveal increased solidity of microglial cells treated with 10ng/ml IFN γ ($p=0.0408$) and 50ng/ml IFN γ ($p=0.0056$) compared to vehicle-treated controls (Figure 6.29e).

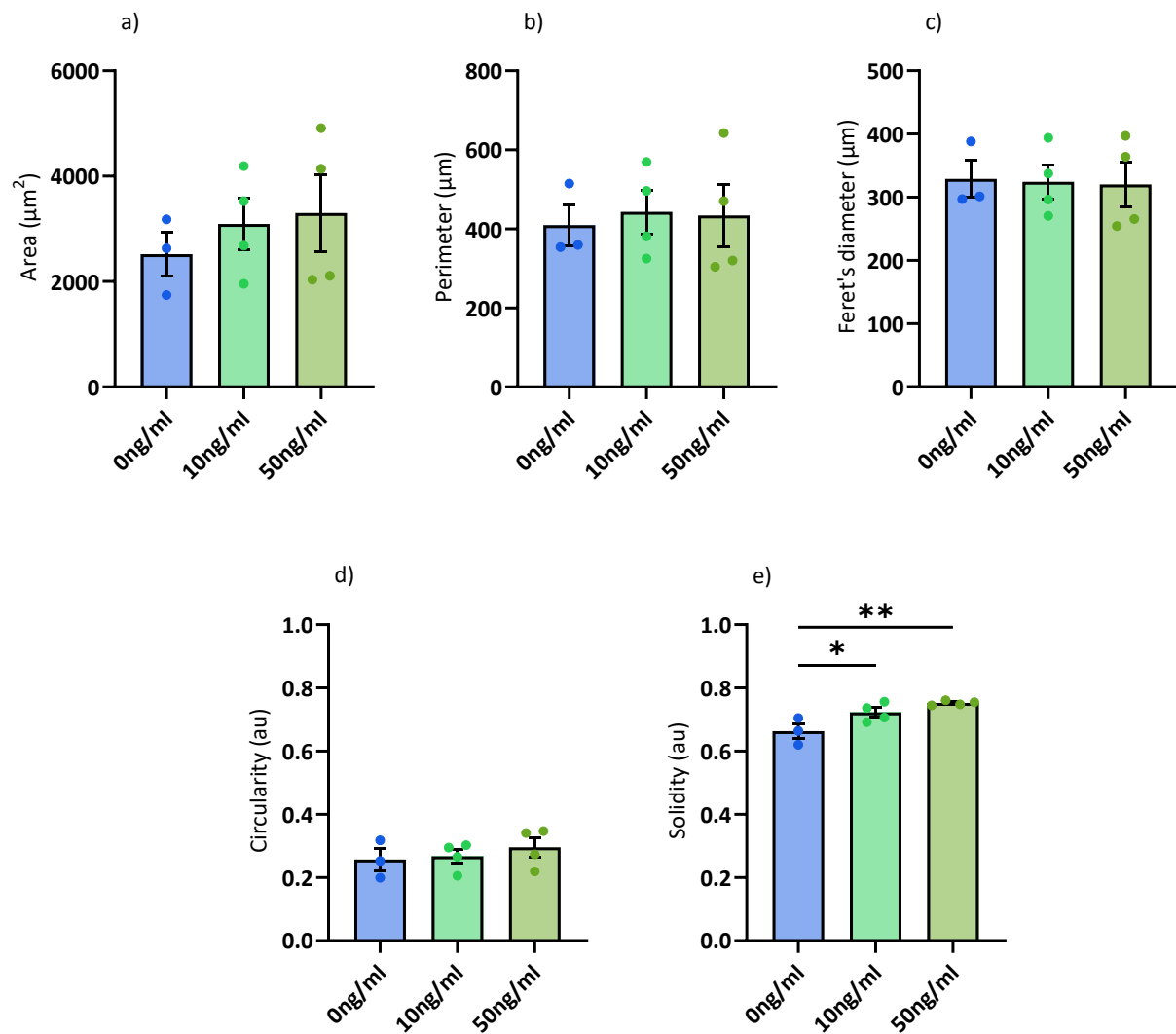


Figure 6.29. Morphology of Iba1+ microglia in primary enriched microglial cultures treated with 0-50ng/ml IFN γ for 24 hours. Cells were fixed for Iba1 immunofluorescence. Image analysis was performed on ImageJ to quantify cell a) area, b) perimeter, c) Feret's diameter, d) circularity, e) solidity. Data are expressed as mean $\pm$ SEM. One-way ANOVA followed by Dunnett's test for multiple comparisons. $n=3$. *= $p \leq 0.05$, **= $p \leq 0.01$.

One-way ANOVA of IL-1 α mRNA expression shows no effect of IFN γ treatment [$F(2,15)=1.029$, $p=0.3823$] (Figure 6.30a). One-way ANOVA of TNF α mRNA expression shows no effect of IFN γ treatment [$F(2,15)=2.232$, $p=0.1417$] (Figure 6.30b).

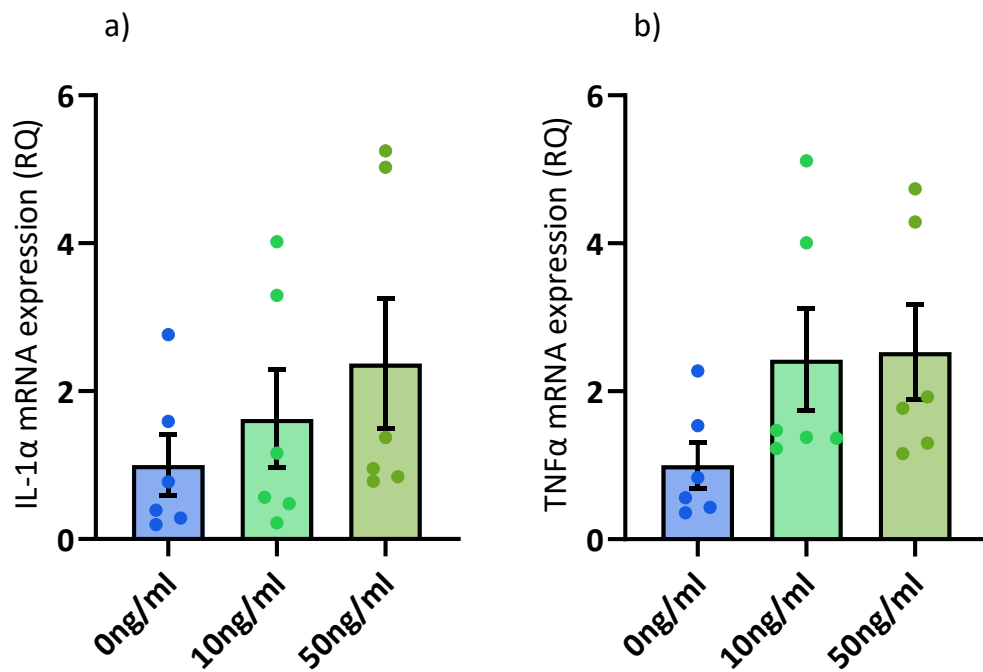


Figure 6.30. mRNA expression of target cytokines in enriched microglial cells treated with 0-50ng/ml IFN γ for 24 hours. Enriched microglial mRNA expression of a) IL-1 α and b) TNF α expressed as relative quantity compared to 0ng/ml IFN γ -treated controls. GAPDH was used as an internal control. Data are expressed as mean $\pm$ SEM. One-Way ANOVA followed by Dunnett's test for multiple comparisons. $n=6$.

6.5.12. Neuronal response to treatment with conditioned media transferred from enriched microglial cultures treated for 24 hours with IFN γ .

A one-way ANOVA of neuronal LDH activity expressed as a % of controls shows no effect of conditioned media treatment [$F(2,9)=0.6944$, $p=0.5243$] (Figure 6.31).

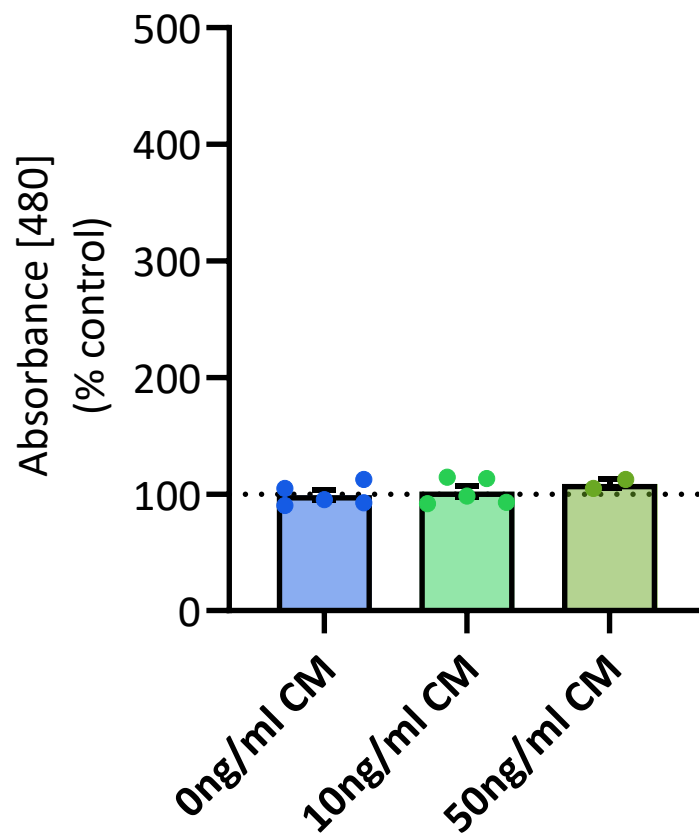


Figure 6.31. LDH activity in primary neuronal cultures treated with conditioned media transferred from enriched microglial cells treated for 24 hours with 0-50ng/ml IFN γ . LDH activity expressed as percentage of 0ng/ml IFN γ conditioned media-treated controls (100%). Values of 100% depict negligible cell death induced by negative control (PBS), values of 1000% depict maximal cell death induced by positive control (H $_2$ O $_2$). Data are expressed as mean $\pm$ SEM. One-Way ANOVA. $n=3-5$.

Independent one-way ANOVAs show no effect of conditioned media treatment on number of primary neurites [$F(2, 6) = 3.444$, $p = 0.1009$], length of primary neurites [$F(2, 6) = 0.6802$, $p = 0.5417$], or number of branches [$F(2, 6) = 0.2755$, $p = 0.7683$] (Figure 6.32a-c). A two-way ANOVA of Sholl profile shows an effect of conditioned media treatment [$F(2, 156) = 9.3$, $p = 0.0001$] and radial distance [$F(25, 156) = 85$, $p < 0.0001$], and no interaction between treatment and radial distance [$F(50, 156) = 0.22$, $p > 0.9999$]. Post hoc comparisons show no differences in Sholl profiles between treatment groups (Figure 6.32d).

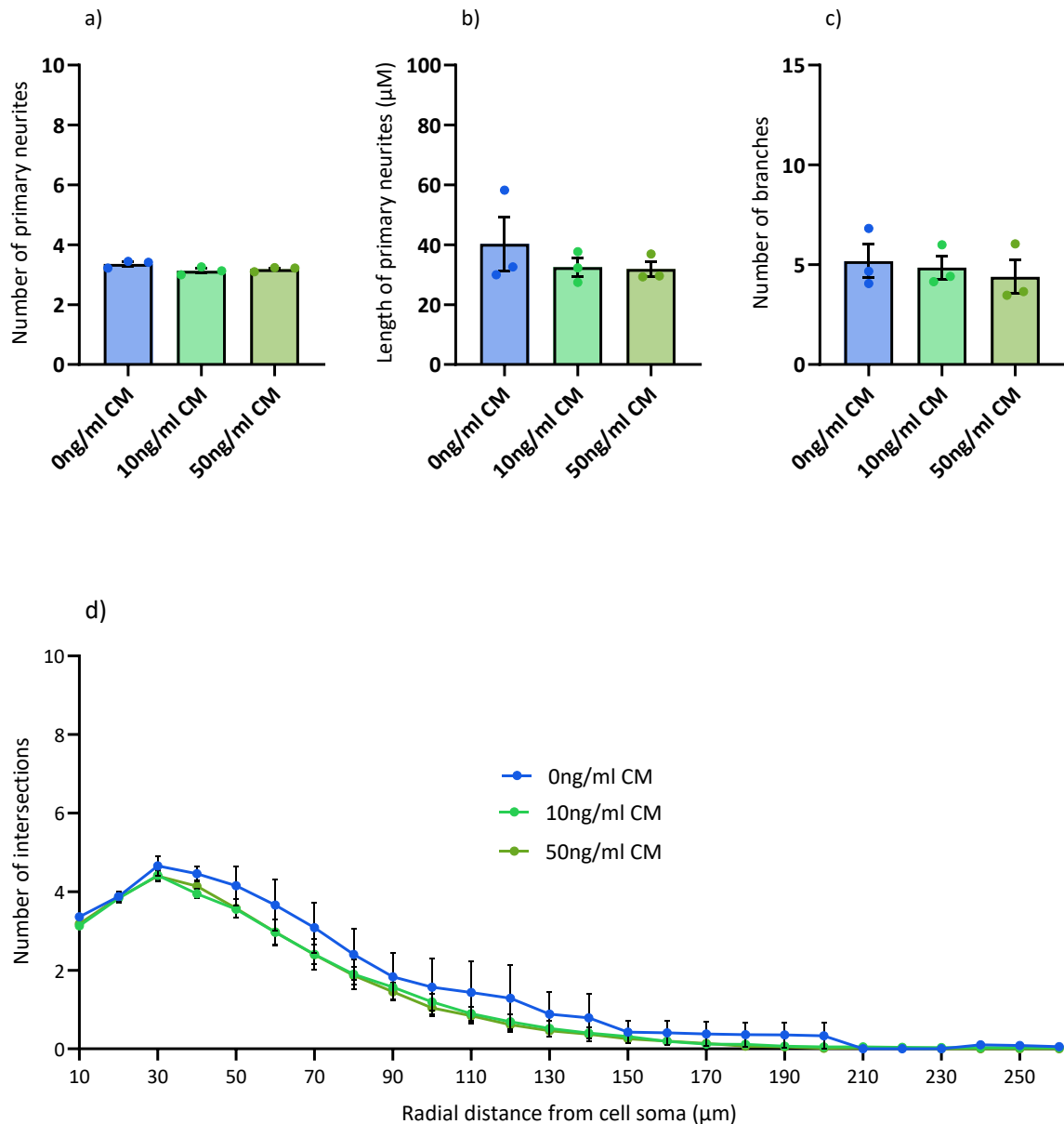


Figure 6.32. Sholl profile of MAP2+ primary neurons treated for 24 hours with culture media from enriched microglial cells conditioned with 0-50ng/ml IFN γ for 24 hours. a) Number of primary neurites, b) length of primary neurites, c) number of secondary branches, d) Sholl profile. Data are expressed as mean $\pm$ SEM. a-c) One-way ANOVA, d) two-way ANOVA followed by Dunnett's post hoc. $n = 3$.

6.5.13. Neuronal response to treatment with conditioned media transferred from enriched microglia pre-treated with A53T PFF for 24 hours prior to treatment with IFN γ .

One-way ANOVAs of neuronal complexity measures show no effect of conditioned media treatment on number of primary neurites [$F(3,8)=1.882$, $p=0.2110$], length of primary neurites [$F(3,8)=0.4295$, $p=0.7375$], or number of branches [$F(3,8)=1.780$, $p=0.2286$] (Figure 6.33a-c). Two-way ANOVA of Sholl profile shows no effect of conditioned media treatment [$F(3,208)=2.4$, $p=0.0702$], an effect of radial distance [$F(25,208)=748$ $P<0.0001$], and no interaction between treatment and radial distance [$F(75,208)=0.42$, $p>0.9999$]. Post hoc comparisons reveal no differences in Sholl profile between treatment groups (Figure 6.33d).

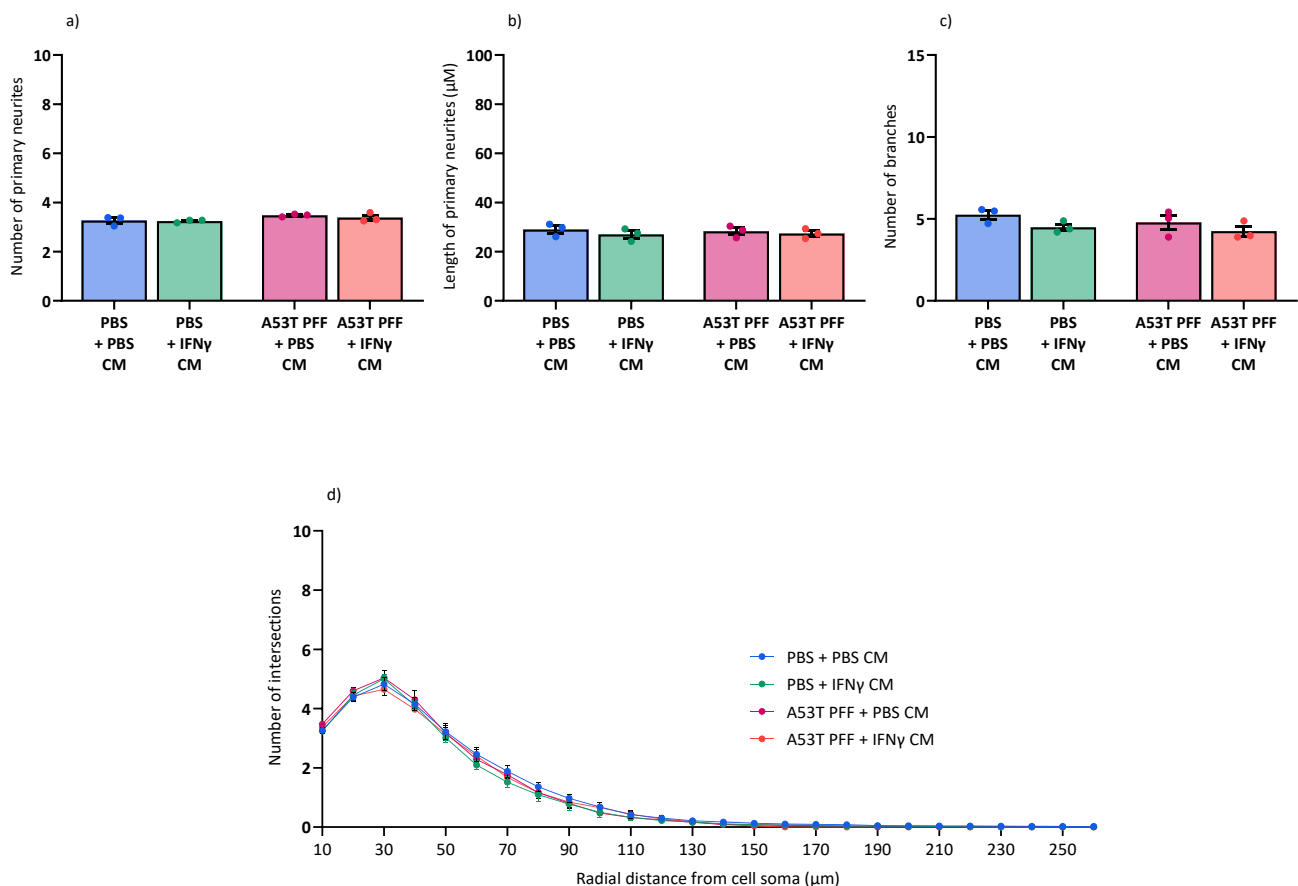


Figure 6.33. Sholl profile of MAP2+ primary neurons treated with conditioned media transferred from enriched microglial cultures pre-treated with A53T PFFs for 24 hours prior to treatment with IFN γ . a) Number of primary neurites, b) length of primary neurites, c) number of secondary branches. Data are expressed as mean $\pm$ SEM. a-c) One-way ANOVA, d) two-way ANOVA followed by Tukey's test for multiple comparisons. $n=3$.

6.5.14. Neuronal response to treatment with conditioned media transferred from enriched microglia treated with IFN γ in DMEM.

Unpaired t-tests show a reduction in the number of primary neurites of MAP2+ neurons treated with conditioned media from enriched microglial treated with IFN γ (50ng/ml) compared to vehicle DMEM controls [t(6)=2.452, p=0.0496] (Figure 6.34a). IFN γ -treated DMEM conditioned media had no effect on length of primary neurites [t(6)=2.335, p=0.0583] or number of branches [t(6)=2.373, p=0.0553] (Figure 6.34b-c). Two-way ANOVA of Sholl profile shows an effect of treatment [F(1,156)=88, p<0.0001], an effect of radial distance [F(25,156)=61, p<0.0001], and an interaction between treatment and distance [F(25,156)=1.6, p=0.0418]. Post hoc comparisons reveal reduced number of intersections of neurons treated with IFN γ -treated DMEM conditioned media at 30-120 μ m from the cell soma, compared to DMEM controls (Figure 6.34d).

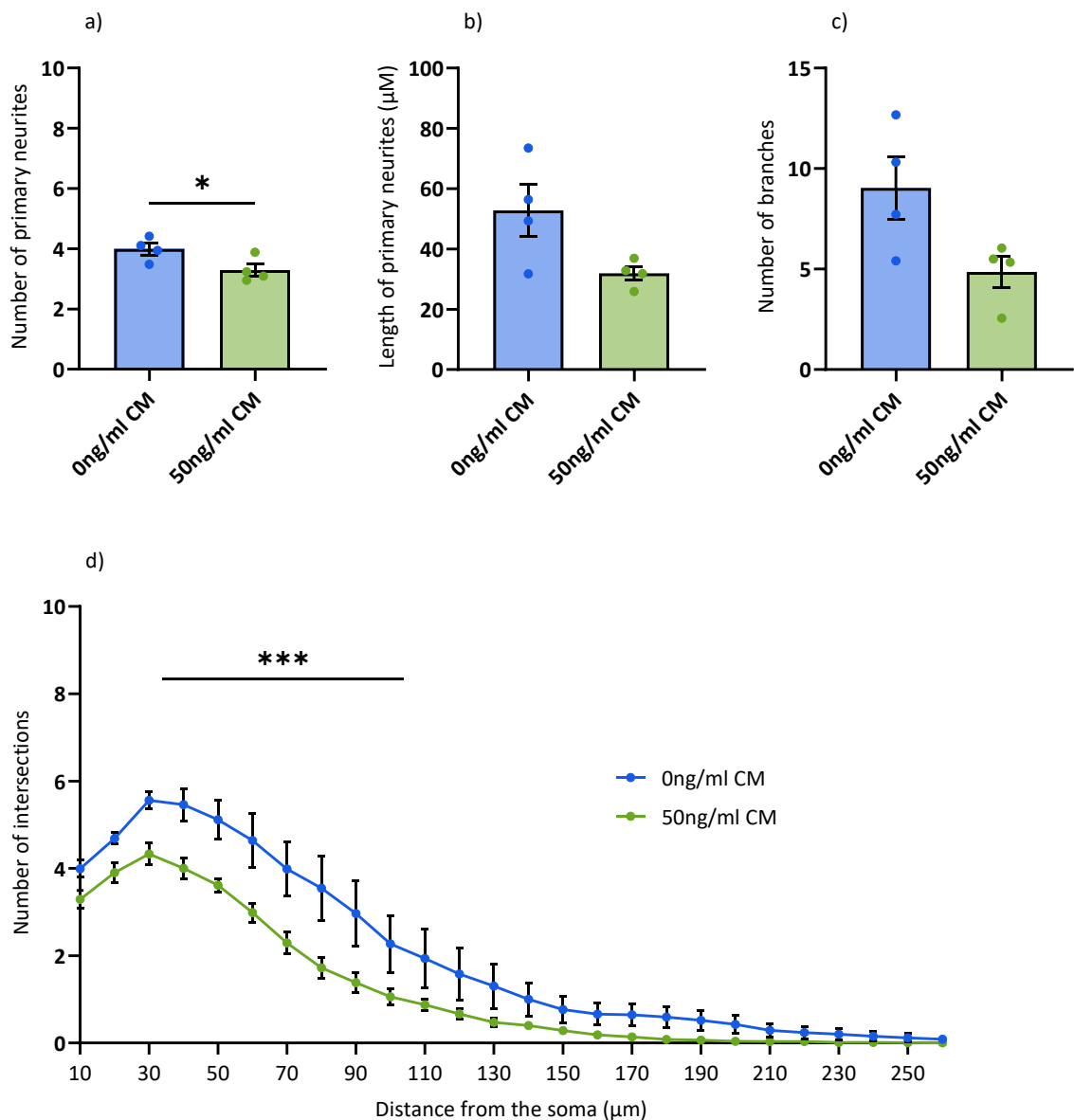


Figure 6.34. Sholl profile of MAP2+ primary neurons treated with conditioned media transferred from enriched microglial cultures treated with IFN γ in DMEM. a) Number of primary neurites, b) length of primary neurites, c) number of secondary branches. Data are expressed as mean $\pm$ SEM. a-c) Unpaired t-test, d) two-way ANOVA followed by Tukey's test for multiple comparisons. n=3.

6.6. Discussion

The collective aim of the present *in vitro* investigations was to assess a role for glial cells as mediators of α Syn-induced neuronal atrophy, and explore variant forms of α Syn in producing these effects *in vitro*. Primary cortical glial and neuronal cultures proved unavailing for the study of α Syn-mediated microglial activation, and associated neuronal atrophy following transfer of glial conditioned media.

24-hour treatment of mixed glial cultures with α Syn filaments, oligomers, WT PFFs, or A53T PFFs (the same PFFs as those employed in the *in vivo* investigations described previously) failed to induce changes in glial mRNA expression of target cytokines, or morphology of Iba1+ microglia. Conditioned media from glia treated with these constructs had no impact on neuronal complexity when transferred to primary cortical neurons in culture.

Application of α Syn filaments, oligomers, WT PFFs, or A53T PFF (4 μ g/ml) were non-toxic to mixed glial cultures comprising approximately 25% Iba1+ microglial cells. The structural morphology of microglia can be used as a proxy to gauge their activation state (Davis et al., 1994). Through analysis of cell area, perimeter, Feret's diameter, circularity, and solidity, the present studies examined the degree to which primary Iba1+ microglia assumed ameboid-like or ramified morphologies in culture (Soltys et al., 2005; Soltys et al., 2001). Morphologically, Iba1+ microglia treated with filamentous, oligomeric, or fibrillar α Syn constructs assumed comparable shapes and sizes to those of vehicle-treated cells (representative images in Figure 6.1). The absence of architectural changes of Iba1+ microglia 24 hours following α Syn stimulation indicated little transition from ramified to ameboid morphology. While cultured microglia underwent no morphological changes, Iba1+ cells displayed greater fluorescence intensity 24-hours post treatment with EGCGO compared to PBS-treated controls. Increased Iba1 fluorescence intensity demonstrates enhanced Iba1+ protein expression, a microglial response which typically follows cellular interaction with an immunogenic stimulus (Gou et al., 2021; Ito et al., 1998). Analysis of mRNA expression from mixed glial cell lysate indicated increased IL-1 β and IL-1 α expression after 24-hour treatment with DSO. Although significance was not reached, a trend of increased IL-10 mRNA expression was also observed in response to treatment with DSO, compared to vehicle-treated controls. The absence of changes in mRNA expression following treatment with PFFs was surprising, given PFFs have been shown previously to drive IL-1 β and IL-6 expression following 24-hour treatment in primary glial cultures (Gordon et al., 2018). Further, these findings contradict previous reports which show fibrillar α Syn to be more immunogenic than oligomeric α Syn (Gustot et al., 2015). Nonetheless, transfer of conditioned media from DSO-treated mixed glia (which displayed enhanced IL-1 β , IL-1 α , and IL-10 mRNA expression) to primary neurons had no effect on neuronal complexity, despite altering immune response in glia (representative images in Figure 6.3). From this result, it can be inferred that enhanced IL-1 β and IL-1 α mRNA expression following DSO treatment was likely not accompanied by enhanced protein secretion into glial media. The increase in Iba1+ immunoreactivity in microglial cells following treatment with EGCGO was not accompanied by changes in mRNA expression of cytokines selected.

The precursor form of IL-1 β is not biologically active in culture. It requires enzymatic cleavage to elicit its inflammatory activity, and as such, is not likely to contribute directly to glial-mediated changes in neuronal complexity *in vitro* when transferred via conditioned media (Garlanda et al., 2013; Rebe & Ghiringhelli, 2020). IL-1 α , however, exhibits inflammatory activity both in its precursor and cleaved states, and may prove a more relevant candidate for assessing pro-inflammatory response of glial cells to α Syn-treatment (Di Paolo & Shayakhmetov, 2016).

Changes in IL-10 expression in response to α Syn has not been as widely characterised. As a cytokine typically recognised for its anti-inflammatory properties, IL-10 mRNA expression at increased levels may indicate a counteracting response initiated by activated microglia in attempts to combat a cytotoxic environment driven by IL-1 β and IL-1 α (Iyer & Cheng, 2012). Transfer of conditioned media from dopamine-stabilised oligomer-treated mixed glia to primary neurons had no impact on neuronal complexity. This outcome likely suggests that changes in mRNA transcription were not reflected by changes in protein expression or secretion into the conditioned media at this timepoint. Analysis of protein content within cell supernatant would be necessary to confirm this. Should IL-1 β , IL-1 α , and IL-10 protein expression all be enhanced in response to this form of α Syn oligomers, the absence of neuronal damage may be explained by anti-inflammatory properties of IL-10, which has been shown to confer protection against IL-1 β and IL-1 α -associated neuronal damage.

In contrast, IL-10 has also been shown to exacerbate α Syn aggregation in mice. In an apparent shift away from its well-established immunosuppressive state, IL-10 expression induced microglial activation, exacerbated α Syn aggregation, and impaired autophagy in midbrain and spinal cord neurons of PFF-seeded mice. IL-10 expression also upregulated microgliosis in transgenic mice expressing human A53T-mutant α Syn, and led to early death (Cockey et al., 2021). In demonstrating deleterious effects of enhanced IL-10 expression in both A53T-mutant α Syn transgenic mice and PFF-seeded mice, this study bolsters a theory in support of a bidirectional relationship between IL-10 and α Syn-associated neurodegeneration. These findings raise the interesting question around the multiple characteristics which IL-10 can present in pathogenic conditions. Further investigation may elucidate whether increased IL-10 expression in α Syn-treated mixed glial cultures represent a neuroprotective immunosuppressive response for combating α Syn, or a pathogenic phenotype to further exacerbate its pathology. Nonetheless, despite the trend of increased IL-10 expression in the present *in vitro* investigations, the transfer of conditioned media from DSO-treated glia to primary neurons incurred no cytotoxicity to neurons, and had no effect on neuronal complexity. An assessment of protein expression in conditioned media will be necessary to assess whether altered mRNA expression was paralleled by protein secretion.

EGCG, a flavonoid, has been exploited in research to stabilise the conformational assembly of recombinant proteins like α Syn and amyloid β . Despite recent advances in PD-related research with the use of EGCG-stabilised α Syn preformed proteins, the structural basis explaining the effective mechanism of interaction remains unknown. While maintaining the same size and morphology as non-treated oligomers, α Syn oligomers formed in the presence of EGCG display an altered β -sheet structure, and are reportedly less toxic than pristine α Syn oligomers (Gronnemose et al., 2022). Through interacting with and immobilising the proteins' C-terminal region, EGCG can both direct the aggregation of α Syn monomers towards an oligomeric state, and remodel amyloid fibrils into disordered oligomers. In doing so, EGCG inhibits α Syn oligomers' ability permeabilise cellular and vesicular membranes, thereby reducing their cytotoxic profile. In addition, EGCG-stabilised oligomers are unable to stimulate production of reactive oxygen species or reduce mitochondrial activity. EGCG also shows particular affinity for the N-terminal region of α Syn, the binding of which may prevent spontaneous fibrillation (Lorenzen et al., 2014). The use of EGCG to stabilise recombinant α Syn oligomers does not alter the size or secondary structure of the protein, and is therefore not thought to have a direct effect on the environment in which they are being explored in the laboratory (ie. cells or animals). Oxidised dopamine is also widely used in research to kinetically stabilise oligomeric α Syn assemblies, through noncovalent, reversible interaction with c-terminus

residues of α Syn, dopamine substoichiometrically prevents oligomeric maturation into fibrils. Without altering the proteins higher order structure, stabilisation by dopamine is also not thought to confound treatment-related results (Mor et al., 2017). A major shortcoming of the present investigation was the omission of a Thioflavin-T assay to confirm the aggregatory state of constructs immediately before use. With a Thioflavin-t-assay, the experimenter would have been able to confirm whether the constructs were still in oligomeric or fibrillar confirmation at the time of use, and had not degraded or aggregated into smaller or larger constructs. This assay should be employed in all future experiments.

Although 24-hour treatment with recombinant oligomeric α Syn elicited more observable immunogenic changes in mixed glial cultures than PFFs, *in vitro* investigations continued to employ PFFs following interesting findings from the previous *in vivo* investigations. Delivery of WT PFFs into the rat substantia nigra showed capacity to increase the number of Iba1+ microglia in the ipsilateral substantia nigra. To examine this finding on a cellular level, mixed glial cells were extracted from Wistar rat neonates, grown to maturity in culture, and treated with WT PFFs for an extended timeframe of 24-96 hours.

LDH activity in mixed glial supernatant is defined by absorbance, which is expressed as a percentage of 24-hour PBS-treated controls to achieve a value representative of cytotoxicity. A value of 100% signifies the absence of LDH activity, while values within the range of 800-1000% represent high levels of cytotoxicity, as defined by an LDH positive control substrate provided within the assay. Mixed glial cultures treated with 0.01% hydrogen peroxide exemplify levels of LDH comparable to those of the internal positive control. Analysis of LDH activity in mixed glial cultures revealed an increase in LDH activity from mixed glia following a 96-hour treatment regime. Albeit significant, this increase is negligible relative to positive controls included in the experiment, and thus, treatment can be interpreted as non-cytotoxic.

Surprisingly, application of WT PFFs at a concentration of 4 μ g/ml induced no changes to the morphology of Iba1+ microglia or Iba1+ immunofluorescence over 24, 48, 72 or 96 hours of exposure. Low-range circularity values (0.1-0.3 au) typically define cells with a ramified microglial morphology. With the average observed circularity values lying at approximately 0.2-0.4 au both with and without PFF treatment, the data imply that microglial cells in mixed glial cultures exhibit a resting state following WT PFF treatment throughout the 96-hour time course. Given the widely reported finding that microglial morphology can be assessed to gauge glial activation state, and that WT PFFs at this concentration have shown immunogenic properties in primary cultures, these results were unexpected.

Time-dependent patterns of IL-6, IL-1 α , IL-1 β , TNF α , and IL-10 mRNA expression were observed in this study. These patterns, however, were observed consistently in control cultures and WT PFF-treated cultures, further showing the inability for WT PFFs to alter glial mRNA expression, even following extended exposure. In both vehicle-treated and WT PFF-treated groups, IL-1 β , IL-1 α , and IL-6 mRNA expression levels fell over time, showing highest levels of expression at 24 hours, the earliest time point measured. WT PFF treatment may have accelerated the reduction in IL-1 β and IL-1 α expression at 48 hours compared to vehicle, although this implication was minor. In both groups, TNF α expression increased over the course of 72 hours, by which point, the expression increased to 4-fold that of its expression at 24 hours, and then gradually declined. Comparable time-dependent cytokine gene expression patterns were observed in both WT PFF- and vehicle-treated cells,

although a trend indicated the possibility that WT PFFs amplified the increase in TNF α expression from 24-72 hours to a greater extent than controls. As these changes in mRNA expression were time-dependent and not treatment-dependent, it may be possible that the proportion of Iba1+ cells in these cultures were too low to elicit a significant reaction following PFF treatment. The transfer of glial conditioned media onto primary neurons had no effect on cell viability or neuronal complexity, as assessed by Sholl analysis.

Although the inflammasome was not central to this investigation, inflammasome priming experiments have brought to light the role of microglial priming in driving neurotoxic α Syn-mediated inflammation. Previous research has shown, that while α Syn fibrils alone can induce IL-1 β release from primary murine microglial cells at 24 hours, glial response to α Syn fibrils is enhanced by approximately 35% in primed cultures. Previous research in monocytic and immortalized microglial cell lines have shown an ability for α Syn fibrils to trigger NLRP3 inflammasome activation and upregulation of both IL-1 β mRNA and protein expression (Codolo et al., 2013). Notably, these results differentiate α Syn fibrils from other inflammatory stimuli or particulate structures which show propensity to induce IL-1 β secretion, but not its synthesis (Halle et al., 2008; Hornung et al., 2008). In primary murine microglia primed with LPS and activated with fibrillar α Syn, a time-dependent secretion of IL-1 β was observed over 18 hours. After 24 hours of treatment with α Syn, the quantity of secreted IL-1 β was comparable to that obtained one hour after treatment with potent NLRP3 activator ATP (adenosine 5'-triphosphate). Cleaved IL-1 β was also confirmed in glial conditioned media 24 hours after stimulation. In complete contradiction to results reported here, this finding suggested that fibrillar α Syn mediates the delayed, but nonetheless robust activation of the inflammasome pathway in cultured primary microglia, prompting the transduction and translation of IL-1 β and its cleaved counterpart. For these reasons, IFN γ , a classic experimental microglial stimulant, was employed to explore activity of primed microglia in culture, and assess their response to α Syn.

An investigation was first conducted to explore the effects of IFN γ alone, on mixed glial cultures (representative images in Figure 6.3). In this investigation, rat IFN γ at doses from 1-100ng/ml were non-toxic to mixed glial cultures, while 500ng/ml of rat IFN γ increased LDH release from mixed glial cells to approximately 250% of controls, implying mild cytotoxicity at this dose. A dose-related response to rat IFN γ was not observed through Iba1+ cell morphology analysis, but was apparent following analysis of Iba1+ immunofluorescence. Iba1+ immunofluorescence was increased in cells treated with IFN γ from 10ng/ml to 500ng/ml. These changes in Iba1+ immunoreactivity in response to higher doses of IFN γ likely depict more reactive microglia in culture. Though not significant, IL-1 β and IL-1 α expression gradually fell in response to IFN γ in a concentration dependent manner, with the lowest levels of mRNA expression observed following treatment at a dose of 100ng/ml. A dose-dependent trend of TNF α mRNA expression was observed in the opposite direction of IL-1 β and IL-1 α expression, showing greater expression in response to higher IFN γ concentrations. IL-10, a cytokine associated predominantly with anti-inflammatory properties, was measured in this study to determine if treated glial cells in culture expressed IL-10 to counteract changes in the expression of pro-inflammatory factors (Wolf et al., 2017). IL-10 mRNA expression appeared to decline in response to treatment with increasing concentrations of IFN γ . IL-6 mRNA expression in mixed glial cells appeared unchanged upon exposure to IFN γ . Upon application of conditioned media from IFN γ -treated mixed glia to neuronal cultures, treatments proved non-toxic to neurons, with LDH activity

values falling below 100% in all treatment groups. Neuronal complexity was not affected following transfer of conditioned media from IFN γ -treated mixed glia (representative images in Figure 6.3).

Under these conditions, mixed glial response to α Syn filaments, oligomers, fibrils, and IFN γ , were negligible (Figure 6.1). Selected glial treatments failed to produce a conditioned media which impacted complexity of primary neurons following the transfer of conditioned media. In aiming to continue the exploration of the cellular interactions triggered by α Syn, mixed glia were pretreated with α Syn, and subsequently treated with IFN γ to introduce an immunogenic stimulus which may be sufficient to enhance the glial response to α Syn. For the following priming experiments, a dose of 10ng/ml IFN γ was chosen. 10ng/ml IFN γ was the lowest dose which elicited changes in Iba1+ immunoreactivity. This concentration also showed early signs of eliciting mild changes in mRNA expression of target cytokines. A low concentration was of interest in this experimental design to allow the cells to become primed in response to initial introduction of IFN γ , without causing an overt and excessive immunogenic response. Moreover, as reports from chapters 4 and 5 demonstrated an ability for A53T PFFs, in combination with A53T AAV, to increase Iba1+ immunoreactivity and induce neurodegeneration six weeks following intranigral injection, A53T PFFs were applied to mixed glial cultures in combination with IFN γ to determine a response.

Pre-treatment of mixed glial cells with A53T PFFs for 24 hours prior to treatment with IFN γ did not alter LDH activity. mRNA expression of IL-1 β was reduced in PBS-treated mixed glial cells following subsequent treatment with IFN γ on day 2, compared to controls treated with PBS on day 2. IL-1 α mRNA expression was reduced in both PBS-treated and PFF-treated mixed glial cells following subsequent treatment with IFN γ , compared to cells subsequently treated with PBS on day 2. Changes in IL-1 α expression suggest an impact of IFN γ treatment in mixed glial cultures, irrespective of the priming agent. IL-6 and TNF α expression were unaffected by treatment regime. Transfer of conditioned media from A53T PFF-primed glia had no effect on neuronal complexity or morphology.

The protocol was subsequently reversed, such that IFN γ was initially applied as a microglial stimulus, and A53T PFF was applied 24 hours later, to assess the effects of α Syn on microglia previously primed by an immunogenic stimulus. In this experiment, increased LDH release was observed in mixed glia pre-treated with IFN γ , irrespective of secondary treatment, possibly indicating higher levels of stress in glia exposed to IFN γ (10ng/ml) for a total of 48 hours. However, these changes in LDH activity are mild compared to positive controls. IL-1 β mRNA expression was reduced in PBS-treated cells following subsequent PFF treatment. IL-1 β mRNA expression was reduced in IFN γ -treated cells, regardless of secondary stimulus. Together, these results suggest a greater likelihood that changes in IL-1 β are associated with extended IFN γ exposure, and not necessarily its ability to prime the glial response to PFF application. IL-1 α mRNA appear reduced in IFN γ -treated cells following subsequent treatment with vehicle, but not PFF, suggesting a combative effect of PFF on IFN γ -primed glia. IL-6 mRNA expression was reduced in all cultures treated initially with IFN γ , regardless of secondary stimulus, further suggesting changes in IL-6 to be associated with extended IFN γ exposure, and not necessarily its ability to prime glia prior to PFF exposure. TNF α expression was reduced only in vehicle-treated cells following subsequent PFF treatment, while no changes were observed in IFN γ -primed glia following subsequent PFF treatment. Together, changes in mRNA expression of target cytokines (namely IL-1 β and IL-6) depict the mild immunogenic potency of IFN γ at 10ng/ml in mixed glial cultures, but no overt ability for this dose to prime an enhanced response following subsequent exposure to α Syn PFFs.

Although still negligible compared to positive controls, glial response extended IFN γ exposure was observed through a mild increase in LDH production. Despite changes in mRNA expression and LDH production, conditioned media transferred to neuronal cultures from IFN γ -primed glia subsequently treated with A53T PFF had no effect on neuronal complexity.

With closer resemblance to the true cellular environment within the brain, mixed glial cultures exhibit suitable experimental conditions for assessing the interplay between microglia, astrocytes, and oligodendrocytes *in vitro*. Mixed glial cultures are, with the exception of bi-weekly media changes, relatively-self maintaining. They are capable of endogenously generating sufficient nutritive support to promote survival of cells in the culture. Mixed glial cultures used in the present investigations contained approximately 25% microglia and 75% astrocytes. Other cell types, namely macrophages and oligodendrocytes, were likely present, although proportions were not assessed in these cultures. In line with findings from *in vivo* investigations, the current *in vitro* hypothesis proposed that α Syn PFFs (WT or A53T PFF) stimulate microglial activation and promote the release of pro-inflammatory cytokines IL-1 β , IL-1 α , IL-6, and TNF α into glial cell supernatant, which may drive dysfunction or degeneration of neurons in culture following the transfer of conditioned media. However, given microglia comprised only 25% of cells in mixed glial cultures (Figure 6.1), it may be possible that the absence of an observable microglial-driven response to α Syn PFFs was due to insufficient microglial numbers in these cultures. On the contrary, Zhang and colleagues reported significant glial activation from treatment with A53T and WT α Syn in co-cultures comprising 48% astrocytes and 11% microglia (other cell types also present for the purpose of the model) (Zhang et al., 2007).

IFN γ has repeatedly been used as a positive control for microglial activation, stimulation of pro-inflammatory cytokine production, transition to ameboid morphology, and alterations in gene expression (Barcia et al., 2012; Mamane et al., 1999; Mount et al., 2007; Papageorgiou et al., 2016). Such responses were not observed in the present *in vitro* investigations. The absence of a clear response in mixed glial cultures prompted the substitution of mixed glial for enriched glial cultures, although less representative of the brain's cellular milieu. Mixed glial cultures were enriched with gmCSF to obtain microglial cultures with a purity of approximately 80% Iba1+ cells (representative images in Figure 6.2). Upon application of IFN γ to enriched microglial cultures, slight changes in Iba1+ microglial morphology were observed, with cells exhibiting more ameboid-like morphology, as measured by solidity, compared to vehicle-treated controls. This was observed in response to both low dose (10ng/ml) and mild dose (50ng/ml) of rat IFN γ . Glial response to both IFN γ doses were observed through increased IL-1 α and TNF α mRNA expression, although significance was not achieved following statistical analysis. Changes in mRNA expression were likely not accompanied by protein secretion, as transfer of conditioned media to neurons had no effect on neuronal complexity. Moreover, conditioned media from PFF-primed enriched microglia subsequently treated with IFN γ , has no effect on neuronal complexity when transferred to primary neurons in culture (representative images in Figure 6.4). Together, it was concluded that altering the proportion of microglial cells in culture did not overtly influence glial response to treatment with IFN γ , A53T PFFs, or combined treatments.

Wang and colleagues reported the unexpected finding of the dose-related inhibitory effects of WT and A53T α Syn (conformation not specified) on IFN γ mRNA and protein expression in primary mouse astrocyte cultures (Wang et al., 2018). Likewise, co-exposure of mixed glial and enriched microglial cultures to both IFN γ and IL-1 β inhibited IFN γ -induced microglial activation and production of NO by

87%. This study proposed a modulatory effect in which IL-1 β -induced production of oxidative stress could be modulated by IFN γ in an immune-regulating and cyto-protective manner (Tichauer et al., 2007). Moreover, IFN γ , in partnership with its receptors, has been shown to possess anti-inflammatory properties in transgenic mouse EAE models (Mangalam et al., 2014). Considering these reports, it is not unreasonable to suggest that a highly inflammatory environment was achieved following treatment with α Syn (or other immunogenic agents), but subsequent treatment with IFN γ contributed to immune suppression. Although, the absence of immunogenic stimulation of glial cells by α Syn alone, irrespective of prior or subsequent treatment with IFN γ , indicates that this is an unlikely explanation for the current findings.

In experiments which involved the transfer of glial conditioned media to primary neuronal cultures, treatment was prepared and applied to glial cultures in neuronal culture media. This ensured that following the transfer of glial supernatant onto primary cortical neurons, neuronal cultures continued to thrive in media supplemented with nutrients required for dendritic outgrowth and neuronal survival. Due to their robust nature in culture, mixed glial cultures are largely unaffected by differences between DMEM and NBM when applied for short periods of time (i.e. 1–2-day treatments). In contrast, enriched microglia have little nutritive support from astrocytes, oligodendrocytes, or other glial cells present in mixed glial cultures to aid their survival. As such, enriched microglial cultures can be more vulnerable to changes in culture media conditions, and require specific components of glial culture media, including FBS, L-glutamine, and glucose, in order to thrive.

As shown in Figure 6.19, the transfer of conditioned media from mixed glial cultures treated with IFN γ (1-500ng/ml) in NBM to primary neurons had no effect on neuronal complexity. As shown in Figure 6.32, the transfer of conditioned media from enriched microglial cultures treated with IFN γ (10 and 50ng/ml) in NBM to primary neurons had no effect on neuronal complexity. As shown in Figure 6.34, however, an exploratory analysis shows a reduction in the complexity of MAP2+ mature primary neurons in culture in response to conditioned media transferred from enriched microglial cells treated with IFN γ (50ng/ml) in DMEM, compared to vehicle treated DMEM controls (representative images in Figure 6.4). Together, these results suggest that the generation of a glial conditioned media capable of influencing neuronal complexity is dependent on culture media conditions. While this result may show promising results for generating neuron-altering conditioned media, the use of non-neuronal media to maintain or treat neuronal cultures should be undertaken with caution. Should NBM be substituted with DMEM as the vehicle through which conditioned media is transferred to neurons, it should also be considered that minor differences in glutamine, glucose, and serum concentrations may also affect experimental outcomes. Additional supplements may be required in order to maintain neuronal cultured where experimental conditions are applied through DMEM. Further work must be conducted using this altered experimental protocol to assess the prolonged effects of various conditioned medias to primary neurons. These findings may open a gateway to finding an effective means by which the glial and neuronal interactions can be explored in relation to α Syn.

In order to continue the investigation, the use of alternative cell types, alternative α Syn materials, and/or amendments to cell culture protocols will be necessary to complement findings reported in the present *in vivo* experiments. The absence of overt response from mixed or enriched glia to α Syn PFFs, which showed propensity to increase Iba1+ cells *in vivo*, raises the question of the aggregatory abilities of these constructs *in vitro*. Confirmation of the aggregatory abilities of α Syn in culture could

be conducted through a Thioflavin-T assay, while electron microscopy could also be employed to explore the structural characteristics between filaments, oligomers, and fibrils, in order to further explain the absence of overt interaction with glial cells in culture. A shortcoming of this experiment was It may be of interest to investigate glial reactivity to rat PFFs, as primary rat glial cells may be better equipped with cell surface receptors to react to protein constructs of their own species, than human protein constructs.

The present work questions whether or not preconditioning with aggregated α Syn PFFs could affect the innate immune response of microglia under conditions of immunogenic challenge with IFN γ . This investigation is of great relevance specifically to sporadic forms of synucleinopathic diseases, as it may provide information on the microglia mediated functional innate response upon infection at the very initial stages of disease onset. Extracellular α Syn could potentially act as a priming factor for microglia, to produce an altered TLR response compared to the same challenge in the absence of such priming. To further advance the exploration of microglial priming in the α Syn-burdened CNS environment, alternate priming stimuli may be required. Roodveldt and colleagues addressed this question by examining the response of enriched microglia to a range of TLR ligands following α Syn administration, and found negligible changes in cytokine secretion profile following treatment with LPS (a TLR4 ligand), despite treatments being applied in DMEM (Roodveldt et al., 2013).

Similar inflammatory reactions were induced by LPS independently of the presence of α Syn in both transgenic mice overexpressing wild-type α Syn (Gao et al., 2008) and mice injected with non-aggregated WT α Syn into the substantia nigra (Couch et al., 2011). In contrast, WT α Syn-primed microglia have been shown to affect the immune response mediated by TLR2/1 and TLR7, either by increasing the secretion of the pro-inflammatory cytokines IL-6 and TNF α , or by reducing the expression IL-13, an anti-inflammatory cytokine (Roodveldt et al., 2013). Interestingly, IL-13 has shown propensity to protect against dopaminergic neuronal cell death under physiological conditions, but exacerbate cell loss under conditioned of oxidative stress (Morrison et al., 2012). Furthermore, intranigral injection of a TLR3 viral mimetic, Poly I:C, in rats induced glial reactivity within the nigra, predisposed rats to exacerbated midbrain dopaminergic neuronal loss, and striatal nerve terminal degeneration, following subsequent exposure to a low dose of 6-OHDA (Deleidi et al., 2010). A series of experiments aimed to address the question of microglial priming by examining the consequences of a systemic LPS injection on microglia in the α Syn-primed rat brain (Appendix III). The primary aim of this investigation was to assess the impact of a systemic injection of LPS on pSer129+ α Syn accumulation, microglial activation, nigral dopaminergic neuronal integrity, and motor function following WT PFF delivery to the substantia nigra, and assess the propensity for the β_2 AR agonist formoterol to mitigate PD-related pathologies. Human recombinant WT PFFs were administered to the rat substantia nigra by a single stereotactic unilateral injection. Three weeks later, a systemic LPS challenge was administered via an i.p. injection. Commencing four hours post-LPS delivery, the β_2 AR agonist formoterol was administered once daily for 10 days, to assess its potential to influence α Syn accumulation, microglial activation, dopaminergic neuronal cell counts, and motor behaviours in LPS-challenged animals following WT PFF delivery. Skilled motor function, limb-use asymmetry, and fine motor balance were assessed through application of the stepping, cylinder, and rotarod tests, respectively. pSer129 immunofluorescence, Iba1 immunohistochemistry, and TH immunohistochemistry were subsequently conducted on cryopreserved midbrain tissue sections to assess α Syn deposition, glial cell activation and dopaminergic neuronal integrity, respectively, in the substantia nigra. Unexpectedly, pSer129+

immunofluorescence was not detected in brain sections of WT PFF-treated animals from this investigation (Figures A3.8). As the presence of pSer129+ α Syn could not be confirmed, the results from this study are limited by the lack of evidence confirming an α Syn burden driven by PFF delivery in the substantia nigra. Various histological approaches were employed to unveil the presence of α Syn in the substantia nigra, but no approach provided confirmatory results (Figures A4.1-A4.4). As such, results from these experiments were deemed inconclusive, and included in the appendix (Appendix III, IV). The experimental design remains, nonetheless, a suitable one for exploring the consequences of primed microglia in the rat brain, and can be extrapolated to investigate the cellular interactions between primed microglia and dopaminergic neurons *in vitro* following a systemic challenge of LPS. Future investigations may employ alternative immunogenic stimuli to trigger microglial priming, and address the question of glial response to inflammatory stimuli in the α Syn-burdened brain.

A limitation to the present *in vitro* investigation was the absence of analysis of protein secretion into the conditioned media. Future investigations could utilize ELISA assays to assess cytokine secretion profiles, which may provide further clarity of media components which do or do not affect neuronal complexity when transferred via conditioned media. Further, the involvement of certain chemokines, the functional polymorphisms in the genes encoding interleukins and chemokines, and their links with the disease onset or severity, could be further investigated in culture. Future investigations may highlight the possible implications of locally affected cytokine and/or chemokine environments generated by primed microglia as a result of infection or cellular insult, and elucidate the involvement of immune cells in promoting neuronal cell death in cases of synucleinopathy.

With a better understanding of the mechanisms by which α Syn drives microglial activation and neuronal dysfunction in culture, the potential for a β_2 AR agonist to alleviate inflammation-related pathology in a cell culture model of synucleinopathy could be assessed.

Chapter 7: Discussion

7.1. Summary of findings

This thesis details the experimental approaches employed to develop a preclinical model in which the effects of α Syn on microglial activation and early-stage neurodegeneration could be assessed. The model was further employed to assess the capacity for formoterol, a long-acting brain penetrable pharmacological β_2 AR agonist, to regulate the microglial phenotype, and provide neuroprotection in response to an induced α Syn burden in the adult rat substantia nigra. Glial-neuronal interactions associated with α Syn were subsequently assessed *in vitro* to determine whether microglial cells respond to recombinant α Syn in culture, and whether the resulting glial conditioned media affects the complexity of cultured neuronal cells in a manner indicative of *in vitro* of early-stage neurodegeneration.

Human recombinant WT or A53T-mutant α Syn PFFs were unilaterally delivered, either alone, or in combination with AAV vector expressing WT or A53T-mutant α Syn, via stereotactic surgical injection into the substantia nigra of adult male and female Wistar rats. WT PFF, when injected alone, induced limb-use asymmetry, promoted phosphorylation of endogenous α Syn, and stimulated an increase in Iba1+ microglial reactivity, without incurring changes to the number of nigral TH+ dopaminergic neuronal cell bodies in the substantia nigra, six weeks post-intranigral delivery. WT PFF+AAV delivery further drove limb-use asymmetry, α Syn phosphorylation, and a rise in Iba1+ microglial reactivity in the ipsilateral substantia nigra, where nigral TH+ dopaminergic neuronal cell body counts remained unchanged. Intranigral delivery of A53T PFF alone also induced limb-use asymmetry, but failed to affect pSer129, Iba1 or TH immunoreactivity in the nigra. In contrast, unilateral intranigral delivery of A53T PFF, in combination with AAV expressing human A53T-mutant α Syn, promoted α Syn phosphorylation at Ser129, increased Iba1+ microglial reactivity, and reduced TH+ dopaminergic neuronal cell bodies in the nigra six weeks post-delivery. The cellular changes induced by A53T PFF+AAV delivery were coupled with reduced contralateral limb-use in tests of skilled motor behaviour within 3-4 weeks of delivery.

The structural, biochemical, and pathological homology between fibrillar aggregates in the human PD brain, and synthetic recombinant PFFs utilized in the present rodent model, gives credence to their validity in model development. Inclusion of genetic overexpression of the protein aided in the generation of a more representative model of synucleinopathy comprising key aspects of pathology. Specifically, intranigral delivery of A53T PFF in combination with AAV-mediated expression of A53T-mutant α Syn in the rat, produced four significant features relevant to PD: 1) limb-use asymmetry 2) phosphorylation of α Syn, 3) microglial activation, and 4) a loss of dopamine neuronal cell bodies in the substantia nigra.

To assess the potential anti-inflammatory and neuroprotective effects of a β_2 AR agonist in the A53T PFF+AAV combination model of synucleinopathy, animals were treated with formoterol two weeks following α Syn delivery. Formoterol (0.1mg/kg, i.p., once daily for 7 days) failed to alter A53T PFF+AAV associated α Syn phosphorylation or Iba1+ microglial reactivity, but appeared to reduce TH+ neuronal cell body loss in the substantia nigra six weeks after α Syn delivery. Behaviourally, treatment with formoterol appeared to partially influence limb-use asymmetry, by minimising a bias against contralateral limb-use in spontaneous motor tasks following A53T PFF+AAV delivery. Further investigation will be required to confirm these findings.

As cell culture can be used to assess underlying disease mechanisms, primary mixed glial and enriched microglial cultures were employed to assess a role for glial cells as likely mediators of α Syn-induced neuronal atrophy, and to explore variant forms of α Syn in driving a glial response. WT PFF and A53T PFFs (the same PFFs as those employed in the *in vivo* investigations) failed to induce changes in glial mRNA expression of target cytokines, or morphology of Iba1+ microglia in either mixed glial or enriched microglial cultures. Conditioned media from glia treated with these constructs had no impact on neuronal complexity when transferred to cultured primary cortical neurons. Alternative α Syn conformers, including oligomers and soluble filaments of α Syn, also proved ineffective in driving a microglial response capable of inducing changes to neuronal complexity following conditioned media transfer.

Microglia in the amyloidogenic brain are thought to become “primed” following interaction with α Syn aggregates. With the aim of continuing the exploration of the cellular interactions triggered by α Syn, glial cultures were primed with α Syn PFFs, and subsequently treated with IFN γ , to introduce a stimulus capable of activating a glial response. The objective was to determine whether priming glial cells *in vitro* with α Syn would enhance the response to IFN γ . Transfer of conditioned media from PFF-primed glia subsequently treated with IFN γ , however, had no effect on the complexity of MAP2+ neurons in culture. The reverse protocol also gave rise to negligible results. Exploratory analysis of MAP2+ Sholl analysis following the transfer of glial conditioned media to neurons through non-neuronal media, indicated promising results for the continuation of this investigation. Alternative cell types, protein constructs, and/or cell culture medias will likely be required in future investigations of α Syn-associated cellular interactions *in vitro*.

Taken together, unilateral injection of A53T PFF+AAV into the Wistar rat substantia nigra generated an experimental model of hemi-Parkinsonism in which the efficacy of anti-inflammatory and potentially neuroprotective pharmacological agents could be tested. Clinically, pharmacological agents which target the pro-inflammatory effects of α Syn on microglia in early stages of neurodegeneration may prove useful for the treatment of neurodegenerative synucleinopathic conditions. Furthermore, mechanisms by which adrenergic receptor activation may afford protection against α Syn-associated neuronal cell loss should be further assessed. To date, complementary *in vitro* investigations did not aid in the elucidation of underlying glial-neuronal interactions associated with an α Syn-burden *in vivo*. Additional investigation is warranted, and will require alternative cell culture protocols to optimise conditions whereby the transfer of conditioned media from treated glia may impact neuronal outgrowth and complexity.

7.2. The prion hypothesis: using preformed fibrils as seeds for accelerating prion-like pathology in models of synucleinopathy

The prion-like seeding hypothesis, which describes the process through which misfolded α Syn serves as a template for the conversion of naïve α Syn, is a suggested mechanism for the spread of α Syn (Brundin & Melki, 2017). This hypothesis is supported by *in vivo* work, which showed the transfer of α Syn between host cells and grafted dopaminergic neurons in mice overexpressing human α Syn. α Syn propagation from α Syn-expressing SH-SY5Y neuroblastoma cells to co-cultured naïve SH-SY5Y cells also demonstrated this process. Prion-like seeding is thought to involve an endocytic process, as cellular uptake of α Syn has been inhibited both in cultured cells and in the rat cortex by dynasore, an endocytosis inhibitor (Hansen et al., 2011). Moreover, in human patients grafted with primary

foetal ventral mesencephalic tissue, α Syn from host cells has been shown to spread to transplanted cells (Kordower et al., 2008; Kordower et al., 2011; Li et al., 2008).

The progression of PD pathology from prodromal stages, through early-stages, to eventual late-stages has been mapped by the propagation of α Syn beginning in the vagal nerve and brainstem, continuing throughout the midbrain, and eventually reaching cortical regions, where the regional distribution correlates with progressive symptomatology (Braak et al., 2003). pSer129+ immunostaining in rat midbrain tissue collected 4, 8, and 24-weeks post-striatal delivery of α Syn PFFs, indicated the initial spread of α Syn to several brain regions which innervate the striatum (frontal and insular cortices, amygdala, and substantia nigra pars compacta). This suggests that α Syn pathology initially extends to multiple brain regions distal from, but that project to, the injection site. Intraneuronal accumulation of hyper-phosphorylated α Syn were not observed in regions downstream of the striatum (substantia nigra pars reticula, globus pallidus, or entopeduncular nucleus) until 24 weeks post-injection. At this time point, hyper-phosphorylated α Syn persisted in regions in which they were originally observed, albeit less abundantly. Reported findings indicate that the propagation of phosphorylated α Syn, likely seeded by PFFs, commences initially in the retrograde direction, but shifts to anterograde transport after an extended time period. Furthermore, this study observed a shift in the presentation of striatal α Syn at the same time point at which the axial shift was observed. Initial α Syn aggregates posed as neuritic accumulations prior to progressing into intraneuronal Lewy body-like inclusions. These findings indicate a slow progression in the development of intraneuronal α Syn pathology in the striatum, resulting possibly from a secondary seeding effect of PFFs at nigrostriatal terminals. It is possible that intraneuronal hyper-phosphorylated α Syn inclusions are released from degenerating nigral neurons into the extracellular space after death, further driving the progressive propagation and accumulation of α Syn along neurons of the nigrostriatal pathway (Paumier et al., 2015).

Although the prion-protein hypothesis has been debated, substantial evidence supporting its validity has been widely demonstrated in experimental models of progressive α Syn pathology and PD-like neurodegeneration. *In vitro* studies have shown that administering α Syn PFFs to SH-SY5Y neuroblastoma cells, which overexpress α Syn, induces α Syn seeding and leads to the formation of insoluble, Lewy body-like inclusions (Luk et al., 2009). The seeding propensity of α Syn PFFs, which drive classic progressive PD-like pathology in rats and mice, has been well documented *in vivo* (Jimenez-Ferrer et al., 2021; Jimenez-Ferrer et al., 2017; Luk et al., 2012b; Rey et al., 2016).

Of note, PFF-induced microglial activation and MHC-II upregulation have been shown to precede dopaminergic neurodegeneration, and to correlate with pSer129+ α Syn accumulation in the rat substantia nigra (Duffy et al., 2018). The application of AAV vectors, which serve to express substrate α Syn, and act as seeds for locally administered human PFFs, has been demonstrated (Thakur et al., 2017). AAV-mediated models of synucleinopathy replicate many of the key pathophysiological features observed in PD, including p α Syn inclusions, microgliosis, dopaminergic cell loss, and motor impairment. Compared to α Syn-based rat PD models which use viral vectors alone (Decressac et al., 2012a), models combining viral vectors with PFFs offer the benefit of physiologically relevant transgene expression and seeding material for the recruitment, aggregation, and propagation of endogenous α Syn. Unlike central administration of PFFs alone, the combination of fibril and vector delivery allows for the study of mechanisms underlying seeded pathology on locally produced human α Syn. Additionally, the treatment combination enhances and accelerates the formation of

α Syn inclusions, dopaminergic neuron degeneration, and associated behavioural deficits (Paumier et al., 2015).

7.3. Considerations for intranigral injection protocols used for the development of unilateral rodent models of synucleinopathy

The PFF+AAV combination model was based on a theory that in human synucleinopathic conditions, α Syn fibrils act as seeds to trigger the pathological conversion of naïve α Syn into propagating aggregates. In using recombinant PFFs to represent fibrillar seeds, and AAV-mediated α Syn expression to simulate enhanced levels of α Syn expression in the diseased-brain, the combination model is thought to more accurately represent progressive synucleinopathy. Moreover, the combination of low titre AAV to overexpress human α Syn prior to the injection of human PFFs has previously been shown to result in an efficient model for inducing pSer129+ α Syn, compared to either transgenes or PFFs alone (Jimenez-Ferrer et al., 2021; Luk et al., 2012a; Luk et al., 2012b; Paumier et al., 2015; Thakur et al., 2017).

Throughout this body of work, two injection protocols were used to administer A53T PFF in combination with A53T AAV to the rat substantia nigra. In Chapter 4, 2 μ L of A53T PFF and 2 μ L of A53T AAV were delivered unilaterally through two independent injections into two different loci within the substantia nigra. In Chapter 5, 2 μ L of A53T PFF and 2 μ L of A53T AAV were combined in a 4 μ L injection volume, which was delivered unilaterally through a single injection into the substantia nigra. In both protocols, injections were administered within the same surgery. The structural integrity and stability of fibrillar α Syn are maintained at room temperature (37°C). In contrast, AAV1/2-human A53T α Syn requires cold storage (-20°C; thaw on ice). Given the sensitivity and vulnerability to denaturation, materials were independently maintained throughout the surgical process to ensure optimal construct integrity at the point of stereotactic delivery.

The choice to initially administer treatments through two independent injections was further driven by published injection protocols, which achieved PD-like dopaminergic degeneration in the midbrain, by independently injecting α Syn PFFs and AAVs into two or three midbrain sites. In one study, Thakur and colleagues observed pSer129+ α Syn accumulation, TH+ cell loss, and behavioural deficits in rats following delivery of AAV α Syn through two injections into medial and lateral regions of the substantia nigra, and another two injections of PFF into the same sites four weeks later. Similar pathology was achieved in rats following one injection of AAV into the substantia nigra, and another to ventral tegmental area, followed four weeks later by subsequent PFF injections into both regions (Thakur et al., 2017). Others have designed injection protocols which deviate from those presented by Thakur and colleagues, to reduce surgery-related inflammatory responses in the substantia nigra. Humanoid synucleinopathy-like pathology has been achieved in adult rats following delivery of a single intranigral injection of rAAV6 expressing α Syn (3 μ L; 3.6×10^{14} vg/ml), followed two weeks later by two striatal injections of human recombinant α Syn PFF (1 x 2 μ L injections containing 5 μ g protein per site; 10 μ g protein total) (Jimenez-Ferrer et al., 2017).

The intent of the *in vivo* studies presented in the current report was to compare the effects of unilateral α Syn injection on the injected side of each animal to the non-injected side of the same animal. Use of the non-injected side as an internal control provides a basis upon which cross-animal variability can be controlled. Use of the non-injected side as an internal control is also valuable in datasets which include both female and male cohorts. Furthermore, without the inclusion of independent animal controls in the current experiments, the non-injected side was the only viable

means by which α Syn-associated changes could be distinguished from normal behaviour or histopathology.

Injection protocols were optimised for the induction of histopathology ipsilateral to the injection, coupled with behavioural impairment in the limb contralateral to the injection.

A comparison between single and double injection of mouse PFFs unilaterally into the striatum was made in adult male Sprague Dawley rats. PFF, non-fibrillar recombinant α Syn, or sterile NaCl was injected into the left striatum at one site (1 x 4 μ l injection containing 8 μ g protein) or two sites (2 x 2 μ l injections containing 4 μ g protein per site; 8 μ g protein total). Both approaches led to similar unilateral accumulation of pSer129+ α Syn in the substantia nigra, and distribution across the striatum, pre-frontal cortex, and connected cortical regions. Despite unilateral injection, both approaches resulted in bilateral dopamine neuron loss, however, the magnitude of degeneration appeared greater following the two-site injection protocol (Paumier et al., 2015). Bilateral dopaminergic dysfunction following use of the double injection protocol led to misleading behavioural outcomes in the cylinder and amphetamine-induced rotations tests. The results imply motoric asymmetry following injection, where true functional impairments resulting from unilateral injection were likely masked by the bilateral distribution of injected materials. Although the double injection presented in Chapter 4 did not elicit bilateral behavioural dysfunction or histopathology, Paumier and colleagues associated a double injection protocol with a greater risk of driving bilateral motor deficit. Crossover of PFF-associated pathology has the potential to confound interpretation of behavioural data collected with the intention of using the ipsilateral limb as an internal control.

Although not explicitly reported, Paumier and colleagues raise concern for the possibility that PFF administration through two injections into the rat brain could result in greater neuroinflammation than a single-site injection, thus contributing to greater nigrostriatal degeneration. With inflammation at the core of the hypothesis under investigation, refining the injection protocol to include a single injection was employed to reduce such a risk of driving incidental microglial activation associated with the injection, and not explicitly with α Syn. Moreover, behavioural tasks employed in these studies are valuable explicitly for examining unilateral motoric asymmetry subsequent to a unilateral lesion. Bilateral pathology would therefore influence, and possibly confound, outcomes from behavioural studies which are employed to explicitly assess unilateral behavioural deficits.

7.4. Inclusion of appropriate controls in *in vitro* and *in vivo* investigations of alpha-synuclein preformed fibril and/or adeno-associated viral vector associated synucleinopathy

Prior to generating the combination model, exploratory investigations were conducted to examine α Syn-associated pathology driven by PFF alone (Chapter 3) and AAV alone (Appendix I), six weeks post-delivery to the rat substantia nigra. In experiments in which animals received either AAV or PFF alone, compounds were delivered to the rat substantia nigra through one 2 μ l stereotactic injection. In experiments in which animals received AAV and PFF together, compounds were delivered either through two 2 μ l injections or one 4 μ l injection. As such, direct comparisons between independent and combination treatments cannot be made without taking these experimental variances into account.

AAV-WT α Syn alone did not induce motor impairment six weeks post unilateral intranigral delivery. No histological analysis was conducted on midbrain tissue from these animals (Burke-Mullaly, 2021).

Intranigral injection of WT PFFs alone, promoted the phosphorylation of endogenous α Syn, and increased the number of Iba+ microglia in the ipsilateral substantia nigra six weeks post-unilateral intranigral delivery. No change in the number of TH+ dopaminergic neurons in the substantia nigra was observed post WT PFF delivery, although mild limb-use asymmetry was observed in comparison to PBS-treated controls. In combination with AAV-WT α Syn, WT PFFs produced α Syn phosphorylation, and increased Iba1+ microglial cell counts, but induced no change in the number of nigral TH+ dopaminergic neuronal cell bodies. Skilled motor function, as measured in the stepping test, and spontaneous limb-use, as measured in the cylinder test, were impacted by the combined delivery of WT PFF+AAV.

Complementary investigations were conducted with an A53T-mutant construct of α Syn, to examine α Syn-associated pathology driven by A53T AAV or A53T PFFs when delivered independently to the rat substantia nigra. Delivery of AAV-A53T α Syn alone, did not alter skilled or spontaneous motor impairment, or nigral TH+ dopaminergic cell counts. Delivery of AAV-A53T α Syn alone, did result in local pSer129+ α Syn immunoreactivity and increased Iba1+ glial immunoreactivity in the substantia nigra, six weeks post-delivery. A53T PFFs alone, did not seed phosphorylation of endogenous α Syn, or induce changes to Iba1+ microglial or TH+ dopaminergic neuronal cell numbers in the substantia nigra. Skilled motor function and symmetrical limb-use were also unaffected. In the presence of AAV vector expressing A53T α Syn, A53T PFFs expanded pSer129+ α Syn distribution, and increased Iba1+ microglial immunoreactivity to a greater extent than AAV alone. Nigral TH+ cell counts, which were not affected following delivery of AAV-A53T α Syn or A53T PFFs alone, were reduced in the substantia nigra six weeks following the combined delivery of A53T PFFs with A53T AAV.

Experimental controls for PFFs range from naïve animals or cultures to the application and use of non-fibrillar forms of α Syn. Naïve animals or cultures are advantageous in providing baseline measures representative of healthy environments, against which pathological effects of treatment can be compared. Naïve animals or cultures are limited in that they do not consider or control for auxiliary aspects of an experimental protocol which are not related directly to treatment. In models of synucleinopathy, monomers, filaments, and other non-fibrillar forms of α Syn are suitable controls for identifying pathologies associated specifically with oligomeric or fibrillar α Syn in highly controlled experimental environments, but are limiting in that they can undergo spontaneous conformational changes if not regulated carefully, or if implemented in experiments with extensive timeframes. Mouse α Syn PFFs have been shown to trigger a neurodegenerative cascade observable to various extents within 4, 8, and 24 weeks post-delivery to the rat striatum. Non-fibrillar recombinant α Syn proved to be an effective control at early timepoints, during which intrastriatal delivery failed to drive pSer129+ α Syn immunoreactivity. After 24 weeks, non-fibrillar constructs ultimately induced mild pSer129+ α Syn aggregation within the substantia nigra and cortex, raising questions about construct stability. Paumier and colleagues discussed the possibility that pathology induced by non-fibrillar α Syn may have resulted from the oligomerisation or fibrillisation of α Syn following injection of such a large quantity into a micro-environment conducive to polymerisation and aggregation (37°C). The large injection quantity (8 μ g) may also have contributed to the production of an α Syn burden which was comparable to that observed through AAV-mediated genetic overexpression. The specific conformation of non-fibrillar α Syn used in this study was unknown to the researchers, but analysis of the α Syn preparations injected confirmed the presence of high molecular weight α Syn in PFF solutions, and the absence of high molecular weight α Syn in non-fibrillar solutions. (Paumier et al., 2015). These findings demonstrate that, although non-fibrillar, α Syn conformers lacking high

molecular weight protein are nonetheless capable of inducing pathology representative of pathogenic α Syn aggregation, and suggest this be considered when designing experimental protocols in which non-fibrillar constructs are employed as controls.

To explicitly confirm the seeding capacity of PFFs in the presence of local expression of human α Syn, a fully factorial experimental design needs to be conducted. The proposed experiment would require a series of controls to account for both components of the combination injection, in addition to injection volume and number of injections, in the same experimental setup. A well-controlled experiment would include the following groups: PBS+PBS, PFF+PBS, PBS+AAV, PFF+AAV. Additional controls must also account for both the gene expression factor (AAV-eGFP or AAV-empty to account for the effects of AAV-mediated gene expression) and the seed (FILL or DSO to account for the effects of protein injection). Together, additional groups might include FILL/DSO+PBS, PBS+eGFP, and perhaps FILL/DSO+eGFP. With these additional groups, the non-injected hemisphere would serve as an internal control.

Complementary work published in the same year as the experiments described in this thesis provides additional rationale for the continued use of the combination treatment in developing a rodent model of microglial-mediated neurodegeneration. AAV-mediated eGFP expression was used to account for AAV-mediated α Syn expression, and BSA was used to account for recombinant α Syn fibrils. The effects of PFF alone (seeding effect), and α Syn-overexpression alone (substrate effect) on the aggregation and propagation of pSer129+ α Syn pathology were assessed. In rats overexpressing human A53T-mutant α Syn, pSer129+ α Syn immunoreactivity was observed within TH+ cells in the substantia nigra. In rats expressing human α Syn and seeded with PFFs (PFF+AAV), the A53T strain promoted greater pSer129+ α Syn immunoreactivity and distribution compared to WT. While AAV alone produced round inclusions, combination treatment promoted Lewy-body and Lewy-neurite-like aggregates located within the substantia nigra and across the greater midbrain (Jimenez-Ferrer et al., 2021).

Here, exploratory investigations were conducted to examine α Syn-associated pathology driven by AAV alone, six weeks post-delivery to the rat substantia nigra (Appendix I). This work demonstrated the absence of motor impairment in Wistar rats following unilateral injection of AAV5-Human WT α Syn (2 μ l; 1×10^{13} vg/ml) or AAV1/2-Human A53T α Syn (2 μ l; $>5 \times 10^{12}$ vg/ml) into the substantia nigra (Burke-Mullaly, 2021). AAV1/2-A53T induced pSer129+ α Syn (Figure A1.1, 1.2) and Iba1+ microglia immunoreactivity (Figure A1.1, A1.3) in the substantia nigra six weeks post-delivery. In line with the absence of behavioural changes, TH+ dopaminergic cell counts were unaffected by intra-nigral delivery of AAV1/2-A53T (Figure A1.1, A1.4).

Throughout these investigations, AAV1/2-empty (2 μ l; $>5 \times 10^{12}$ vg/ml), and AAV5-eGFP (2 μ l; 9.5×10^{13} vg/ml) were employed to control for AAV-mediated protein expression. While neither eGFP nor empty vector affected immunoreactivity of pSer129+ or Iba1+, eGFP-injected animals did present with pathology indicative of a unilateral lesion, including limb-use asymmetry in cylinder test four weeks post-delivery, rotational asymmetry in amphetamine-induced rotations test six weeks post-delivery, and unilateral TH+ neuronal cell loss in the substantia nigra six weeks post-delivery (Burke-Mullaly, 2021). The high viral titre of the eGFP-expressing vector used likely explains these observed effects, as similar loss of TH+ immunoreactivity has been reported in cellular and animal studies which utilized comparably high titres (1×10^{10} vg/ml- 1×10^{13} vg/ml) of eGFP-expressing vector (Klein et al., 2006; Klein et al., 2002; Koprach et al., 2010). Considering reports of titre-dependent neuronal

loss following AAV vector delivery, it has been proposed that although transgene-carrying vectors are more appropriate controls than empty vectors for AAV-associated animal models, eGFP is likely not the appropriate gene to utilize (Klein et al., 2006; Koprich et al., 2011). The pronounced vulnerability of dopaminergic neurons towards transgene overload is a challenge in establishing an appropriate control (Albert et al., 2017).

A limitation to the present investigations, specifically those presented in Chapter 4, was the use and comparison of two different viral vector serotypes when developing WT and A53T-mutant models of α Syn-associated synucleinopathy in the rat. Differences in outcomes associated with WT and A53T-mutant AAV delivery may, in part, be accounted for by differing serotypes and/or viral titres. In a comparative analysis between AAV-mediated α Syn expression in rodents, rAAV2/8 and rAAV2/5 were employed to promote WT or S129A-mutant α Syn in the rat substantia nigra. Both rAAVs show greater transduction efficiency than rAAV2/1 in the rodent nigrostriatal system. In fact, rAAV2/8 resulted in higher transgene expression than other rAAV serotypes, including 2/1 and 2/5 (Gorbatyuk et al., 2008; McFarland et al., 2009a). In the present study, it is possible that elevated α Syn expression following delivery of differing vector serotypes and tit2res may have masked differences in toxicity between WT and A53T-mutant constructs. Future investigations should carefully consider AAV serotype for assessment in experiments and model development.

Naïve controls were omitted from experiments in Chapters 4 and 5, following the understanding that the unaffected limb and hemisphere could be used as adequate internal controls for behavioural and histological analysis, respectively. This experimental design was chosen with an aim of reducing the number of animals used in experiments at such early stages of exploratory investigation. In future experiments involving the development of a unilateral animal model, both naive controls and internals must be used. Where naive controls were included in animal experiments, PBS was, at large, the primary control used. Although limited in its inability to control for the effects of injecting/applying any exogenous protein or vector, its isotonic and non-toxic nature makes it an ideal substrate for maintaining pH and osmotic balance within the experimental environment, thus minimizing adverse effects on cells or tissues. PBS is believed to have an effect not dissimilar to a sham (mock) lesion, and is therefore a safe and strong control which can be used during exploratory investigation to mimic the administration of a volume of substrate and confirm the absence of adverse effects of stereotactic surgery. PBS was used as a control throughout experiments presented in Chapter 3, Appendix II, and Appendix III, to account for the effects of an intranigral injection. Its use provided a large dataset confirming negligible adverse effects of a stereotactic intranigral injection on behavioural or histological outcomes, which can now be used to expand experiments and justify the use of controls which are more representative of experimental materials, like protein (eg. monomer, oligomer) or vectors (eg. empty vectors, eGFP). These controls should be selected with caution, as the use of these materials have been associated with higher rates of variability.

7.5. Optimization of *in vitro* models to further assess glial-neuronal interactions associated with the application of alpha-synuclein to glia and neurons in culture

Primary rat glial cultures have been shown to express TLR2 and TLR4, two membrane bound receptors through which α Syn may activate microglia (Codolo et al., 2013; Li et al., 2018; Marinelli et al., 2015; Roodveldt et al., 2013). MHC-I and MHC-II expression in primary glial cells also indicate a capacity for antigen presentation (Harms et al., 2017; Ponomarev et al., 2005; Sanchez-Guajardo

et al., 2010). Extracellular α Syn has repeatedly been shown to be taken up by neurons and glia in primary cultures through TLR-mediated endocytosis, before undergoing endocytic trafficking for degradation in lysosomes (Lee et al., 2014a). In primary murine microglia primed with LPS and treated with fibrillar α Syn, a time-dependent secretion of IL-1 β was observed within 18 hours (Gordon et al., 2018). Polinski and colleagues have shown more effective α Syn PFF seeding in primary hippocampal neuronal cultures than mesencephalic neuronal cultures. Some have recommended the use of mouse primary cultures over rat primary cultures, as the seeding of mouse or human α Syn PFFs has shown to be consistently dampened in rat primary neuronal cultures. It is unclear as to whether this is due to species homology issues, lower levels of endogenous α Syn expression, or other factors (Polinski et al., 2018).

Initially, the negligible response of mixed glia to treatment with α Syn PFFs was thought to be related to low numbers of microglia in cultures, relative to higher numbers of astrocytes. Glial cultures were further enriched with the microglial-growth factor gmCSF to promote microglial proliferation in mixed glial cultures. These cultures also showed a negligible response to treatment with either α Syn PFF or IFN γ , even though similar treatments have been reported elsewhere to induce morphological changes in Iba1+ microglia, and increase mRNA expression of pro-inflammatory cytokines (Barcia et al., 2012; Mamane et al., 1999; Mount et al., 2007; Papageorgiou et al., 2016). Another possible explanation for the lack of glial response may relate to species specificity. In comparison to the human α Syn constructs used in the present investigation, rat α Syn constructs may drive a response from primary rat cultures should species-matched materials be more optimal. Further optimization of glial cultures is warranted in future investigations to assess glial-neuronal interactions associated with the application of α Syn *in vitro*.

Historically, monocytes and immortalized microglial cell lines have shown clear responses to α Syn fibrils, which trigger NLRP3 inflammasome activation, and concomitant IL-1 β and IL-18 mRNA and protein expression (Codolo et al., 2013; Halle et al., 2008; Hornung et al., 2008). Some have reported a preferential use for monocytic cultures over immortalised microglial cultures to assess α Syn-associated inflammasome activation and IL-1 β expression, as macrophage-like cells, such as microglia, can sometimes require priming with LPS before robust production of pro-IL-1 β can be observed (Dostert et al., 2008; Gordon et al., 2018; Mariathasan et al., 2006; Martinon et al., 2006). Generally, the extraneous use of LPS in inflammation investigations is thought to hamper the translatability of experiments to the study of synucleinopathy, as PD patient brains are not explicitly exposed to LPS. Those with opposing viewpoints argue that the use of LPS more accurately directs the cultured cells towards a representative phenotype typical of the human condition, in that prior exposure to inflammogen may in fact exacerbate pre-existing PD pathology. Monocyte cultures have also been favoured over microglial cultures considering their relevant behaviours *in vivo*. Blood circulating monocytes are recruited during inflammatory events in the CNS, and are responsible for microglial cell replenishment. Taken together, monocyte cultures make for plausible models for the continued study of α Syn-associated glial interactions *in vitro*, as they likely represent a target of α Syn *in vivo* (Codolo et al., 2013).

The vast majority of the studies of microglial involvement in neurodegenerative disease rely on rodents and cultured rodent tissue. Differences between rodent and human microglial receptor profiles and responses have been well documented. *In vitro*, rodent microglia proliferate at greater speeds than cultured human microglia (Smith & Dragunow, 2014). Similarly, CNS microglia do not proliferate in humans with Alzheimer's disease, but do in mouse models of this disease (Streit et al.,

2014). The LPS receptor TLR4 is a primary mediator through which mouse microglia become activated by immunogenic stimuli, but seems to be of less importance in human cells. Neuroinflammatory mediators, including TGF β for example, show greater significance in mediating the inflammatory response in mice, compared to adult human microglia. Another principal component of microglial activation is the production of NO through the activity of iNOS. Whereas mice respond with high production of NO upon interaction with inflammatory stimuli, this induction is much lower in human microglia (Wolf et al., 2017). NO production is used in many screening models as a proxy for monitoring drug-associated microglial activation, but its varied production between glia of different species has major implications for the translatability of experimental research involving drug development.

Neuronal atrophy, intraneuritic swelling, and cellular inclusions all feature following AAV-mediated A53T α Syn expression in primary mesencephalic neuronal cultures (Zach et al., 2007). As such, future investigations may involve the application of α Syn-expressing AAVs, with or without PFFs, to cultured cells. AAV vectors also have been used to demonstrate gene silencing, as was shown through downregulation of SNCA expression in SH-SY5Y neuroblastoma cell lines (Han et al., 2011). AAV-mediated genetic expression in microglia has proved more challenging than in neurons (Maes et al., 2019). Su and colleagues, however, have suggested the AAV2 vector to be better suited for microglial transduction, considering its lower immunogenicity profile compared to AAV5, 6, 8, or 9. (Su et al., 2016). In addition to serotypes being cell specific, AAV-mediated gene delivery may differ when delivered *in vivo*, with recent work indicating differential efficiencies of gene delivery by the same vector when used *in vitro* compared to *in vivo* (Ambrosi et al., 2019). Careful consideration is warranted when selecting vectors for use in *in vitro* and *in vivo* experimental models, and in models which utilize animals or cells derived from different species.

There is a need to confirm results obtained from rodent cells in human microglia. Human microglia can be isolated from resection material obtained from glioma or epilepsy patients following surgical removal. Monocytic cells from brains of glioma patients are, however, a mixture of intrinsic microglia and monocytes which have entered the brain from the periphery. A caveat to this model is that the derived microglia are likely representative of those of a disease state, and not physiological conditions. An advanced method for obtaining human-specific microglia (or other cells of the CNS for that matter) is through the generation of iPSC. As first described by Yamanaka and colleagues in 2006 following induction of pluripotency in mouse embryonic fibroblasts, iPSC are cell lines generated from somatic cells of a living organism which have been reprogrammed back to a pluripotent state (Takahashi & Yamanaka, 2006). With this method, iPSC can be utilized to achieve, in theory, any cell type of interest, and therefore present an array of opportunities for the study of disease mechanisms in patient-derived cells (Kouroupi et al., 2020; Park et al., 2008a; Schweitzer et al., 2020; Shuvalova et al., 2020; Soldner et al., 2009). In the study of microglial involvement in synucleinopathic conditions, mouse embryonic and human iPSC remain the most commonly used, although it remains unclear how well they represent the properties of naïve microglia in the mouse or human brain, respectively (Beins et al., 2016; Muffat et al., 2016).

Of specific relevance to the research presented in this thesis, disease-specific iPSC can be derived from PD patients with known genetic mutations (Grigor'eva et al., 2023; Kawatani et al., 2021; Schmid et al., 2021; Schondorf et al., 2014; Shuvalova et al., 2020). The use of this technology could be of value in future investigations of the glial-neuronal interactions of cells expressing either the WT or A53T-mutant form of α Syn. As iPSC can be maintained as co-cultures, tri-cultures, and multi-

cellular systems, they may be used to further elucidate the relevance of mixed glial cultures compared to enriched glial cultures, in the study of α Syn-associated neuroinflammation. iPSC-derived astrocytes, generated from skin biopsies of sporadic PD patients, have shown varied mRNA and protein expression of pro-inflammatory signalling proteins in response to treatment with microglial secreted cytokines TNF α , IL-1 α and C1q, following 24-hour treatment, compared to treated cells derived from healthy siblings (Liddel et al., 2017; MacMahon-Copas, 2024). Should these cells be more reactive to recombinant forms of α Syn protein in culture, they may generate a conditioned media capable of altering the neuronal phenotype.

iPSC also provide a robust platform for testing potential therapeutics *in vitro* (Cappella et al., 2021; Chehelgerdi et al., 2023; Ko & Gelb, 2014). Specifically, the ability to assess the cellular response to immune-modulating drugs in cellular systems derived from specific idiopathic or familial cases of disease, may pave the way for defining precision-based, patient-specific medicines which are tailored for specific patient subsets who may respond better or worse to certain therapeutics. Clinical trials from recent years have applied iPSC technology as a therapeutic approach for treating patients by replacing disease-specific cells with regenerated healthy replacements (Schweitzer et al., 2020; Yamanaka, 2020).

iPSC technology remains limited by an incomplete understanding of the function of iPSC derived cells. In having to reprogram and revert the cellular machinery back to non-differentiated myeloid cells before regenerating cells of interest *in vitro*, iPSC are stripped of all original epigenetic alterations that initially contributed to disease phenotypes observed in their host (Horvath, 2013; Kerepesi et al., 2021; Maherali et al., 2007; Mertens et al., 2015). Some have aimed to bypass this, by directly reprogramming the initial somatic cells to cell type of interest, thereby preventing the cells from undergoing epigenetic erasure (Ladewig et al., 2012; Liu et al., 2013). This process, however, has yet to rectify the problem. Furthermore, like cell lines and primary cultures, iPSC do not recapitulate the CNS as a whole. Nonetheless, iPSC-derived neuronal and glial cultures would be welcomed vessels for the future development of the current investigation.

7.6. Differences in preformed fibril origin, generation, and purification protocols may account for variation in cellular response

Results from the present investigation of intranigral delivery of WT PFF or A53T PFF to rats fall in line with previously published reports of pSer129+ α Syn intraneuronal accumulation, glial activation, TH+ neuronal cell loss, and/or motoric asymmetry in rodent models carrying A53T-mutant α Syn, compared to WT or E46K (Teravskis et al., 2018; Wang et al., 2018; Winner et al., 2011). Initially, *in vivo* experiments may have suggested that A53T α Syn-associated pathology more accurately represented late-stages of progressive synucleinopathic conditions, which constitute neuronal damage and consequential motor impairment, while pathology driven by WT α Syn (or other mutations which are reportedly less toxic) better recapitulated prodromal disease states. However, in failing to observe microglial responses to neither WT nor A53T PFF in either mixed glial or enriched microglial cultures, the *in vivo* observations failed to generalise.

The absence of microglial reactivity following PFF stimulation *in vitro* may be explained, although seemingly antithetical, by excessive purification of α Syn constructs. Winner and colleagues demonstrate different seeding capabilities, fibrillisation capacities, and neurotoxic properties of identical recombinant α Syn constructs following different purification protocols e.g. inclusion or exclusion of a streptomycin sulphate precipitation step in the protocol used to purify protein

aggregates (Winner et al., 2011). WT, A53T-, A30P-, and E46K-mutant α Syn all formed amyloid fibrils as expected, when prepared by an established purification procedure which omits streptomycin sulphate precipitation (Der-Sarkissian et al., 2003). α Syn single-point mutants E35K and E57K showed significantly reduced tendencies to form fibrils compared to WT, as evidenced by time-resolved amyloid formation measured by Thioflavin-T binding. Neither E35K- nor E57K-mutant constructs showed any capacity to fibrillise, even after one year of incubation. These mutants did eventually form ring-like structures, which resemble the morphology of neuronal cytoplasmic α Syn inclusions found in the hippocampus of patients with atypical MSA (Aoki et al., 2015). A more rigorous purification protocol, which includes a streptomycin sulphate precipitation step, has shown to alter the aggregatory and cytotoxic profiles of these variants. Using a lentivirus-mediated approach to express the same α Syn variants in the rat, α Syn variants E35K and E57K induced a loss of TH+ cells of proximately 50%, variants A30P and E46K induced a loss of approximately 40%, while the A53T variant induced a loss of approximately 17%, despite displaying the highest seeding propensity prior to the change in protocol. Consistent with the hypothesis that the neurotoxic properties of α Syn relate to its aggregate-forming ability, E35K and E57K showed a greater ability to fibrillise. Following these experiments, reports from lentivirus PD rat models have ranked α Syn variants from most toxic to least toxic: E57K > E35K $\geq$ A30P $\geq$ E46K $\geq$ WT > A53T (Winner et al., 2011). PFF constructs used in this investigation were provided by Stressmarq Biosciences, and were ion-exchange purified to >95% pure confirmed by SDS-PAGE analysis (endotoxin levels <5 EU/mL at concentration of 2mg/mL). The protocol for generating these monomers was not shared by the manufacturer. Variation in results (or absence of glial response) may be due to alterations in PFF generation, whereby both the WT and A53T PFFs tested may have reduced aggregative abilities, compared to those tested and reported in the literature.

PFF-associated pathology in rat models of synucleinopathy differ significantly from that in mouse. Mouse α Syn PFFs have been extensively characterized, but produce inconsistent pathologies in different host species (Luk et al., 2012a; Volpicelli-Daley et al., 2014; Volpicelli-Daley et al., 2011). Rat α Syn ([Rat SNCA Uniprot](#)) differs from mouse α Syn ([Mouse SNCA Uniprot](#)) only by a single amino acid at the 121st residue (Rat; Serine121, Mouse: Glutamine121). Unilateral injection of mouse PFFs into the rat striatum appears to drive slower and more regionally limited pathology than unilateral injection of proportional quantities of mouse PFFs into mice. Intracellular pSer129+ accumulation and fibrillar aggregation are dependent on sufficient levels of endogenous α Syn (Luk et al., 2012a). Different endogenous expression levels between rats and mice likely contribute to varying capacities for injected PFFs to seed pathology, thereby affecting rates of aggregation and subsequent glial activation and/or neurodegeneration. Larger scale of the rat brain compared to mouse may also contribute to longer timelines required to observe pathology. Although the reasons are not fully understood, some argue that PFFs generated from mouse α Syn produce more inclusion pathology in rats than PFFs generated from rat α Syn (Howe et al., 2021). One of the first known studies investigating the impact of mouse α Syn PFFs in Sprague Dawley rats involved delivery of 8 μ g of PFFs into to either one or two striatal loci (Paumier et al., 2015). Interestingly, unilateral injection of PFFs led to bilateral expression of α Syn in the cortex, but not the substantia nigra. As time progressed, α Syn pathology was observed in the insular, cingulate, and motor cortical regions, the thalamus, and the amygdala, most abundantly from 30-60 days post-injection. TH+ cell loss was observed in the substantia nigra in both the injected and non-injected hemispheres, while α Syn inclusions were only

expressed ipsilateral to the injection site. Skilled motor behaviours were reportedly unaffected by α Syn delivery.

Other studies have found that unilateral administration of mouse PFFs into either the Sprague Dawley rat substantia nigra or striatum leads to sufficient levels of α Syn inclusions and loss of TH immunoreactive cells in the ipsilateral substantia nigra six months post-delivery, without the bilateral spread of pathology (Abdelmotilib et al., 2017). Similar observations, which show AAV-mediated expression of human α Syn in the substantia nigra to produce greater neurodegeneration than AAV-mediated expression of rat α Syn have been reported (Landeck et al., 2017). On the contrary, others suggest the delivery of PFFs from species which do not match the host reduces the likelihood of the onset of pathology, compared to injection of species-matching α Syn, as has been observed in mouse models (Fares et al., 2016; Luk et al., 2016).

7.7. Astrocytic involvement in synucleopathic disease

The investigations in this report explored the effects of α Syn aggregates on microglia in primary cell cultures and in the rat brain. While the effects of α Syn on astrocytes (or other glial cell types) were not directly assessed, their involvement in mediating the inflammatory response to α Syn is nonetheless highly influential. A worthwhile future direction for this research would assess the astrocytic response to α Syn fibrils (with or without AAV) following intranigral delivery to the rat brain. *In vitro* investigations with enriched astrocytic cultures may also prove useful.

In partnership with microglia, astrocytes are thought to contribute largely to the neuroinflammatory process seen in PD and other synucleinopathies (MacMahon-Copas et al., 2021). Astrocytes are the most abundant non-neuronal cell type in the vertebrate nervous system (Azevedo et al., 2009). They provide structural and metabolic support to surrounding neuronal network, promote synaptogenesis, and control of the permeability of the blood brain barrier. Astrocytic production of neurotrophic factors GDNF and MANF have shown neuroprotective effects when applied *in vitro* and *in vivo*. Unlike microglia, astrocyte TLR expression is limited to TLR3, with minimal expression of TLR1, TLR4, TLR5, and TLR9 (Jack et al., 2005). This renders them limited in their capacity to respond directly to pathogens or protein aggregates. As such, astrocytes are reliant on crosstalk from microglia to initiate and facilitate their response upon recognition of insult, injury, or inflammatory stimuli (Pascual et al., 2012). Astrocytes adopt a pro-inflammatory reactive phenotype in response to stimulation by α Syn-mediated microglial production of IL-1 α , TNF α , and C1q, specifically through the activation of NF κ B signalling (Kirkley et al., 2017; Liddelow et al., 2017). α Syn released by neurons can further drive IL-1 α , IL-1 β , and IL-6 release from astrocytes following endocytosis of neuron-released α Syn deposits.

α Syn accumulation in *in vitro* models of human astrocytes induce cellular stress, driving changes in the function of mitochondria, lysosomes, and endoplasmic reticulum. iPSC models have shown the involvement of nanotubules as a likely mechanism through which astrocytes can drive cell-to-cell propagation of aggregate α Syn (Rostami et al., 2017). Yun and colleagues demonstrated the neuroprotective effects of upregulated astrocytic cAMP by a glucagon-like peptide-1 receptor (GLP-1R) agonist in PFF mouse model of PD (intrastratial injection of 2 μ l of PFF; 2.5mg/ml), which prevented astrocyte stimulation (by activated microglia), thereby alleviating damage to dopamine neurons coupled with behavioural deficits (Yun et al., 2018).

Noradrenergic activation of glial-expressed β_2 -adrenoceptors promotes secretion of NGF and BDNF, which in turn activate neuronal TrkA and TrkB receptors, respectively, to provide protection against amyloid (Counts & Mufson, 2010; Liu et al., 2015). Noradrenaline mediates upregulation of NGF and BDNF in astrocytic cultures via interaction with glial expressed α_1 and/or $\beta_{1/2}$ AR activation (Furukawa et al., 1989; Juric et al., 2008; Mele et al., 2010). Noradrenaline may also increase astrocytic anti-inflammatory production of cytokines and chemokines including IL-10, gmCSF and TNF (Braun et al., 2014).

In rats, the noradrenaline reuptake inhibitor atomoxetine inhibits intranigral LPS-induced microglial activation and midbrain TNF α , IL-6, and IL-1 β mRNA expression, whilst up-regulating BDNF, CDNF, and GDNF, thereby attenuating LPS-induced loss of nigral TH⁺ dopamine neurons, preserving striatal nerve terminal degeneration and dopamine depletion, and attenuating motor dysfunction (Yssel et al., 2018). In a rat model of amyloid β -induced inflammation and noradrenaline depletion, representative of amyloidogenic pathology in the LC associated with Alzheimer's disease, cortical injection of amyloid β provoked microglial and astrocytic expression of IL-1 β , iNOS, and IL-6 in the LC. Treatment with noradrenaline or isoproterenol attenuated this production of pro-inflammatory cytokines (Heneka et al., 2002). Treatment with the β_2 AR agonist terbutaline mimicked these effects, while alleviating amyloid β -induced inhibition of long-term potentiation in adult rats (Wang et al., 2009).

Conversely, some astrocyte investigations have reported potential negative effects of β AR signalling. Primary rat astrocytes treated with noradrenaline or isoproterenol showed an increase in both APP mRNA and protein levels, increases which were blocked by treatment with the β AR antagonist propranolol (Lee et al., 1997). Moreover, intra-hippocampal injection of isoproterenol into the rat hippocampus resulted in Alzheimer-like tau hyper-phosphorylation and impaired memory (Sun et al., 2005). Plaque formation has also been shown to become exacerbated in mouse models of Alzheimer's disease following chronic treatment with β_2 AR agonist (Ni et al., 2006). Together, the astrocytic response to β AR activation has proven to be multi-faceted; conferring protective effects through inhibition of chronic inflammation and production of neurotrophic factors, but also driving deleterious mechanisms linked with increased plaque formation and subsequent neurodegeneration. The net effect of microglial-astrocytic interplay in neurodegeneration requires further investigation. The protective effects of β AR antagonists against functional decline in Alzheimer's disease patients, and reduced risk of PD associated with β_2 AR agonist use, give credence to a likelihood that β AR-targeting drives an overall net positive outcome, worthy of further investigation (Rosenberg et al., 2008).

7.8. Sex differences in rodent models of synucleinopathy

Basic applied research often fails to consider sex as a moderating variable. This oversight has undoubtedly impeded understanding of brain disorders, and reduced the translational potential of research efforts to date. There is a need to examine sex as a biological variable in preclinical research. Funding agencies are now mandating balanced representation of sexes when designing preclinical studies. Although the present *in vivo* experiments were not designed with a principal aim of exploring sex-based differences, males and female rats were included considering the relevance of sex differences in preclinical models of synucleinopathy for translating findings to the clinic. The present investigation found no overt differences in behavioural or histological outcomes between males and females included in this study.

After aging, epidemiological studies indicate sex as the second most prominent risk factor for developing PD, in all age and nationality pools in which PD is researched. Large meta-analyses report the risk of developing PD in men to be twice that of woman (Elbaz et al., 2002). The onset of disease is also typically earlier in men compared to women (Cerri et al., 2019). Studies have suggested this sex effect be explained by sexually dimorphic genetic and epigenetic factors, hormones which impact mitochondrial function and inflammatory response, and/or the quantity and topographical organisation of dopamine neurons in the substantia nigra. Specifically, sex differences in PD disease pathology are thought to be closely associated with female gonadal hormones, namely oestradiol, which is reportedly protective against dopamine loss. In women, high cumulative lifetime levels of oestrogen have been linked with a significantly lower risk of developing PD (Gatto et al., 2014). Case reports have complimented this theory by noting a positive effect of 17 β -oestradiol in male PD patients presenting with motor fluctuation and dyskinesia (Adams & Kumar, 2013). Notably, however, sex differences in PD in the human condition typically manifest after menopause, suggesting that estrogenic neuroprotection may be just one aspect by which sex differences in PD pathology may be explained.

17 β -oestradiol attenuates LPS-induced production of proinflammatory cytokines, namely IL-1, TNF α , and IL-6, in mice (Brown et al., 2010). In a study involving an α Syn tetramer-abrogating mouse model of PD, which amplifies the familial E46K α Syn mutation, male mice displayed motor deficits with earlier time of onset and greater severity than females. Males also displayed greater α Syn protein load, and lower dopaminergic fibre length and quality, compared to females, whose α Syn burden and dopaminergic fibre destruction were suggested to be combatted by endogenous oestrogen metabolism. In line with this theory, treatment of males with a brain selective prodrug of 17 β -oestradiol alleviated the reduction in dopaminergic fibre loss and behavioural deficits (Rajsombath et al., 2019).

The *in vivo* study presented in chapter 4 includes an uneven distribution of males and females, a limitation to the study which should be considered when interpreting results. In behavioural datasets, the WT PFF+AAV group comprises 1 male and 5 females, while the A53T PFF+AAV group comprises 6 males and 4 females. In histological datasets, the WT PFF+AAV group comprises 0-1 male and 1-5 females, while the A53T PFF+AAV group comprises 2-5 males and 3-5 females. Uneven distribution of sex across treatment groups in histological datasets was on account of the limited availability of tissue sections for histological staining and analysis.

Although the present dataset is limited by this confounding variable, there remains no evidence that males and females responded differently to intranigral delivery of A53T PFF+AAV, nor did the uneven distribution of sexes in the dataset contribute to conclusions. Generally, with smaller bodies and shorter forearms, female rats tend to take more steps along the length of the stepping test table in order to complete the stepping test, compared to males who make longer strides. In this dataset, however, steps made by males and female were evenly distributed in the data set. Of specific note, both males and females contributed to the high average % contralateral score measured in WT-treated animals in the stepping test at baseline (Figure 4.1c and 4.2c). Exploratory analysis of cylinder test results from animals within the A53T PFF+AAV treatment group indicates that male rats may be more susceptible to a contralateral motor deficit six weeks post A53T AAV+PFF delivery, compared to females. This is not apparent, however, in either the stepping or amphetamine-induced rotations tests. Analysis of pSer129+ immunofluorescence, Iba1+ microglial cell numbers, and TH+ cell body

numbers in tissue sections obtained from males alone, show comparable changes to those observed in tissue sections obtained from females, irrespective of treatment group.

Sex differences in behavioural outcomes were not apparent in the experiments presented in chapters 3 or 5. In behavioural tests where uneven baseline scores made interpretation of treatment effects more challenging, both male and females contributed equally to the variability in these datasets. Males and females also show similar distributions of pSer129+ immunofluorescence, Iba1+ cell counts, and TH+ cell counts across treatment groups within chapters 3 and 5.

An important caveat to consider with respect to interpretation of the amphetamine-induced rotations test is that female rats behave differently in response to amphetamine injection during different phases of the oestrous cycle (Becker et al., 2001). Female rats tend to display enhanced rotational behaviour in response to amphetamine on the day of oestrous compared to other days. In future investigations, it may be helpful to track the oestrous cycle of females, and schedule behavioural tasks accordingly, in order to further standardise test results. Of further interest would be how pharmacokinetic and pharmacodynamic properties of formoterol administration in the adult rat differ between sexes, thereby contributing to differing drug responses. Overall, with insufficient evidence from the present *in vivo* investigations to justify the separation of data derived from females and males, the continued inclusion of both sexes in forthcoming experiments should be encouraged.

7.9. Considerations for the use of non-steroidal anti-inflammatory drugs as post-surgical analgesics in animal models of neuroinflammation

Buprenorphine, a potent opioid analgesic, is the standard post-operative analgesic used in the context of stereotactic rodent brain surgery (Reifenrath et al., 2022). All animals were administered buprenorphine (0.05mg/kg, s.c.) every 12 hours for 72 hours post-surgery. Although an effective pain alleviant, the strong side effect profile of opioids when used as post-operative analgesics have been well documented.

In the context of improving animal welfare in experimental studies, animal welfare experts encourage the substitution of opioid use with alternative analgesics. Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of organic acids with potent analgesic, antipyretic, anti-inflammatory, and platelet-inhibiting properties. Many support the replacement of opioids with NSAIDs as post-operative analgesics in rodents, due to their lower side-effect profiles and stress-inducing risks.

In these experiments, however, buprenorphine was employed due to uncertainty relating to the influence of other analgesics on the inflammatory response. Here, it was unknown whether use of NSAIDs, including meloxicam or carprofen, would interfere with the neuroinflammatory processes relating to α Syn delivery into the rat substantia nigra. As the investigation of neuroinflammation and microglial activation were central to the research question, the risk of interference by using NSAIDs was high.

The primary mechanism of action of NSAIDs involves the inhibition of COX activity, leading to a reduction in the synthesis of prostaglandins, which play a key role in mediating inflammation. Two enzymes are involved in this process. COX-1 is constitutively expressed throughout the brain, and contributes mainly to normal physiological function and immune homeostasis. COX-2, expressed in neurons, astrocytes, and microglia, is enriched primarily in the hippocampus and cortex. An inducible

enzyme, COX-2 drives the pathological production of prostaglandins in response to foreign insult or proinflammatory stimuli. Most NSAIDs commonly used by humans, such as ibuprofen and naproxen, are nonselective, and inhibit both COX-1 and COX-2. Celecoxib and valdecoxib are selective COX-2, and preferentially inhibit COX-2 over COX-1. Additional COX-2 selective NSAIDs, carprofen and meloxicam are used in rodent and animal studies, and have shown to be effective in reducing pain and inflammation. Meloxicam and celecoxib, however, can directly influence TNF α , IL-1 β and IL-6 expression, and Iba1+ microglial reactivity in the rat brain (Gou et al., 2021; Saito et al., 2014). As such, their use in the present animal studies, where inflammation was central to the research question, was avoided.

In attempting to justify avoidance of the use of certain analgesics due to the potential effects that they may have on the immune system, and thus experimental results, researchers should equally consider the effects that unalleviated pain or stress may have on animals, and therefore experimental outcomes. It must be considered that, in some cases, the effects of unalleviated pain in rodents can interfere more significantly with experimental outcomes than treatment with NSAID analgesics. Pain and stress are major welfare concerns in animal research, requiring careful management and minimization. If left untreated, pain can significantly and unpredictably impact neuronal processing in the CNS, and animal behaviour, leading to increased variability in research data. Unrelieved pain or unmanaged stress can alter complex cognitive behaviours like decision-making, attention and learning, and motivation. Pain and stress can alter circadian rhythms, impair sensory processing through allodynia and hyperalgesia, and affect physiological and endocrine regulation through HPA axis activation. Other effects of unalleviated pain include activation of the sympathetic nervous system, hypertension, tachycardia, systemic vascular resistance, increased oxygen demand, vasoconstriction of coronary arteries, spleen ischemia, adverse renal effects, increased aldosterone output, increased sodium and water retention, release of endogenous opioids, release of endogenous glucocorticoids, increased platelet aggregation, and/or anxiety (Jirkof, 2017). Untreated pain can trigger upregulation of TNF α , IL-1 β and IL-6, activation of the NLRP3 inflammasome, cellular apoptosis, and other cytotoxic and immunogenic cascades (Jirkof, 2017; Martuscello et al., 2012; Meng et al., 2021; Sebba, 2021). Importantly, pain itself constitutes a research variable. Consequently, effective management of pain and stress is not only essential for ethical reasons, but also for ensuring consistency in experimental outcomes.

7.10. Prospects of repurposing beta2 adrenergic receptor agonists for the treatment of Parkinson's disease and other synucleinopathies

Currently, treatment options for synucleinopathies are of limited efficacy. Of those that are available, effective symptomatic treatments are associated with undesirable side effects. The failure of any existing PD treatment regime to prevent the progression of the disease indicates an unmet clinical need for novel therapeutic strategies. While the majority of currently available PD therapeutics target dopaminergic degeneration, none to date directly address α Syn burden or inflammation as central components of disease progression.

Pharmacologically modulating microglial activation may prove efficacious in ameliorating nigrostriatal dopaminergic neurotoxicity associated with α Syn-induced inflammation. While a classical drug discovery approach to identifying novel therapeutic agents to combat these diseases may prove successful, an alternative approach, using drug repurposing, may be viable operation with a faster lab-to-shelf turnaround time, lower cost output, and higher therapeutic potential.

The classical drug discovery pipeline is an expensive, time-consuming, and high-risk process. Development strategies typically involve multiple stages: discovery, preclinical studies, clinical trials, development, and post-marketing safety surveillance. In recent years, great interest has been placed on drug repurposing, or the use of existing approved drugs in indications other than the one(s) for which the drug was originally marketed.

Recent examples of drug repurposing have been both commercial and therapeutic successes, although historically, drug repurposing has been largely an unintentional process following the discovery of off-target effects, or previously unrecognized on-target effects associated with drug use. The analgesic NSAID, acetylsalicylic acid (aspirin), was repurposed at lower doses for combatting platelet aggregation and associated cardiovascular disease. The rationale for its multimodal use followed early identification of adverse effect reports of increased bleeding following its use for pain relief. Aspirin inhibits platelet COX-1 and, as a consequence, prevents the formation of the pro-aggregatory enzyme, thromboxane A₂ (TXA₂) (Ornelas et al., 2017; Ting et al., 2010).

Thalidomide, an immunomodulatory drug (IMiD) used for the treatment of nausea in pregnant women throughout the 1950's, developed a highly contentious reputation following the discovery of its teratogenicity. Although withdrawn as an antiemetic from market in 1961, thalidomide has since been repurposed for effective treatment of cutaneous erythema nodosum leprosum, a long-term adverse effect of chronic leprosy medication, through its potent regulation of local expression of IFN γ , TNF α , TGF β , IL-6, and other pro-inflammatory cytokines associated with T-cell proliferation (Perri & Hsu, 2003). Furthermore, thalidomide has since been approved as an anti-angiogenic treatment for certain cancers, including multiple myeloma (Bartlett et al., 2004).

The development of sildenafil, a phosphodiesterase inhibitor, is arguably one of the most successful commercial examples of drug repurposing. Initially under development for the treatment of hypertension and angina, sildenafil's potent vasodilative properties led to unexpected reports of adverse erectile effects in men partaking in clinical trials. In filling an unmet clinical need, the trial was redirected to pursue an alternative indication, leading to the subsequent launch of Pfizer's Viagra, the first oral treatment for erectile dysfunction (*Drug Repurposing and Repositioning: Workshop Summary*, 2014). These pipelines demonstrate the potential for drugs (even those with extremely poor safety profiles) to be repositioned, albeit under highly controlled conditions, and typically for the purpose of treating rare disease.

Repurposing clinically approved drugs with well-established safety and efficacy profiles for new indications can lead to lower risk, less expensive development cycles with faster launch times. The potential use of repurposed drugs for treatment of synucleinopathies remains an interesting prospect.

DSP4-mediated degeneration of LC-noradrenergic neurons and ensuing noradrenaline depletion is sufficient to initiate robust microglial activation and oxidative stress in LC-innervated brain regions. This has been shown to drive the sequential, progressive loss of TH+ dopaminergic cells in the substantia nigra, followed by neuronal loss in the primary motor cortex and hippocampus, akin to the discrete caudo-rostral pattern of prion-like propagation and neurodegeneration observed in PD patients (Song et al., 2019). The early loss of LC-noradrenergic cell bodies, and ensuing depletion of central noradrenaline, may be crucial processes promoting neuroinflammation and the ascending pattern of neurodegeneration associated with synucleinopathic progression. Noradrenaline's actions, via regulatory effects mediated via β_2 AR on microglia, may compensate for this loss, and

produce protective effects by ameliorating microglial associated neuroinflammation at early stages of neurodegeneration.

In recent years, the putative neuroprotective properties of noradrenaline mediated via β_2 AR activation have gained traction following discovery of the ability of noradrenaline and β_2 AR agonists to 1) reduce microglial activation and pro-inflammatory gene expression in the degenerating substantia nigra, 2) regulate α Syn expression at both the gene and protein levels, and 3) promote astrocytic production of neurotrophic factors (Mittal et al., 2017). Given the ability for β_2 AR's to regulate three principle contributing elements to synucleinopathic neurodegeneration - α Syn expression, inflammation, and loss of neurotrophic support, the repurposing of β_2 AR agonists, already in clinical use, is of interest for treatment of α Syn associated neurodegenerative diseases.

β_2 AR agonists such as salbutamol, have long been used in the treatment and management of respiratory disorders, as well as muscle-relaxing tocolytic agents. Isoprenaline, the first non-selective β AR agonist developed for clinical use in the 1940s, is a sympathomimetic medication used in the treatment of acute bradycardia, heart block, and sometimes, asthma. Short acting β AR agonists are typically used in first aid and emergency contexts associated with acute exacerbations of respiratory failure or fluctuation. Long acting and ultra long acting β AR agonists approved by the FDA, including formoterol and salmeterol, exert effects throughout the course of a day, without the need for frequent or repeated dosing. Clenbuterol, not currently FDA-approved, has a history of abuse in sport as a performance enhancing drug. It is, however, approved by the European Medicines Agency as a veterinary medication. Like formoterol and salmeterol, clenbuterol has pharmacokinetic attributes which bode well for future testing and repurposing as a potentially clinically useful disease modifying therapeutic.

Aside from the mechanistic aspects that might predict theoretical efficacy of repurposed β_2 AR-targeting agents, additional pharmaceutical considerations are essential. These include 1) the safety profile of the drug for long term use, specifically in elderly the population, 2) proven ability to cross the blood brain barrier – favouring highly lipophilic, uncharged, and relatively small molecular compounds, 3) pharmacokinetic and pharmacodynamic profiles which deliver long duration of effect 4) low adverse-effect profiles, minimising patient compliance challenges, and 5) appropriate bioavailability when delivered via chosen pharmaceutical form (local injection, oral tablet, transdermal patch, intranasal spray, etc). Many of these requirements limit the potential of various β_2 AR-targeting candidates for future repurposing.

Long term use of salmeterol has a well-established safety profile (Calverley et al., 2007). The potential for salmeterol to be repurposed for neurodegenerative conditions is limited in that following inhalation or oral administration, its bioavailability is poor, as it acts mostly locally. Intravenous administration of a single dose of salmeterol has been reported without queries relating to its safety, although long-term use has not been investigated.

Indacaterol, an ultra-long-acting β AR agonist commonly prescribed for alleviating symptoms of COPD, but not asthma, has demonstrated a similar (if not stronger) safety profile compared to salmeterol. Indacaterol is absorbed through a combination of oral and pulmonary absorption, with a total bioavailability of 43-45% (Wedzicha et al., 2016). Indacaterol has a high systemic distribution coefficient, with a larger molecular mass (392.5g/mol) than formoterol (344.4 g/mol) and salbutamol (239.31g/mol), and a higher estimated partition coefficient ($X_{logP3-AA} = 3.3$) than either formoterol (experimentally, $\log P = 2.2$) or carmoterol ($X_{logP3-AA} = 2.2$). If not a direct substrate of P-

glycoprotein (PGP), the efflux transporter involved in transport across the blood brain barrier, indacaterol's high lipophilicity profile may strengthen its permeability potential, but limit its distribution capacity.

Olodaterol, on the other hand, is an ultra-long-acting β_2 AR agonist with a similar safety profile to salmeterol and indacaterol, but reportedly undergoes first-pass metabolism (LaForce et al., 2018). Only one minor metabolite of olodaterol affords agonism at the β_2 AR, while most other metabolites become deactivated following first pass metabolism. Plasma levels of β_2 AR agonist metabolites become reduced significantly following repeated inhalation of olodaterol, further limiting its potential as a repurposed PD therapeutic.

The safety of long-term clenbuterol use has been exemplified in patients with ALS, wherein β_2 AR agonism helps to improve muscle mass. Like other long-acting agonists, the adverse effects associated with repeated clenbuterol use are typically prevalent at a higher dose than those used to induce bronchodilation. Clenbuterol is well absorbed (89-98%), but additional bioavailability reports are highly limited (Bartus et al., 2016).

The safety profile of formoterol is well-documented (Rosenhall et al., 2003; Rosenhall et al., 2002). Its formulation is approved for long-term clinical use in patients with COPD and asthma. In a phase IV clinical trial from 2017, comprising approximately 1000 participants (541 formoterol-treated participants; 530 placebo-treated participants), the use of formoterol reduced-rates of all-cause mortality (0.006%) compared to placebo (0.019%). Formoterol treatment also showed comparable rates of both serious (formoterol: 15.9%; placebo: 16.4%) and non-serious (formoterol: 69.1%; placebo: 69.6%) adverse events compared to placebo treatment. Formoterol-treatment was associated with increased incidence of hypertension (formoterol: 2.4%; placebo: 1.5%) (Hanania et al., 2019; Maltais et al., 2019). Short-term-use-related adverse effects of formoterol are those typical of adrenergic sympathomimetic stimulating agents. With long-term-use, adverse effects align with other β_2 AR agonists, with cardiovascular symptoms being those commonly reported. Additional adverse effects of β_2 AR agonists reportedly subside completely following continued use of the medication. Formoterol has a bioavailability of approximately 60% of delivered dose following inhalation, through combined oral absorption and pulmonary absorption (43%). (Bartus et al., 2016). Formoterol and clenbuterol have both demonstrated blood brain barrier penetrance in humans (Neil-Dwyer et al., 1981). Formoterol is metabolized hepatically, via glucuronidation, by cytochrome P450 isoenzymes 2D6, 2C19, 2C9, and 2A6, and undergoes first pass metabolism when orally administered. For this reason, formoterol use may be contraindicated in patients with cirrhotic liver damage and deficient bilirubin conjugation, or excretion capacity. However, post-trial therapeutic drug monitoring for formoterol patients with liver damage is only recommended when it is co-administered with other pharmacological agents (such as budesonide or glycopyrrolate in the Symbicort® and 'Bevespi Aerosphere® inhalers, respectively) which are not relevant for this repurposing. After inhalation, 6-10% of the dose of formoterol is excreted by urination, unchanged, with the remaining dose excreted as hepatically conjugated metabolites. Although lower than indacaterol ($\approx$ 42-56 hours) and clenbuterol ($\approx$ 35 hours), the half-life ($T_{1/2}$) of formoterol ($\approx$ 10-14 hours) is still reputable. Together, these considerations position formoterol as a favourable agent with potential for future use in neurodegenerative disease.

Furthermore, should drugs for repurpose be chosen, future investigations must also consider realistic methods by which β_2 AR agonists could be administered to patients to directly target the

glial expressed β_2 ARs in the CNS. Ongoing research indicates that intranasal nanoparticulate drug delivery via polysorbate-80 surface modified solid lipid nanoparticles, could improve the stability and distribution of drugs, including formoterol, in the CNS. Further, it has been suggested that intranasally administered nanoparticulate-encapsulated drug delivery could reduce the risk of peripheral side effects. Intranasal administration of formoterol-containing nanoparticles would facilitate drug delivery across the blood brain barrier in a non-invasive manner, in keeping with convenience for the patient and likelihood of compliance with treatment.

Furthermore, it remains ill-understood how β_2 AR activation influences other contributors of PD pathology, including gut dysbiosis, oxidative stress, mitochondrial dysfunction, ubiquitin-proteasome dysfunction, and lysosomal damage. It also remains unknown the varying degrees to which treatment with β_2 ARs effect PD symptomology where genetic mutation plays a role in the onset of pathology. It therefore remains a research interest to evaluate the efficacy by which β_2 AR activation can interfere and/or mitigate additional disease-associated pathways for therapeutic benefit. As β_2 AR agonists are not FDA-approved for the treatment of neurodegenerative disease, further evaluation of their anti-inflammatory and neuroprotective nature in experimental models and in clinical trials may determine their potential for translation to therapeutics for synucleinopathic conditions.

The potential for any agent to slow or stop the progression of a neurodegenerative disease is reliant on the ability to recognize early signs of disease pathology and diagnose before significant neurodegeneration is evident. As such, in early stages of disease progression, targeting microglial activation and central inflammation will likely be of benefit to patients. The continued investigation of non-motor prodromal symptoms of PD provides a window of opportunity wherein the likelihood of disease development and treatment requirement can be evaluated. Similarly, there is a need for the development of diagnostic aids which differentiate between various synucleinopathic disease states, including PD, Lewy body dementia and MSA. CSF and/or blood biomarkers, including pro-inflammatory cytokines such as $\text{TNF}\alpha$, $\text{IL-1}\beta$, and IL-6 , may be helpful in this regard. Additional screening for prodromal symptoms of PD, such as idiopathic olfactory deficits, may be helpful, although these are reportedly subjective and poorly selective indicators of prodromal PD. Isolated rapid eye movement (iREM) sleep behaviour disorders (iRBD), subtle cognitive changes, and reduced movement speed have also been identified as potential signs of early disease, though it is hard to qualify the usefulness of these measures in early stages of diagnosis. With accurate early-disease detection mechanisms, it is not unreasonable to suggest that development of disease modifying interventions with preventative or curative potential, including β_2 AR agonists, might be achievable.

Appendix I: An assessment of PD-related pathologies in the rat substantia nigra six weeks following intranigral delivery of A53T-mutant alpha-synuclein-expressing adeno-associated viral vector

1.1. Background (introduction, aims, objectives, and experimental design)

The establishment of clinically relevant animal models of PD is critical for understanding disease pathology and developing neuroprotective treatments for the human condition. AAV vector tools have been implemented in research as gene-delivery systems which drive cellular expression of a protein of interest over time.

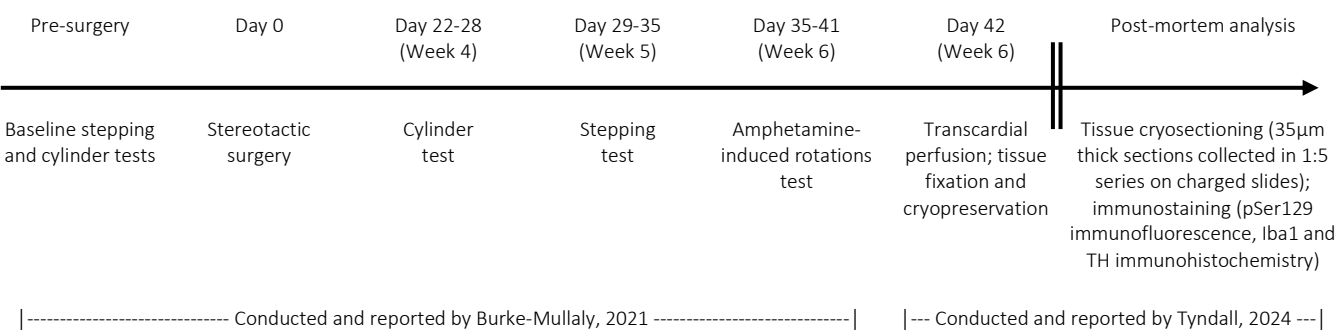
The primary aim of this investigation was to deliver A53T-mutant α Syn-expressing AAV vector unilaterally to the rat substantia nigra, to establish a model of early stage synucleinopathic disease exhibiting motoric dysfunction, α Syn aggregation, microglial activation, and nigral dopaminergic neuronal degeneration within six weeks of delivery.

The objective of this study was to deliver A53T-mutant α Syn-expressing AAV vector into the rat substantia nigra by stereotactic delivery, and assess the effect of AAV-mediated gene expression on skilled motor function, microglial activation, and dopaminergic neuronal degeneration within a period of six weeks. Skilled motor function and limb-use asymmetry were assessed in the stepping and cylinder tests, respectively. Rotational behaviour in the amphetamine-induced rotations test was assessed to functionally evaluate the presence of a unilateral nigral lesion (Burke-Mullaly, 2021). pSer129 immunofluorescence, Iba1 immunohistochemistry, and TH immunohistochemistry were conducted via free floating immunostaining methodology on cryopreserved midbrain tissue sections of 35 μ M thickness to assess phosphorylated α Syn expression, microglial cell activation, and dopaminergic neuronal integrity, respectively, in the substantia nigra six weeks after AAV delivery.

Adult male and female Wistar rats underwent behavioural training and baseline testing before being randomly assigned to a treatment group. Animals in the empty vector treatment cohort received one 2 μ L injection of AAV1/2 empty vector. Animals of the A53T-mutant α Syn-expressing vector treatment cohort received one 2 μ L injection of AAV1/2-human A53T α Syn. The coordinates for AAV infusion in males were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for AAV infusion in females were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. In weeks four, five, and six following AAV delivery, rats were subjected to the cylinder test, stepping test, and amphetamine-induced rotations test, respectively. Behavioural results have previously been reported (Burke-Mullaly, 2021). Six weeks post-AAV delivery, animals were euthanised by sodium pentobarbital overdose and transcardial perfusion/fixation. Cryopreserved brain tissue were serially cryosectioned at a thickness of 35 μ M, and collected in freezing media in preparation for free-floating immunofluorescence and histochemical analysis of pSer129+ α Syn, Iba1+ microglia, and TH+ neuronal cell bodies in the substantia nigra.

Treatment groups (n = 5-9):

- 1. AAV5-CMV-empty (one unilateral injection of 2µl)
 - n = 5-9
 - AAV titre = 5x10¹² vg/mL
- 2. AAV1/2-CMV-human A53T αSyn (one unilateral injection of 2µl)
 - n = 5-9
 - AAV titre: >5x10¹² vg/ml (2µL)



1.2. Results

1.2.1. pSer129+ Immunofluorescence

A two-ANOVA of pSer129 immunofluorescence shows an effect of vector treatment [$F(1, 16)=46$, $p<0.0001$], laterality [$F(1, 16)=57$, $p<0.0001$], and interaction between treatment and laterality [$F(1, 16)=58$, $p<0.0001$]. Post hoc comparisons reveal increased pSer129 immunofluorescence in the ipsilateral hemisphere of A53T AAV-treated animals compared to the contralateral hemisphere of A53T AAV-treated animals ($p<0.0001$), and compared to the ipsilateral ($p<0.0001$) and contralateral ($p<0.0001$) hemisphere of empty vector-treated animals (Figure A1.1a). An unpaired t-test shows increased pSer129 immunofluorescence in the ipsilateral hemisphere when expressed as a percentage of the contralateral hemisphere ($p=0.0627$) (Figure A1.1b).

1.2.2. Iba1+ Immunohistochemistry

A two-ANOVA of Iba1+ cell counts shows an effect of vector treatment [$F(1, 16)=4.6$, $p=0.0479$], no effect of laterality [$F(1, 16)=2.6$, $p=0.1232$], and an interaction between treatment and laterality [$F(1, 16)=5.2$, $p=0.0374$]. Post hoc comparisons reveal increased Iba1+ microglial cells in the ipsilateral hemisphere of A53T AAV-treated animals compared to the ipsilateral hemisphere of empty vector-treated animals ($p=0.0303$) (Figure A1.1c). An unpaired t-test of % contralateral Iba1+ cell counts fails to identify a difference between treatment groups ($p=0.0599$) (Figure A1.1d).

1.2.3. TH+ Immunohistochemistry

A two-ANOVA of TH+ cell bodies shows no effect of vector treatment [$F(1, 32)=2.084$, $p=0.1585$], no effect of laterality [$F(1, 32)=1.538$, $p=0.2240$], and no interaction between treatment and laterality [$F(1, 32)=0.08979$, $p=0.7664$] (Figure A1.1e). An unpaired t-test shows no difference in % contralateral Iba1+ cell counts between treatment groups ($p=0.9278$) (Figure A1.1f).

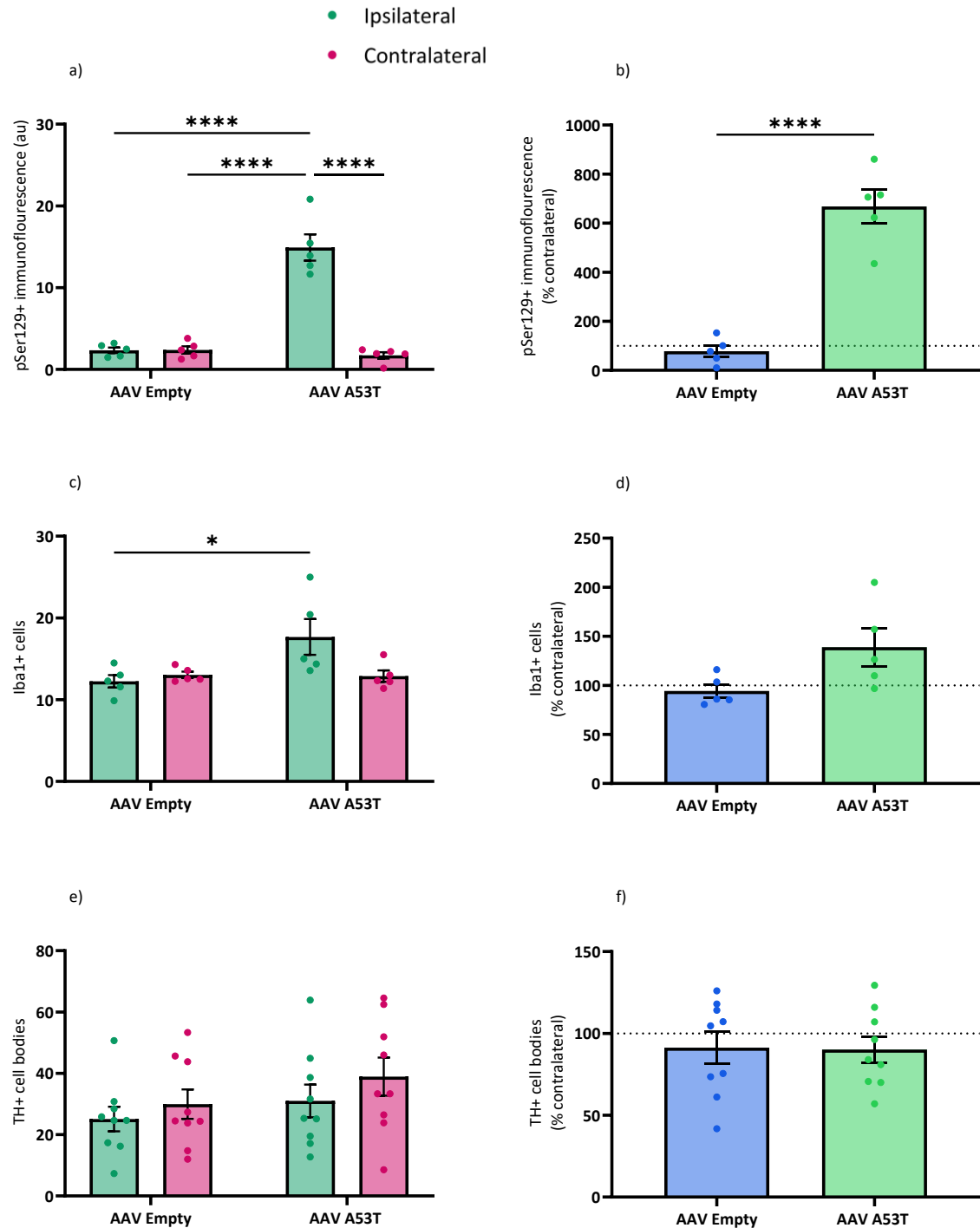


Figure A1.1. AAV-mediated expression of human A53T-mutant α Syn increased pSer129+ immunoreactivity and Iba1+ microglial cell counts in the ipsilateral substantia nigra six weeks following intranigral delivery to the rat. a) Mean grey value of pSer129+ α Syn immunofluorescence, b) mean grey value of pSer129 immunofluorescence in the ipsilateral hemisphere expressed as a percentage of the contralateral hemisphere, c) number of Iba1+ cells, d) number of Iba1+ cells in the ipsilateral hemisphere expressed as a percentage of the contralateral hemisphere, e) number of TH+ cell bodies, b) number of TH+ cell bodies in the ipsilateral hemisphere expressed as a percentage of TH+ cells on the contralateral hemisphere. Data are expressed as mean $\pm$ SEM. a,c,e) Two-way ANOVA followed by with Tukey's test for multiple comparisons, b,d,f) Students unpaired t-test. n=5-9. *p<0.05, ****p<0.0001.

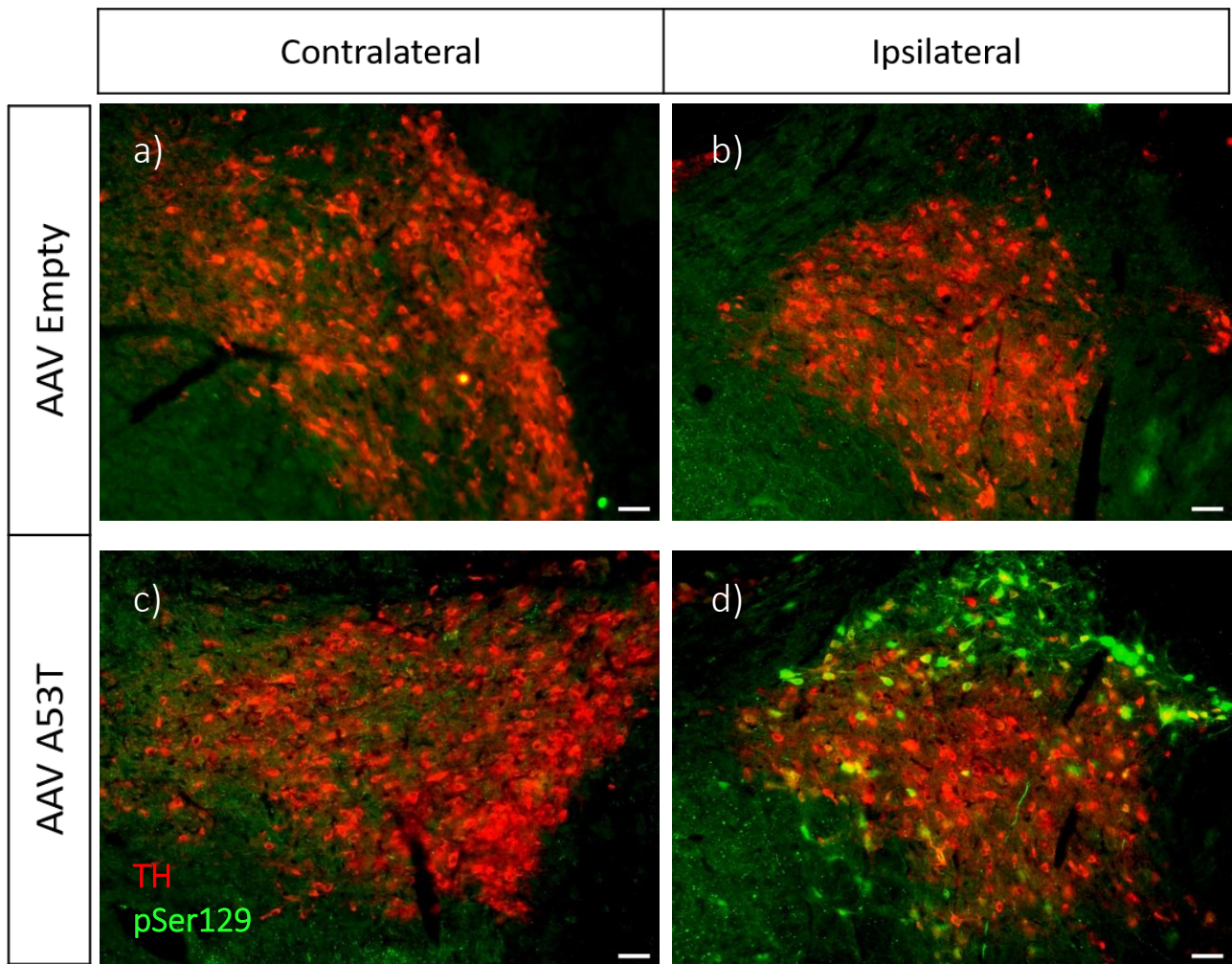


Figure A1.2. pSer129+ α Syn immunofluorescence is increased in the ipsilateral substantia nigra six weeks after intranigral delivery of AAV A53T. Six weeks following intranigral delivery, brain tissue was cryosectioned and fluorescently stained for pSer129+ α Syn and TH to confirm location within the substantia nigra. Representative 10x images show pSer129+ staining in green and TH+ staining in red in the a) contralateral hemisphere of empty AAV group, b) ipsilateral hemisphere of empty AAV group, c) contralateral hemisphere of A53T AAV group, and d) ipsilateral hemisphere of AAV A53T group. Scale bar=50 μ m.

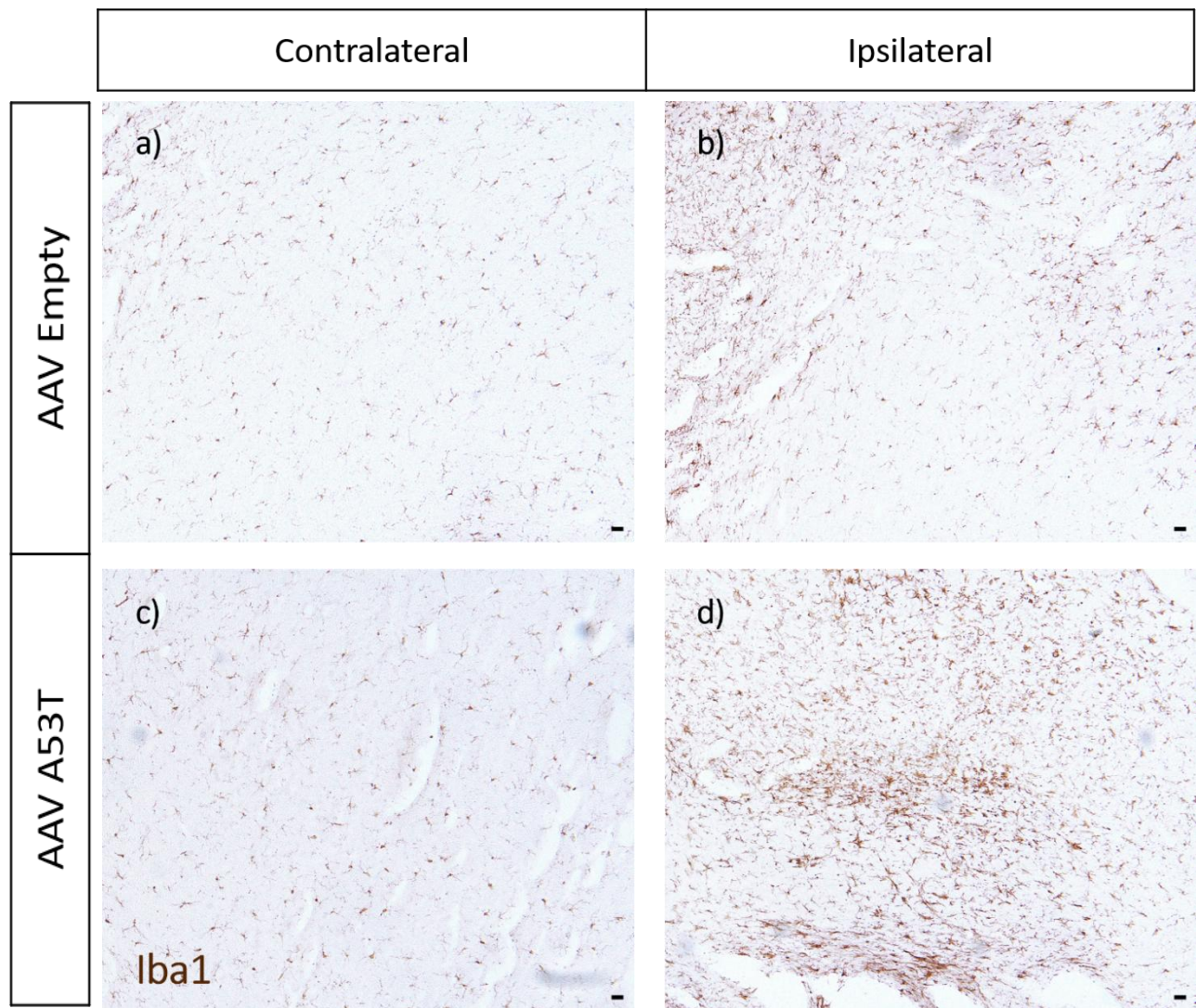


Figure A1.3. Increased Iba1+ microglial cell counts in the ipsilateral substantia nigra six weeks after intranigral delivery of AAV A53T. Six weeks following intranigral delivery, brain tissue was cryosectioned and immunohistologically stained for Iba1 in the substantia nigra. Representative 10x images show Iba1+ microglia in the a) contralateral hemisphere of empty AAV group, b) ipsilateral hemisphere of empty AAV group, c) contralateral hemisphere of A53T AAV group, and d) ipsilateral hemisphere of AAV A53T group. Scale bar=50µm.

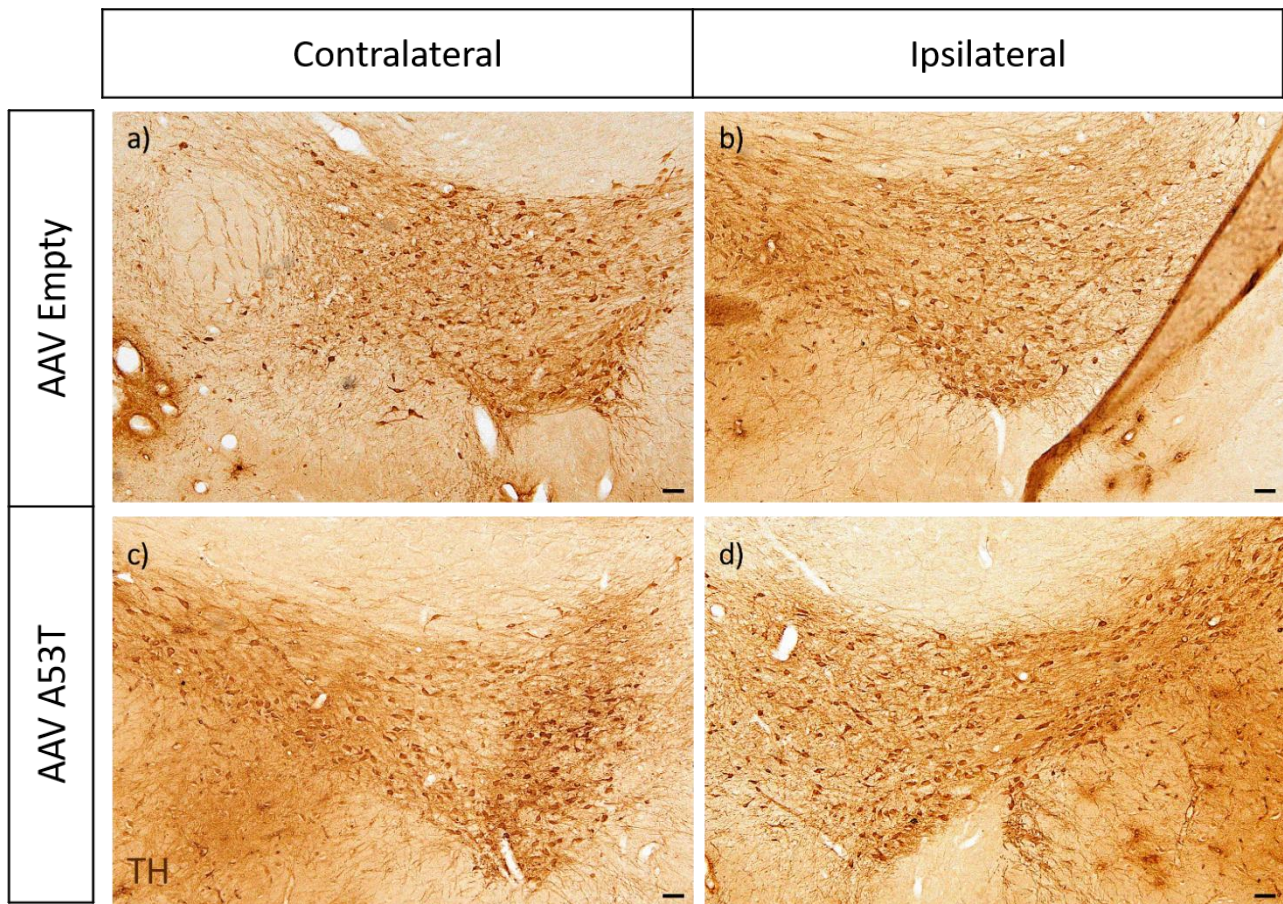


Figure A1.4. TH+ dopaminergic neuronal cells in the substantia nigra six weeks after intranigral delivery of AAV A53T. Six weeks following intranigral delivery, brain tissue was cryosectioned and immunohistologically stained for TH in the substantia nigra. Representative 10x images show TH+ dopaminergic neuronal cells bodies in the a) contralateral hemisphere of empty AAV group, b) ipsilateral hemisphere of empty AAV group, c) contralateral hemisphere of A53T AAV group, and d) ipsilateral hemisphere of AAV A53T group. Scale bar=50 μ m.

1.3. Discussion and conclusion

Prior to generating the combination model, exploratory investigations were conducted to examine α Syn-associated pathology driven by PFF alone (Chapter 3) and AAV alone (Appendix I), six weeks post-delivery to the rat substantia nigra. In the experiments wherein animals received either AAV or PFF alone, compounds were delivered to the rat substantia nigra through one 2 μ l stereotactic injection. In the experiments wherein animals received AAV and PFF together, compounds were delivered either through two 2 μ l injections or one 4 μ l injection. As such, direct comparisons between independent and combination treatments cannot be made without taking these experimental variances into account.

Previous reports conclude that unilateral delivery of AAV1/2 expressing human A53T α Syn has no effects on skilled or spontaneous motor behaviours, as measured in the stepping and cylinder tests, respectively (Burke-Mullaly, 2021). Here, complementary histological analysis shows that AAV-mediated expression of human A53T-mutant α Syn increased pSer129+ immunoreactivity and Iba1+ microglial cell counts in the ipsilateral substantia nigra, six weeks following intranigral delivery. In line with the absence of behavioural changes, TH+ neuronal cell loss was not observed six weeks post-delivery of A53T AAV. In this unilateral model, the non-injected hemispheres served as internal controls within each animal. Intranigral delivery of an empty vector served to control for the effects of vector-mediated gene expression, against which the effects of vector-mediated α Syn expression could be directly compared between each animal.

The cellular changes observed following delivery of A53T-mutant AAV alone and following A53T-mutant PFFs alone, were less pronounced than those observed in the A53T PFF+AAV combination model presented in chapter 4. A53T PFFs alone did not seed phosphorylation of endogenous α Syn, or induce changes to Iba1+ microglial or TH+ dopaminergic neuronal cell numbers in the substantia nigra. Skilled motor function and symmetrical limb-use were also unaffected. In the presence of AAV vector expressing A53T α Syn, A53T PFFs expanded pSer129+ α Syn distribution and enhanced Iba1+ microglial immunoreactivity to a greater extent than AAV alone. Nigral TH+ cell loss, which was not apparent following delivery of AAV-A53T α Syn or A53T PFFs alone, was induced in the substantia nigra 6-weeks following combination delivery. Together, these data suggest that the TH+ cell loss observed six weeks following intranigral delivery of A53T PFF+AAV was dependent on both the presence of A53T PFFs and the overexpression of A53T-mutant α Syn.

While microglial activation is unapparent following A53T PFF delivery, and relatively mild following A53T AAV delivery, a notable increase in the number of Iba1+ microglial cells in the nigra is apparent following delivery of both PFF and AAV together, suggesting a role for α Syn-induced pSer129+ immunoreactivity in glial activation, with knock-on consequences for dopaminergic neuronal loss and ensuing behavioural changes. Addition of AAV to the PFF model to produce a combination treatment model of synucleinopathy might prove more useful for exploring progressive pathology, given the possibility that PFFs may be engulfed, metabolised, or manipulated, before the onset of effects can be observed, while AAV-mediated α Syn expression is believed to remain consistent for an extended period of time.

Contradictory findings have been reported previously. Female Sprague Dawley rats present with insoluble α Syn inclusions six weeks after intranigral delivery of AAV1/2 A53T α Syn (2 μ L, 5.1×10^{12} vg/ml). Vector delivery also reduced nigral TH+ fibres by about 50% across both the substantia nigra and striatum of the injected hemisphere (Koprach et al., 2010). These findings

contradict those reported in the present investigation, which show pSer129+ α Syn deposition, but no TH+ cell loss six weeks following intranigral delivery of AAV expressing A53T-mutant α Syn. Equivocal results between research laboratories beg the question as to whether differences in the efficacy of similar constructs may be dependent on source and manufacturer.

Appendix II: An assessment of the impact of formoterol on PD-related pathologies six weeks following unilateral intranigral delivery of wild-type alpha-synuclein preformed fibrils

2.1. Background (introduction, aims, objectives, and experimental design)

The accumulation of intracellular Lewy body plaques in the brain has been long regarded as the principal hallmark of disease pathology which drives neurodegeneration (Braak et al., 1998). In recent years, chronic microglial activation has been regarded as a detrimental consequence of α Syn aggregation in prodromal stages of synucleinopathy, and is thought to precede Lewy body accumulation and subsequent nigral neurodegeneration. Noradrenaline and β_2 AR agonists can suppress the proliferation of microglia and limit the expression of pro-inflammatory markers in the substantia nigra and affected midbrain regions. Targeting microglial activation with β_2 AR agonists, such as formoterol, may prove to be an effective means for ameliorating α Syn PFF-associated neurodegeneration and associated motor dysfunction in rat models of synucleinopathy.

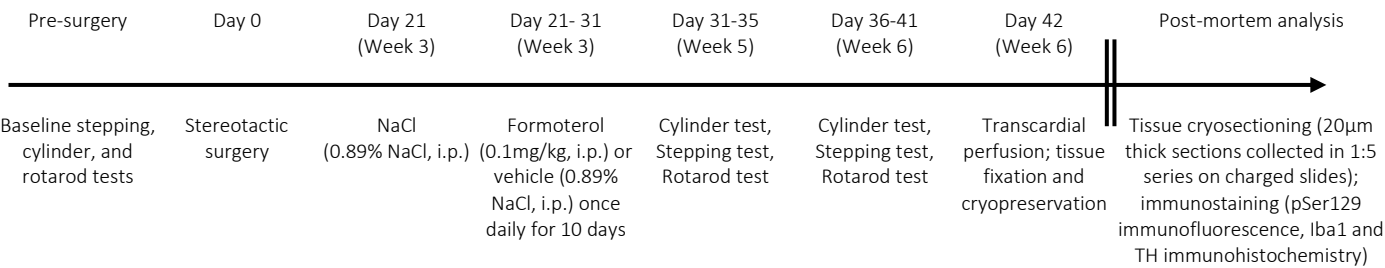
The primary aim of this investigation was to assess the impact of β_2 AR agonist formoterol on pSer129+ α Syn accumulation, microglial cell counts, nigral dopaminergic neuronal cell counts, and motor function six weeks after unilateral delivery of human recombinant WT PFFs into the rat substantia nigra.

The objectives of this investigation were to deliver human recombinant WT PFFs into the rat substantia nigra by a single stereotactic unilateral injection. Three weeks after intranigral delivery of WT PFF, the β_2 AR agonist formoterol was administered once daily for 10 days, to assess its potential to influence α Syn accumulation, microglial and dopaminergic neuronal cell counts, and motor behaviours following WT PFF delivery. Skilled motor function, limb-use asymmetry, and fine motor balance were assessed through the stepping, cylinder, and rotarod tests, respectively. pSer129 immunofluorescence, Iba1 immunohistochemistry, and TH immunohistochemistry were subsequently conducted on cryopreserved midbrain tissue sections to assess α Syn deposition, microglial cell counts, and dopaminergic neuronal cell counts, respectively, in the substantia nigra.

Adult male and female Wistar rats underwent behavioural training and baseline testing before being randomly assigned to a treatment group. Animals received one 2 μ L injection of WT PFF or PBS into the substantia nigra. The coordinates for PFF infusion in males were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for PFF infusion in females were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. Three weeks post-PFF delivery, all animals received an i.p. injection of sterile NaCl to allow for the direct comparison of results from this experiment to a complementary animal experiment where a systemic LPS challenge was employed (Appendix III). Rats received repeated injections of either formoterol (0.1mg/kg, i.p.) or sterile NaCl (0.89% NaCl, i.p.) once daily for 10 days, commencing four hours after NaCl injection. All i.p. injections were administered in volume of 1ml/kg. In weeks five and six following PFF delivery, motor behaviour was assessed in the cylinder, stepping, and rotarod tests. Six weeks post-PFF delivery, animals were euthanised by sodium pentobarbital overdose and transcardial perfusion/fixation. Cryopreserved brain tissue were serially cryosectioned at a thickness of 20 μ M, and collected on glass slides in preparation for slide-mounted immunofluorescence and histochemical analysis of pSer129+ α Syn, Iba1+ microglia, and TH+ neuronal cell bodies in the substantia nigra.

Treatment groups (n = 5-6):

- 1. Intranigral PBS (one injection of 2µl) + systemic vehicle (0.89% NaCl, i.p.)
 - n = 5 (3 females, 2 males)
- 2. Intranigral PBS (one injection of 2µl) + systemic formoterol (0.1mg/kg, i.p.)
 - n = 5 (2 females, 3 males)
- 3. Intranigral WT PFF (one injection of 4µg in 2µl) + systemic vehicle (0.89% NaCl, i.p.)
 - n = 5 (3 females, 2 males)
- 4. Intranigral WT PFF (one injection of 4µg in 2µl) + systemic formoterol (0.1mg/kg, i.p.)
 - n = 5 (2 females, 3 males)



2.2. Results

2.2.1. Cylinder test

A three-way ANOVA of combined ipsilateral placements shows an effect of time [$F(1, 15)=5.961$, $p=0.0275$], no effect of PFF [$F(1, 15)=0.1124$, $p=0.7421$], no effect of formoterol [$F(1, 15)=0.4409$, $p=0.5168$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced combined ipsilateral placements in PFF+formoterol-treated animals at five weeks post-delivery compared to baseline ($p=0.0100$) (Figure A2.1a).

A three-way ANOVA of combined contralateral placements shows an effect of time [$F(1, 15)=5.151$, $p=0.0384$], no effect of PFF [$F(1, 15)=0.2909$, $p=0.5975$], no effect of formoterol [$F(1, 15)=0.1609$, $p=0.6940$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced combined contralateral placements in PBS+formoterol-treated animals at five weeks post-delivery compared to baseline ($p=0.0030$) (Figure A2.1b).

A three-way ANOVA of % combined ipsilateral placements shows no effect of time [$F(1, 15)=0.4061$, $p=0.5336$], PFF [$F(1, 15)=0.02346$, $p=0.8803$], or formoterol [$F(1, 15)=1.904$, $p=0.1879$], but shows an interaction between time and treatment with PFF [$F(1, 15)=6.792$, $p=0.0199$]. Post hoc comparisons reveal reduced % combined ipsilateral placements in PBS+formoterol-treated animals at five weeks post-delivery compared to baseline ($p=0.0200$) (Figure A2.1c).

A three-way ANOVA of ipsilateral wall placements shows an effect of time [$F(1, 15)=7.78$, $p=0.0137$], no effect of PFF [$F(1, 15)=0.200$, $p=0.6615$], no effect of formoterol [$F(1, 15)=0.294$, $p=0.5959$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced ipsilateral wall placements in PFF+formoterol-treated animals at five weeks post-delivery compared to baseline ($p=0.0293$) (Figure A2.2a).

A three-way ANOVA of contralateral wall placements shows an effect of time [$F(1, 15)=11.16$, $p=0.0045$], no effect of PFF [$F(1, 15)=0.09912$, $p=0.7572$], no effect of formoterol [$F(1, 15)=0.8920$, $p=0.3599$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced contralateral wall placements in PBS+formoterol-treated animals at four weeks post-delivery compared to baseline ($p=0.0072$) (Figure A2.2b).

A three-way ANOVA of % ipsilateral wall placements shows no effect of time [$F(1, 15)=0.03048$, $p=0.8637$], PFF [$F(1, 15)=0.7112$, $p=0.4123$], formoterol [$F(1, 15)=1.286$, $p=0.2746$], or interactions between time, PFF, and/or formoterol (Figure A2.2c).

Two-way ANOVA of combined ipsilateral placements expressed as a percentage of baseline shows an effect PFF [$F(1, 15)=7.6$, $p=0.0150$], no effect of formoterol [$F(1, 15)=0.0086$, $p=0.9274$], and no interaction between PFF and formoterol [$F(1, 15)=0.54$, $p=0.4740$]. Post hoc comparisons reveal reduced % ipsilateral placements in PFF+vehicle-treated animals compared to PBS+vehicle-treated animals ($p=0.0302$) (Figure 3.3a). Two-way ANOVA of combined contralateral placements expressed as a percentage of baseline shows no effect PFF [$F(1, 15)=0.16$, $p=0.6983$], no effect of formoterol [$F(1, 15)=2.1$, $p=0.1683$], and no interaction between PFF and formoterol [$F(1, 15)=2.3$, $p=0.1499$] (Figure 3.3b). Two-way ANOVA of ipsilateral wall placements expressed as a percentage of baseline shows no effect PFF [$F(1, 15)=0.86$, $p=0.3683$], no effect of formoterol [$F(1, 15)=0.25$, $p=0.6251$], and no interaction between PFF and formoterol [$F(1, 15)=0.0054$, $p=0.9426$] (Figure 3.3c). Two-way ANOVA of contralateral wall placements expressed as a percentage of baseline shows no effect PFF [$F(1, 15)=0.069$, $p=0.7965$], no effect of formoterol [$F(1, 15)=0.084$, $p=0.7762$], and no interaction between PFF and formoterol [$F(1, 15)=1.8$, $p=0.1946$] (Figure A2.3d).

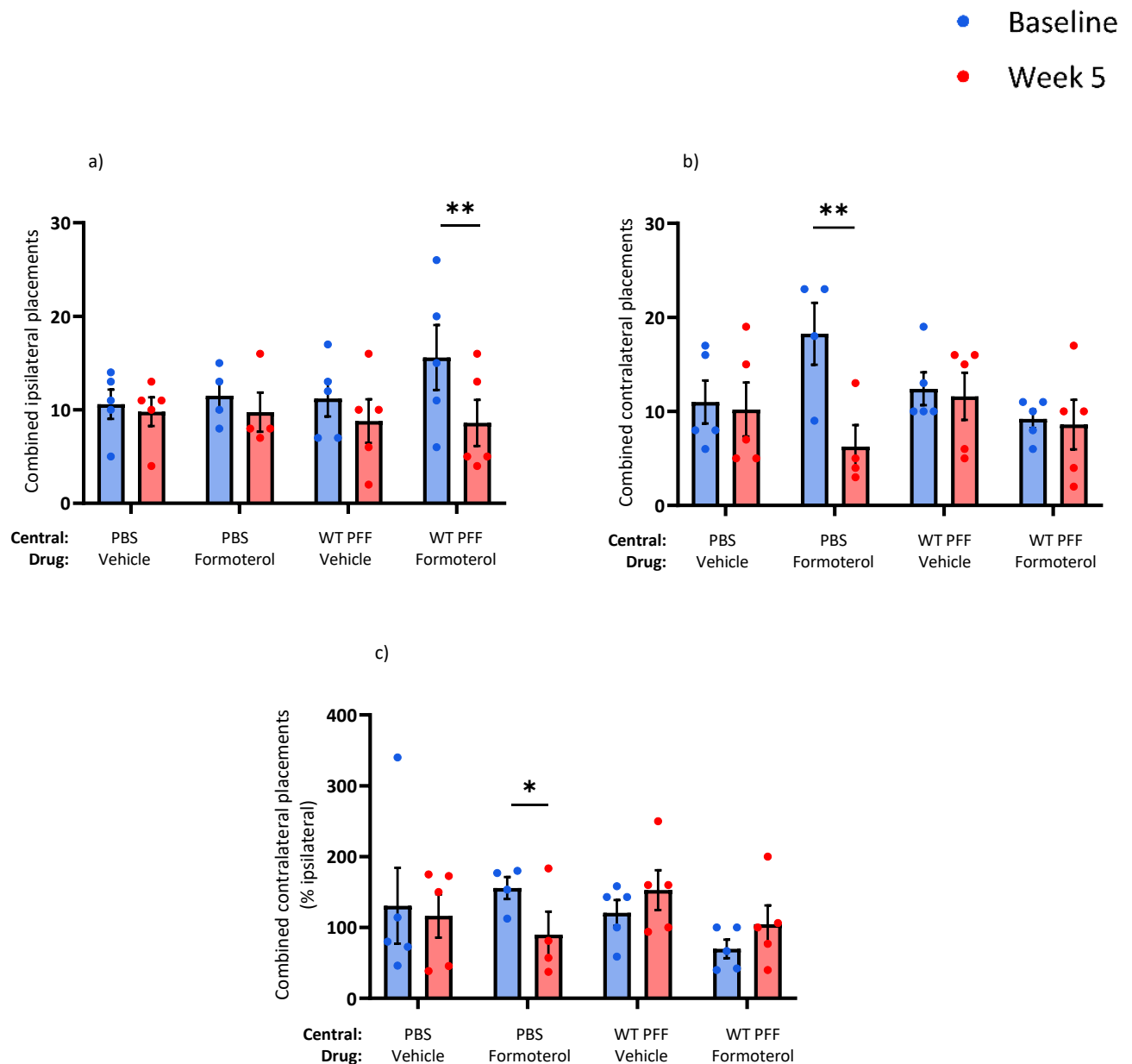


Figure A2.1. Effect of formoterol on combined cylinder test scores five weeks-post intranigral delivery of WT PFF. a) Combined placements made with the limb ipsilateral to the injection, b) combined placements made with the limb contralateral to the injection, c) combined contralateral placements expressed as a percentage of combined ipsilateral placements. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Three-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=5-6$. *= $p \leq 0.05$, **= $p \leq 0.01$.

● Baseline
● Week 5

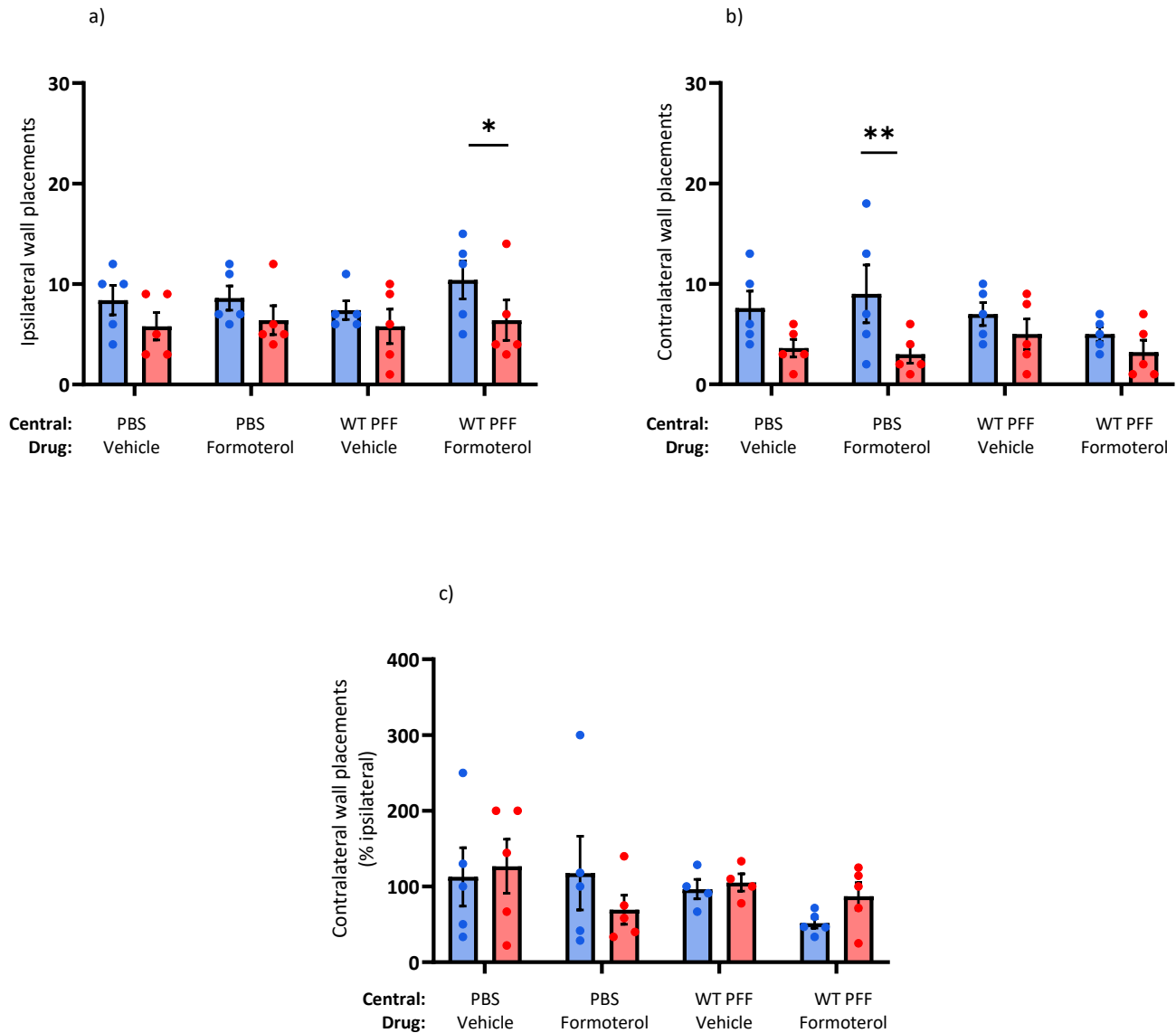


Figure A2.2. Effect of formoterol on wall placement scores in the cylinder test five weeks-post intranigral delivery of WT PFF. a) Combined placements made with the limb ipsilateral to the injection, b) combined placements made with the limb contralateral to the injection, c) combined contralateral placements expressed as a percentage of combined ipsilateral placements. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Three-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=5-6$. *= $p \leq 0.05$, **= $p \leq 0.01$.

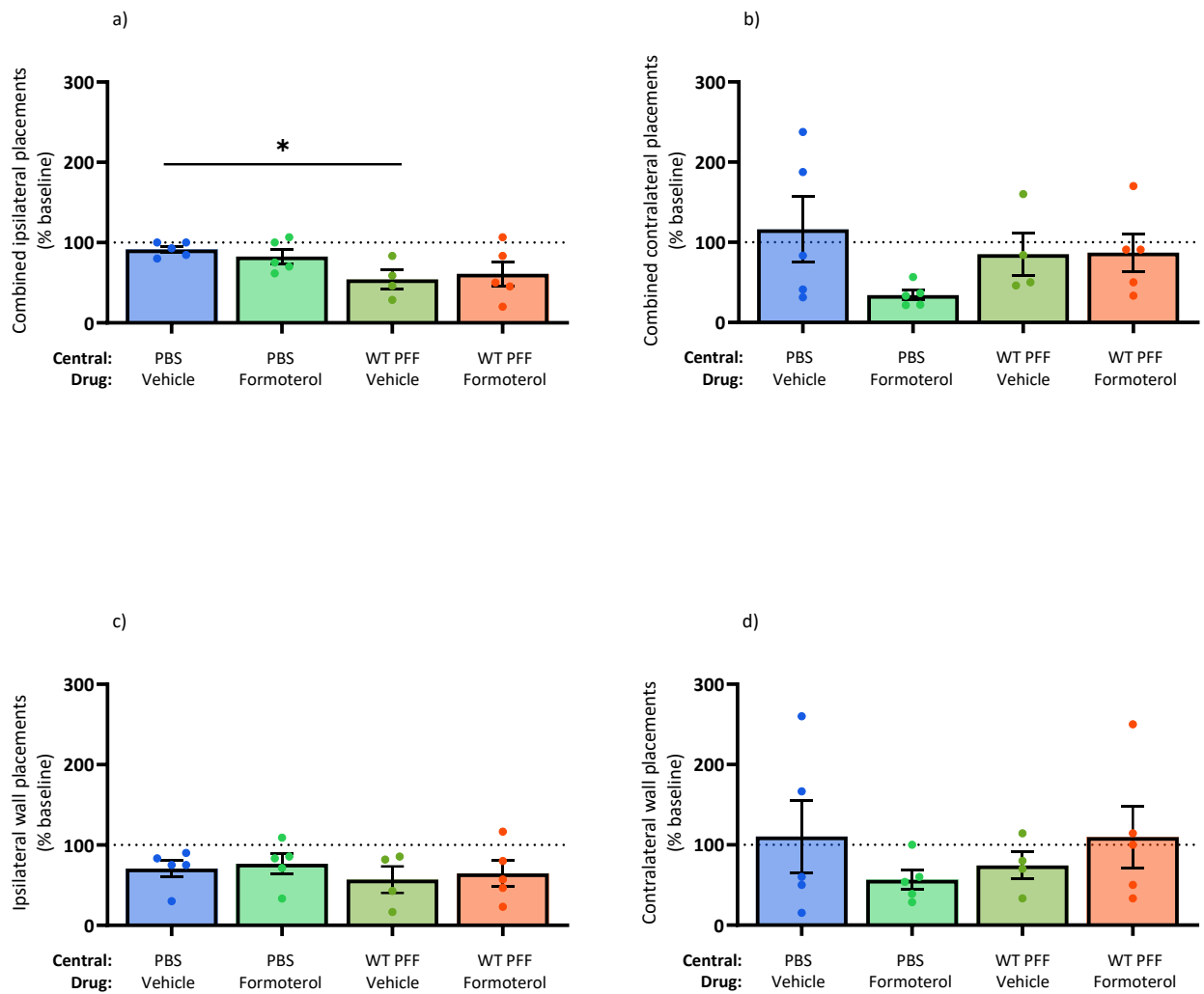


Figure A2.3. Effect of formoterol on cylinder test scores five weeks-post intranigral delivery of WT PFF. Scores of a) combined ipsilateral placements, b) combined contralateral placements, c) ipsilateral wall placements, and d) contralateral wall placements, expressed as % of baseline. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Two-way ANOVA followed by Fisher's test for multiple comparisons. $n=5-6$. $*=p\leq 0.05$.

2.2.2. Stepping test

A three-way ANOVA of combined ipsilateral steps shows an effect of time [$F(1, 16)=49.66$, $p<0.0001$], no effect of PFF [$F(1, 16)=0.01099$, $p=0.9178$], no effect of formoterol [$F(1, 16)=0.6140$, $p=0.4447$], and shows an interaction between PFF and formoterol [$F(1, 16)=4.810$, $p=0.0434$]. Post hoc comparisons reveal reduced combined ipsilateral steps at five weeks post-delivery in PBS+vehicle ($p=0.0009$), PFF+vehicle ($p=0.0013$), and PFF+formoterol ($p=0.0003$) groups compared to respective baselines (Figure A2.4a).

A three-way ANOVA of combined contralateral steps shows an effect of time [$F(1, 16)=56.42$, $p<0.0001$], no effect of PFF [$F(1, 16)=0.04577$, $p=0.8333$], no effect of formoterol [$F(1, 16)=0.7443$, $p=0.4010$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced combined contralateral steps at five weeks post-delivery in PBS+vehicle ($p=0.0488$), PBS+formoterol ($p<0.0001$), PFF+vehicle ($p=0.0033$), and PFF+formoterol ($p=0.0006$) groups compared to baseline (Figure A2.4b).

A three-way ANOVA of % combined ipsilateral steps shows an effect of time [$F(1, 16)=6.987$, $p=0.0177$], no effect of PFF [$F(1, 16)=1.246$, $p=0.2808$], no effect of formoterol [$F(1, 16)=2.547$, $p=0.1301$], and an interaction between time and formoterol [$F(1, 16)=4.934$, $p=0.0411$], and an interaction between PFF and formoterol [$F(1, 16)=4.814$, $p=0.0433$]. Post hoc comparisons reveal reduced % combined ipsilateral steps in PBS+vehicle at five weeks post-delivery compared to baseline ($p=0.0030$). Post hoc comparisons also reveal reduced steps in the PBS+formoterol group at five weeks post-delivery ($p=0.0006$) and PFF+vehicle group at five weeks post-delivery ($p=0.0095$) compared to PBS+vehicle at five weeks post-delivery (Figure A2.4c).

A three-way ANOVA of ipsilateral forward steps shows an effect of time [$F(1, 16)=113.9$, $p<0.0001$], no effect of PFF [$F(1, 16)=1.124$, $p=0.3047$], no effect of formoterol [$F(1, 16)=0.08493$, $p=0.7745$], and an interaction between time, PFF, and formoterol [$F(1, 16)=7.408$, $p=0.0151$]. Post hoc comparisons reveal reduced ipsilateral forward steps five weeks post-delivery in PBS+vehicle ($p<0.0001$), PBS+formoterol ($p=0.0013$), PFF+vehicle ($p=0.0009$), and PFF+formoterol ($p<0.0001$) groups compared to respective baselines (Figure A2.5a).

A three-way ANOVA of contralateral forward steps shows an effect of time [$F(1, 16)=42.51$, $p<0.0001$], no effect of PFF [$F(1, 16)=0.4241$, $p=0.5241$], and formoterol [$F(1, 16)=4.737$, $p=0.0448$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced contralateral forward steps five weeks post-delivery in PBS+vehicle ($p<0.0161$), PBS+formoterol ($p=0.0021$), PFF+vehicle ($p=0.0083$), and PFF+formoterol ($p<0.0021$) groups compared to respective baselines. Reduced steps were also identified in PBS+formoterol treated animals at five weeks post-delivery compared to PBS+vehicle-treated animals at five weeks post-delivery ($p=0.0262$) (Figure A2.5b).

Two-way ANOVA of combined ipsilateral steps expressed as a percentage of baseline shows no effect PFF [$F(1, 16)=0.044$, $p=0.8361$], formoterol [$F(1, 16)=0.11$, $p=0.7428$], or interaction between PFF and formoterol [$F(1, 16)=1.9$, $p=0.1849$] (Figure 3.6a). Two-way ANOVA of combined contralateral steps expressed as a percentage of baseline shows no effect PFF [$F(1, 16)=0.025$, $p=0.8755$], formoterol [$F(1, 16)=6.1$, $p=0.0248$], or interaction between PFF and formoterol [$F(1, 16)=1.2$, $p=0.2972$] (Figure 3.6b). Two-way ANOVA of ipsilateral steps expressed as a percentage of baseline shows no effect PFF [$F(1, 16)=0.12$, $p=0.7330$], formoterol [$F(1, 16)=0.18$, $p=0.6758$], or interaction between PFF and formoterol [$F(1, 16)=2.4$, $p=0.1437$] (Figure 3.6c). Two-way ANOVA of contralateral steps expressed as a percentage of baseline shows no effect PFF [$F(1, 16)=0.26$, $p=0.6166$],

formoterol [$F(1, 16)=2.4$, $p=0.1386$], or interaction between PFF and formoterol [$F(1, 16)=0.32$, $p=0.5811$] (Figure A2.3) (Figure 3.6d).

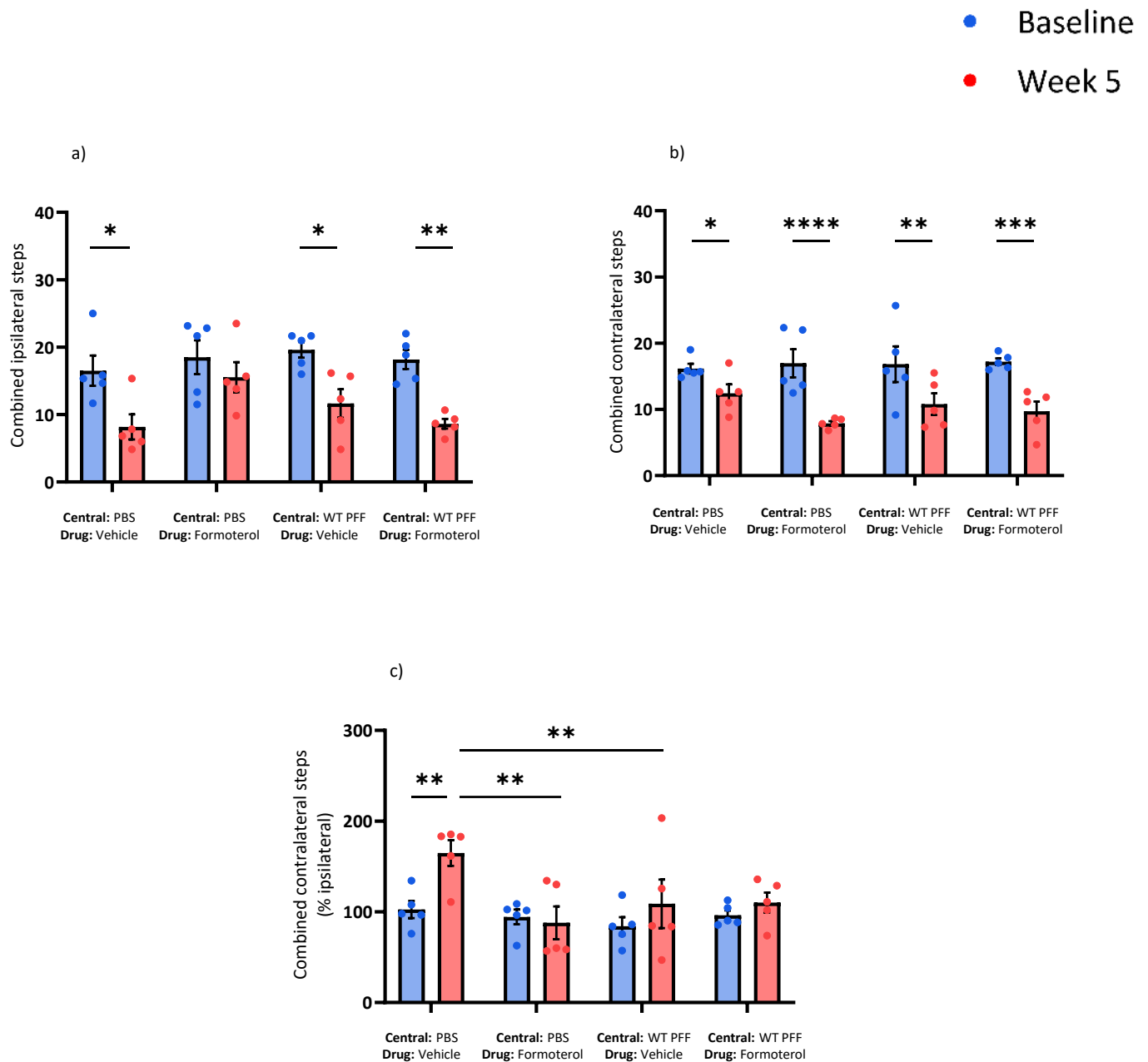


Figure A2.4. Effect of formoterol on combined stepping test scores five weeks-post intranigral delivery of WT PFF. a) Combined steps made with the limb ipsilateral to the injection, b) combined steps made with the limb contralateral to the injection, c) combined contralateral steps expressed as a percentage of combined ipsilateral steps. Data is expressed as mean $\pm$ SEM. Three-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=5-6$. *= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$, ****= $p \leq 0.0001$.

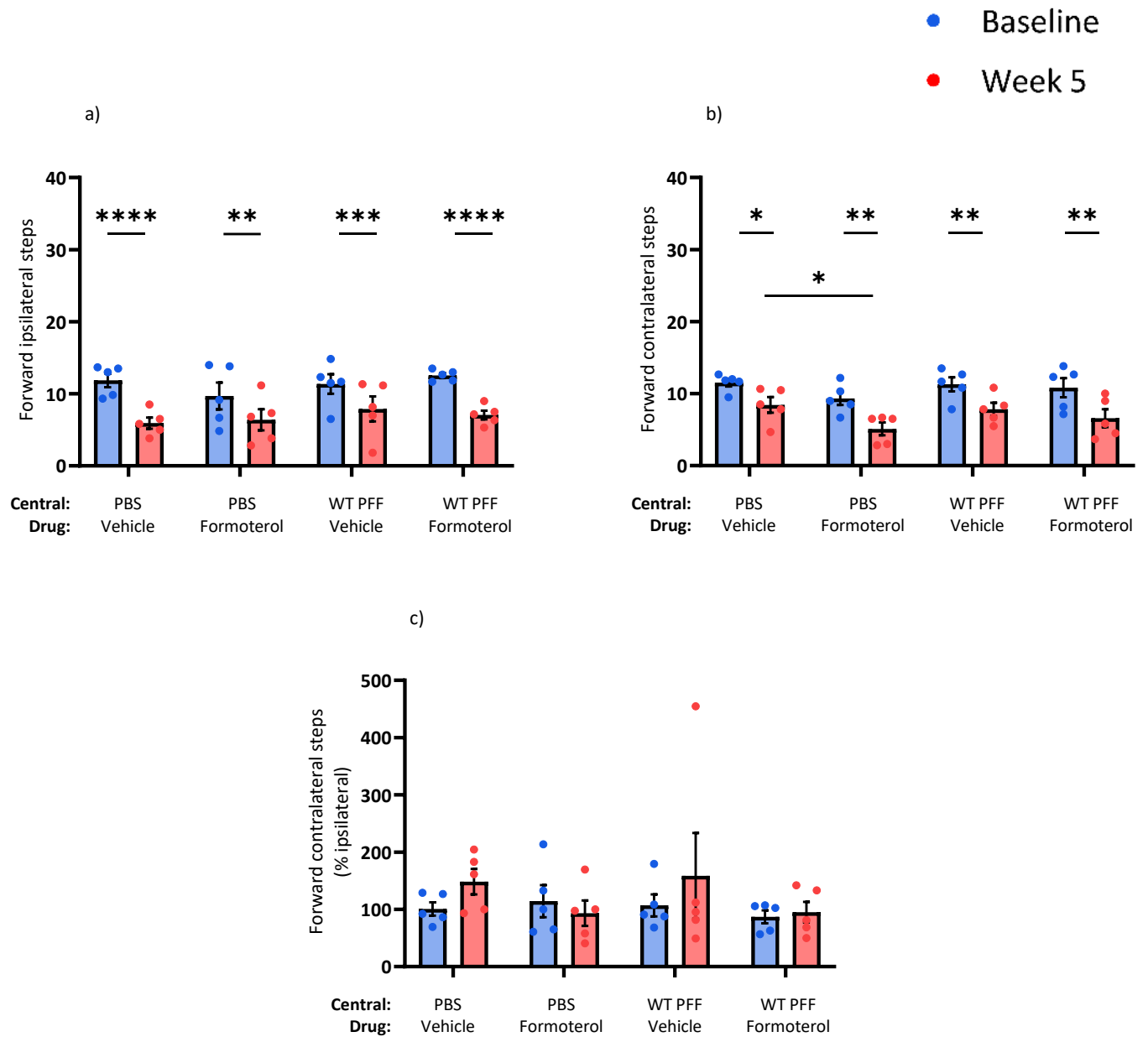


Figure A2.5. Effect of formoterol on forward stepping test scores five weeks-post intranigral delivery of WT PFF. a) Forward steps made with the limb ipsilateral to the injection, b) forward steps made with the limb contralateral to the injection, c) forward contralateral steps expressed as a percentage of forward ipsilateral steps. Data is expressed as mean $\pm$ SEM. Three-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=5-6$. *= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$, ****= $p \leq 0.0001$.

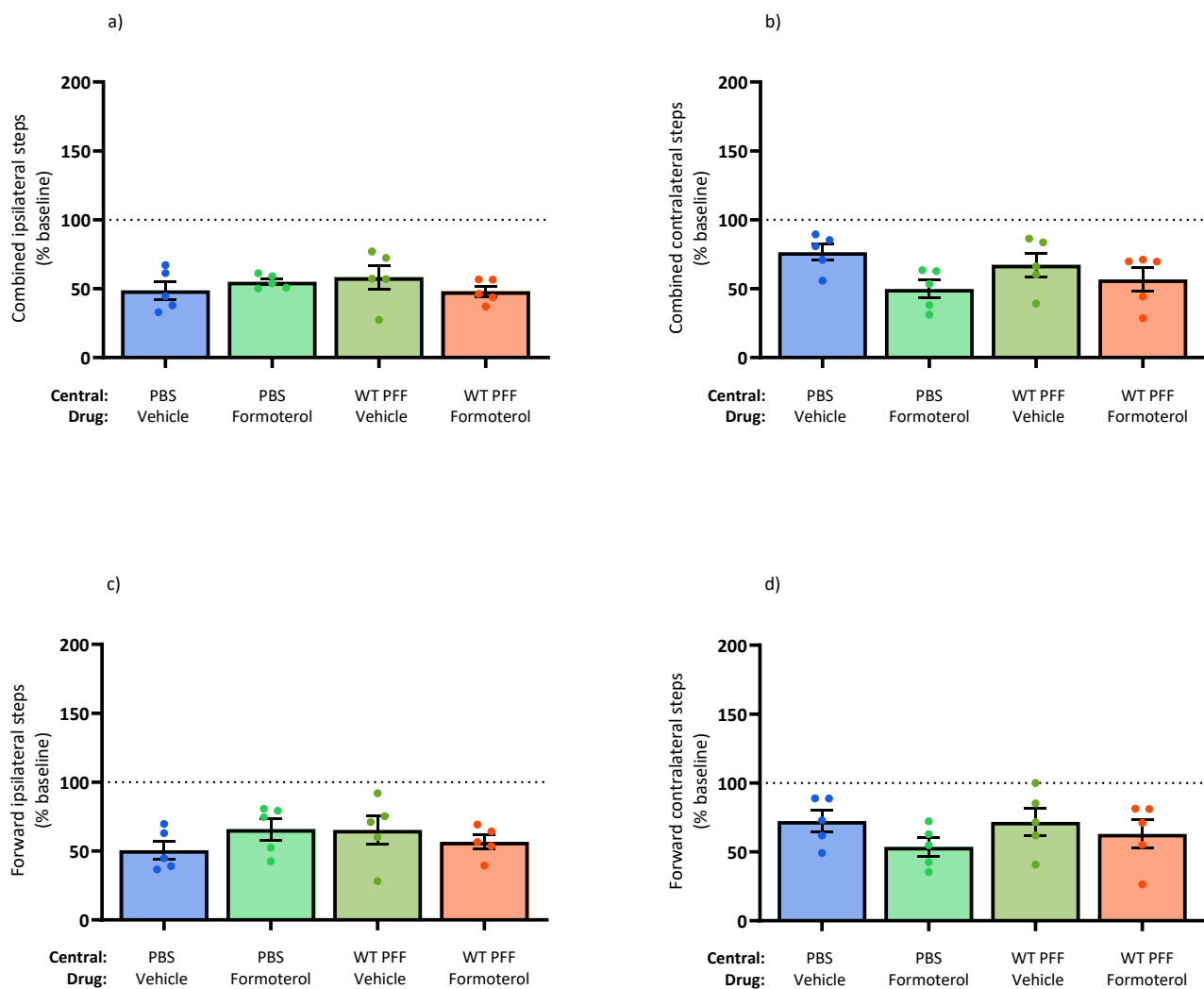


Figure A2.6. Effects of formoterol on stepping test scores five weeks-post intranigral delivery of WT PFF. Scores of a) combined ipsilateral steps, b) combined contralateral steps, c) forward ipsilateral steps, and d) forward contralateral steps, expressed as % of baseline. Data is expressed as mean $\pm$ SEM. Two-way ANOVA. n=5-6.

2.2.3. Rotarod test

A three-way ANOVA of rotarod scores shows no effect of time [$F(1, 15)=0.3611$, $p=0.5568$], PFF [$F(1, 15)=0.07576$, $p=0.7869$], formoterol [$F(1, 15)=0.7508$, $p=0.3999$], or interactions between time, PFF, and/or formoterol (Figure A2.7a). Two-way ANOVA of rotarod scores expressed as a percentage of baseline shows no effect of PFF [$F(1, 15)=0.038$, $p=0.8490$], formoterol [$F(1, 15)=0.98$, $p=0.3378$], or interaction between PFF and formoterol [$F(1, 15)=1.6$, $p=0.2277$] (Figure A2.7b).

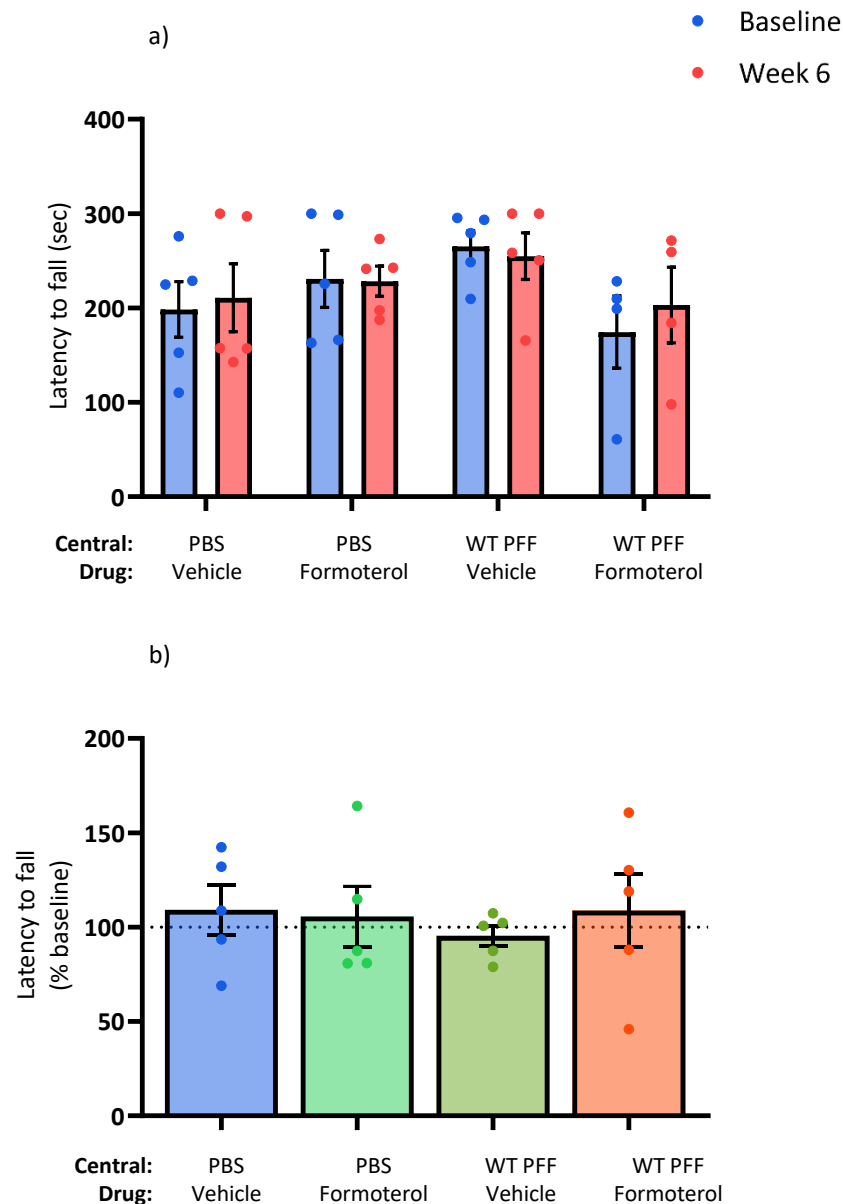


Figure A2.7. Effect of formoterol on rotarod scores six weeks-post intranigral delivery of WT PFF. a) Latency to fall, b) latency to fall expressed as a percentage of baseline. Test duration of 300 seconds. Data is expressed as mean $\pm$ SEM. a) Three-way repeated measures ANOVA, b) two-way ANOVA. $n=5-6$.

2.2.4. pSer129 immunofluorescence

A three-way ANOVA of Iba1pSer129 immunofluorescence shows no effect of laterality [$F(1, 16)=3.104$, $p=0.0972$], PFF [$F(1, 16)=0.6124$, $p=0.4453$], or formoterol [$F(1, 16)=1.305$, $p=0.2700$], and no interactions between laterality, PFF, and/or formoterol (Figure A2.8a). A two-way ANOVA of pSer129 immunofluorescence expressed as a percentage of the contralateral hemisphere shows no effect of PFF [$F(1, 16)=0.31$, $p=0.5831$], formoterol [$F(1, 16)=1.0$, $p=0.3272$], or interaction between PFF and formoterol [$F(1, 16)=1.5$, $p=0.2337$] (Figure A2.8b).

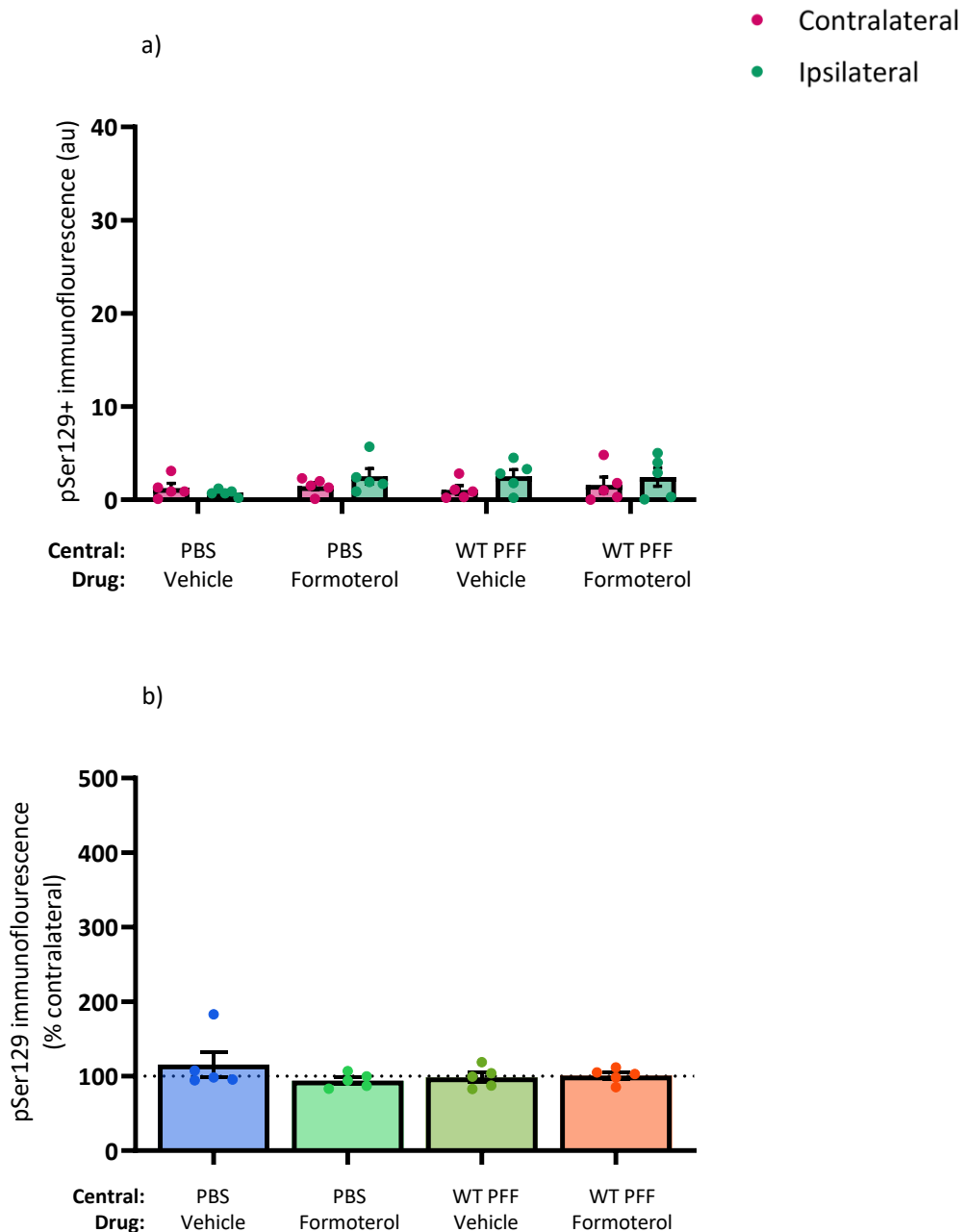


Figure A2.8. pSer129+ α Syn is undetected in the ipsilateral substantia nigra six weeks post intranigral delivery of WT PFF. a) Mean grey value of pSer129 immunofluorescence in the ipsilateral and contralateral substantia nigra, b) mean grey value of pSer129 immunofluorescence in the ipsilateral hemisphere expressed as a percentage of the contralateral hemisphere. Data is expressed as mean $\pm$ SEM. a) Three-way ANOVA, b) two-way ANOVA. $n=5-6$.

2.2.5. Iba1 immunohistochemistry

A three-way ANOVA of Iba1+ cell counts shows an effect of laterality [$F(1, 28)=8.019$, $p=0.0085$], an effect of PFF [$F(1, 28)=4.947$, $p=0.0344$], no effect of formoterol [$F(1, 28)=3.383$, $p=0.0765$], and no interactions between laterality, PFF, and/or formoterol. Post hoc comparisons reveal increased Iba1+ cell counts in the ipsilateral hemisphere in PFF+vehicle ($p=0.0186$) and PFF+formoterol ($p=0.0302$) groups compared to their respective contralateral hemispheres. Increased Iba1+ cell counts are also apparent in the injected hemisphere of WT PFF+vehicle-treated animals compared to the injected hemisphere of PBS+vehicle-treated controls ($p=0.0398$) (Figure A2.9a). A two-way ANOVA of Iba1+ cell counts expressed as a percentage of the contralateral hemisphere shows no effect of PFF [$F(1, 14)=2.8$, $p=0.1145$], formoterol [$F(1, 14)=0.23$, $p=0.6411$], or interaction between PFF and formoterol [$F(1, 14)=0.045$, $p=0.8358$] (Figure A2.9b).

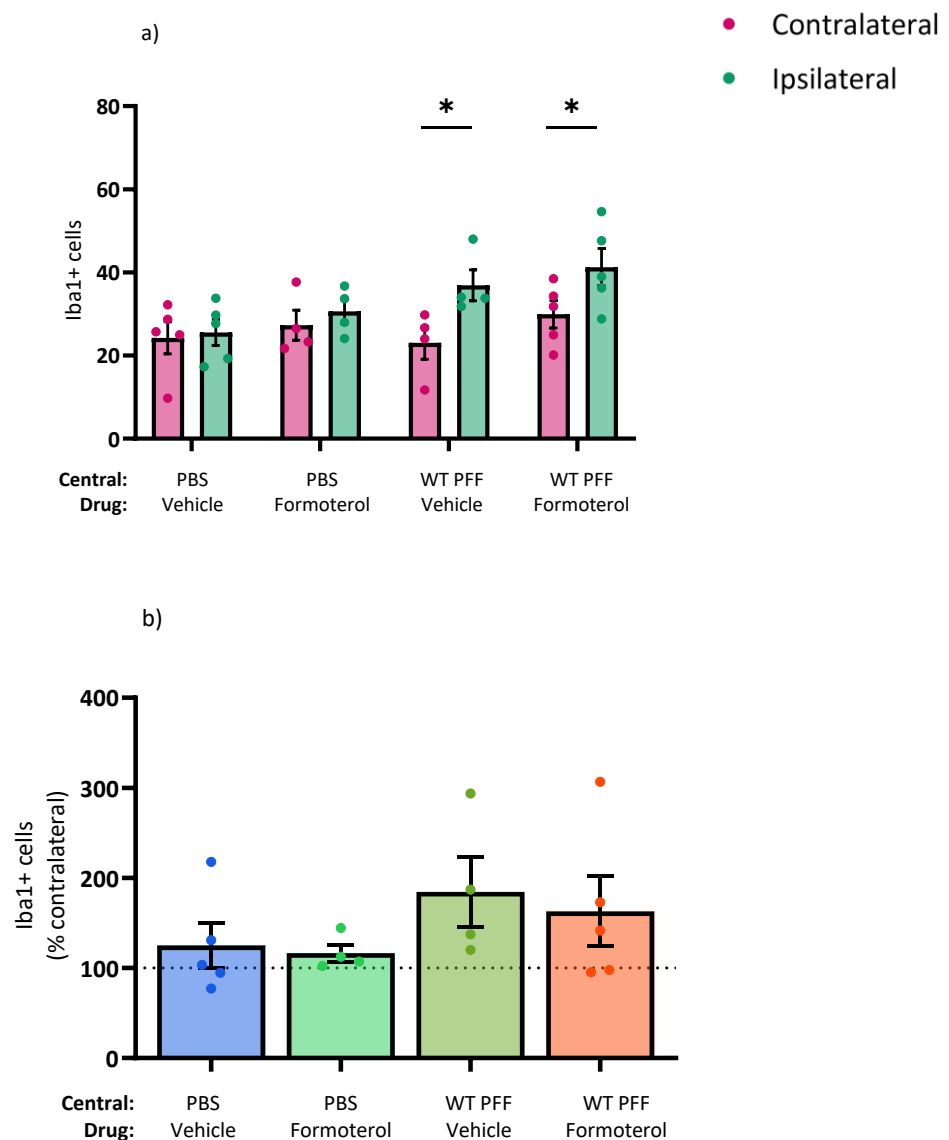


Figure A2.9. Increased Iba1+ microglial cell counts are observed in the ipsilateral substantia nigra six weeks post intranasal delivery of WT PFF. a) Number of Iba1+ cells in the ipsilateral and contralateral substantia nigra, b) number of Iba1+ cells in the ipsilateral hemisphere expressed as a percentage the contralateral hemisphere. a) Three-way ANOVA followed by Fisher's test for multiple comparisons, b) two-way ANOVA. $n=5-6$. $*=p\leq 0.05$.

2.2.6. TH immunohistochemistry

A three-way ANOVA of TH+ cell counts shows no effect of laterality [$F(1, 13)=0.1358$, $p=0.7185$], PFF [$F(1, 13)=0.0009233$, $p=0.9762$], formoterol [$F(1, 13)=0.005223$, $p=0.9435$], or interactions between laterality, PFF, and/or formoterol (Figure A2.10a). A two-way ANOVA of TH+ cell counts expressed as a percentage of the contralateral hemisphere shows no effect of PFF [$F(1, 13)=0.033$, $p=0.8578$], formoterol [$F(1, 13)=0.32$, $p=0.5829$] or interaction between PFF and formoterol [$F(1, 13)=0.97$, $p=0.3437$] (Figure A2.10b).

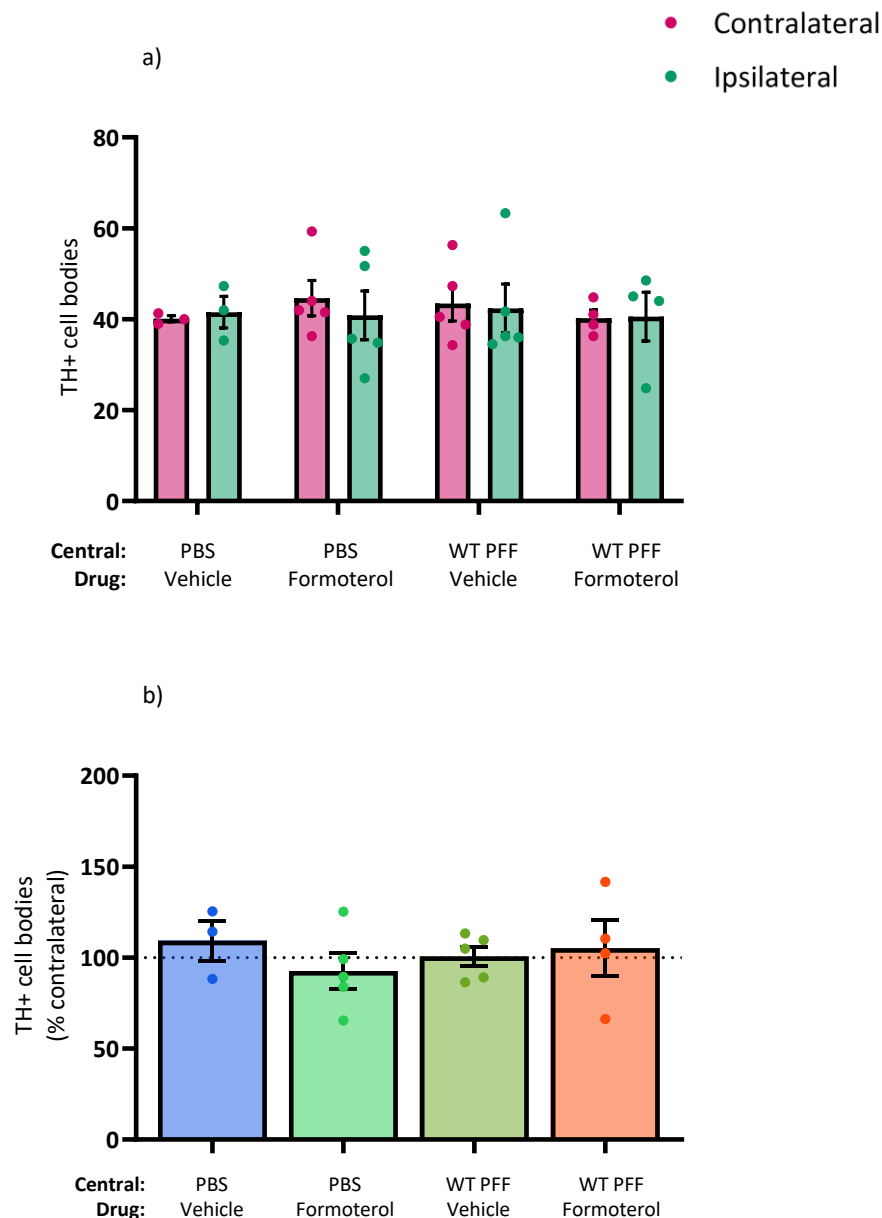


Figure A2.10. Neither WT PFFs nor treatment with formoterol affect the numbers of TH+ dopaminergic neuronal cell counts in the ipsilateral substantia nigra six weeks post intranigral delivery of WT PFF. a) Number of nigral TH+ cells in the ipsilateral and contralateral substantia nigra, b) number of TH+ cells in the ipsilateral hemisphere expressed as a percentage the contralateral hemisphere. a) Three-way ANOVA, b) two-way ANOVA. $n=5-6$.

2.3. Brief conclusion

pSer129+ immunofluorescence was not detected in brain sections of WT PFF-treated animals. As the presence of pSer129+ α Syn could not be confirmed, the results from this study are limited by the lack of evidence confirming an α Syn burden driven by PFF delivery in the substantia. Various histological approaches were employed to unveil the presence of α Syn in the substantia nigra, but no approach provided confirmatory results (see Appendix IV for details). The absence of detectable pSer129+ α Syn in this investigation was unforeseen following successful immunohistochemical detection of pSer129+ α Syn six weeks following injection of the same WT PFF constructs into the substantia nigra in earlier investigations (chapter 3.5.4, chapter 4.5.4). The differing outcomes is not thought to be a result of mal injection, or insufficient sonication prior to injection. These processes were streamlined across experiments and conducted with reproducible technique.

The variation in outcomes is likely a result of variation between the protocols used by the manufacturer to generate the protein constructs used across experiments. It is also possible that minor changes in temperature contributed to varying outcomes with respect to detecting pSer129+ α Syn. In line with observations reported in chapters 3.5.4 and 4.5.4, the present investigation reports an increase in Iba1+ cells six weeks following unilateral delivery of WT PFFs to the substantia nigra. This finding, which was reproduced across three independent *in vivo* studies utilising WT PFFs, suggests that the injected material maintained their immunogenic characteristics, but lost their ability to undergo post-translational phosphorylation at Ser129. Slight changes in the ultra-structure of α Syn PFFs can manifest as major characteristic discrepancies between protein constructs, for example, the post-translational modifications which they can undergo. Failure to reproduce identical structural and biochemical properties of recombinant PFFs has become a major limitation in research which utilizes these materials, and warrants careful consideration when interpreting data. Further work is warranted to confirm these findings.

Appendix III: An assessment of the effect of formoterol on neuronal and glial cellular responses to a systemic lipopolysaccharide challenge following unilateral delivery of wild-type alpha-synuclein preformed fibrils to the rat substantia nigra

3.1. Background (introduction, aims, objectives, and experimental design)

In synucleinopathic disease, it is proposed that the conversion of α Syn from inherent soluble monomers to aggregated amyloid-like insoluble aggregates drives chronic activation of microglia, to promote a pro-inflammatory and cytotoxic environment within the brain (Fellner et al., 2013; Harms et al., 2017; Stefanova et al., 2011). Microglia in the amyloidogenic brain are thought to become “primed” following interaction with protein aggregates. The presence of additional disease-salient stimuli in the α Syn-burdened brain, possibly resulting from systemic infection, appears to boost the prolonged pro-inflammatory response, contributing significantly to disease progression. Findings from the investigations reported in this thesis suggest that while microglial activation associated with intranigral WT PFF delivery may not be sufficient to trigger changes in TH+ dopaminergic cells in the substantia nigra, stimulating α Syn-primed microglia with the bacterial endotoxin LPS may drive a more potent glial response, with downstream consequences for dopaminergic nigral neuronal integrity and motor function. Moreover, while evidence from classic inflammatory models of neurodegenerative disease highlight robust neuroprotective effects of glial β_2 AR modulation on LPS-driven pathology, (O'Neill & Harkin, 2018; O'Neill et al., 2020; Yssel et al., 2018), the ability of β_2 AR agonists to alleviate α Syn-associated inflammation has largely been unexplored.

The primary aim of this investigation was to assess the impact of β_2 AR agonist formoterol on pSer129+ α Syn accumulation, microglial activation, nigral dopaminergic neuronal integrity, and motor function in rats six weeks after unilateral delivery of human recombinant WT PFFs into the substantia nigra, and systemic LPS injection.

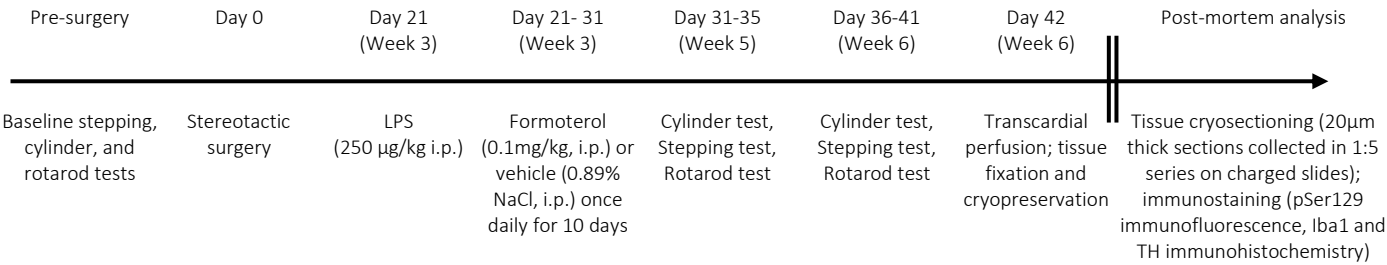
The objectives of this investigation were to deliver human recombinant WT PFFs into the rat substantia nigra by a single stereotactic unilateral injection. Three weeks after intranigral delivery of WT PFF, a systemic LPS challenge was administered via an i.p. injection. Commencing four hours post-LPS delivery, the β_2 AR agonist formoterol was administered once daily for 10 days, to assess its potential to influence α Syn accumulation, microglial activation, dopaminergic neuronal cell counts, and motor behaviours in LPS-challenged animals following WT PFF delivery. Skilled motor function, limb-use asymmetry, and fine motor balance were assessed through application of the stepping, cylinder, and rotarod tests, respectively. pSer129 immunofluorescence, Iba1 immunohistochemistry, and TH immunohistochemistry were subsequently conducted on cryopreserved midbrain tissue sections to assess α Syn deposition, glial cell activation and dopaminergic neuronal integrity, respectively, in the substantia nigra.

Adult male and female Wistar rats underwent behavioural training and baseline testing before being randomly assigned to a treatment group. Animals received one 2 μ L injection of WT PFF or PBS into the substantia nigra. The coordinates for PFF infusion in males were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -8.5 from skull. The coordinates for PFF infusion in females were as follows; AP: -5.3 from bregma, ML: -2.0 from bregma, DV: -7.5 from skull. Three weeks post-PFF delivery, all animals received injection of LPS (250 μ g/kg i.p.). Outputs from NaCl-treated controls are reported in Appendix II. Four hours post-NaCl injection, rats received repeated injections of

either formoterol (0.1mg/kg, i.p.) or sterile NaCl (0.89% NaCl, i.p.) once daily for 10 days. All i.p. injections were administered in volume of 1ml/kg. In weeks five and six following PFF delivery, rats were subjected to the cylinder test, stepping test, and rotarod test. Six weeks post-PFF delivery, animals were euthanised by sodium pentobarbital overdose and transcardial perfusion/fixation. Cryopreserved brain tissue were serially cryosectioned at a thickness of 20µM, and collected on glass slides in preparation for slide-mounted immunofluorescence and histochemical analysis of pSer129+ αSyn, Iba1+ microglia, and TH+ neuronal cell bodies in the substantia nigra.

Treatment groups (n = 5-6):

1. Intranasal PBS (one injection of 2µl) + LPS (250 µg/kg i.p.)
+ vehicle (0.89% NaCl, i.p.)
 - n = 5 (3 females, 2 males)
2. Intranasal PBS (one injection of 2µl) + LPS (250 µg/kg i.p.)
+ formoterol (0.1mg/kg, i.p.)
 - n = 5 (2 females, 3 males)
3. Intranasal WT PFF (one injection of 4µg in 2µl) + LPS (250 µg/kg i.p.)
+ systemic vehicle (0.89% NaCl, i.p.)
 - n = 5 (3 females, 2 males)
4. Intranasal WT PFF (one injection of 4µg in 2µl) + LPS (250 µg/kg i.p.)
+ systemic formoterol (0.1mg/kg, i.p.)
 - n = 6 (3 females, 3 males)



3.2. Results

3.2.1. Cylinder test

A three-way ANOVA of combined ipsilateral placements shows no effect of time [$F(1, 17)=0.4722$, $p=0.4990$], PFF [$F(1, 17)=0.08146$, $p=0.7788$], or formoterol [$F(1, 17)=0.9180$, $p=0.3514$], and no interactions between time, PFF, and/or formoterol (Figure A3.1a).

A three-way ANOVA of combined contralateral placements shows an effect of time [$F(1, 17)=7.050$, $p=0.0167$], no effect of PFF [$F(1, 17)=0.001794$, $p=0.9667$], no effect of formoterol [$F(1, 17)=0.4037$, $p=0.5337$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal no differences between treatment groups (Figure A3.1b).

A three-way ANOVA of % combined ipsilateral placements shows no effect of time [$F(1, 17)=0.4060$, $p=0.5325$], PFF [$F(1, 17)=0.3331$, $p=0.5714$], or formoterol [$F(1, 17)=2.207e-006$, $p=0.9988$], and no interactions between time, PFF and/or formoterol (Figure A3.1c).

A three-way ANOVA of ipsilateral wall placements shows no effect of time [$F(1, 17)=0.3477$, $p=0.5632$], PFF [$F(1, 17)=0.2293$, $p=0.6381$], or formoterol [$F(1, 17)=2.570$, $p=0.1273$], and no interactions between time, PFF, and/or formoterol (Figure A3.2a).

A three-way ANOVA of contralateral wall placements shows an effect of time [$F(1, 17)=20.24$, $p=0.0003$], no effect of PFF [$F(1, 17)=0.006962$, $p=0.9345$], no effect of formoterol [$F(1, 17)=0.2195$, $p=0.6453$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced wall placements at four weeks post-delivery in PBS+formoterol ($p=0.0312$) and PFF+formoterol ($p=0.0051$) groups compared to respective baselines (Figure A3.2b).

A three-way ANOVA of % ipsilateral wall placements shows no effect of time [$F(1, 17)=0.02586$, $p=0.8741$], no effect of PFF [$F(1, 17)=0.3011$, $p=0.5904$], no effect of formoterol [$F(1, 17)=3.966$, $p=0.0628$], and no interactions between time, PFF, and/or formoterol (Figure A3.2c).

Two-way ANOVA of combined ipsilateral placements expressed as a percentage of baseline shows no effect PFF [$F(1, 16)=0.34$, $p=0.5655$], no effect of formoterol [$F(1, 16)=0.53$, $p=0.4778$], and no interaction between PFF and formoterol [$F(1, 16)=1.4$, $p=0.2601$] (Figure A3.3a). Two-way ANOVA of combined contralateral placements expressed as a percentage of baseline shows no effect PFF [$F(1, 16)=0.32$, $p=0.5789$], no effect of formoterol [$F(1, 16)=0.049$, $p=0.8275$], and no interaction between PFF and formoterol [$F(1, 16)=0.045$, $p=0.8340$] (Figure A3.3b). Two-way ANOVA of ipsilateral wall placements expressed as a percentage of baseline shows no effect PFF [$F(1, 16)=0.16$, $p=0.6975$], no effect of formoterol [$F(1, 16)=0.024$, $p=0.8789$], and no interaction between PFF and formoterol [$F(1, 16)=0.61$, $p=0.4465$] (Figure A3.3c). Two-way ANOVA of contralateral wall placements expressed as a percentage of baseline shows no effect PFF [$F(1, 16)=0.43$, $p=0.5219$], no effect of formoterol [$F(1, 16)=0.89$, $p=0.3601$], and no interaction between PFF and formoterol [$F(1, 16)=0.22$, $p=0.6484$] (Figure A3.3d).

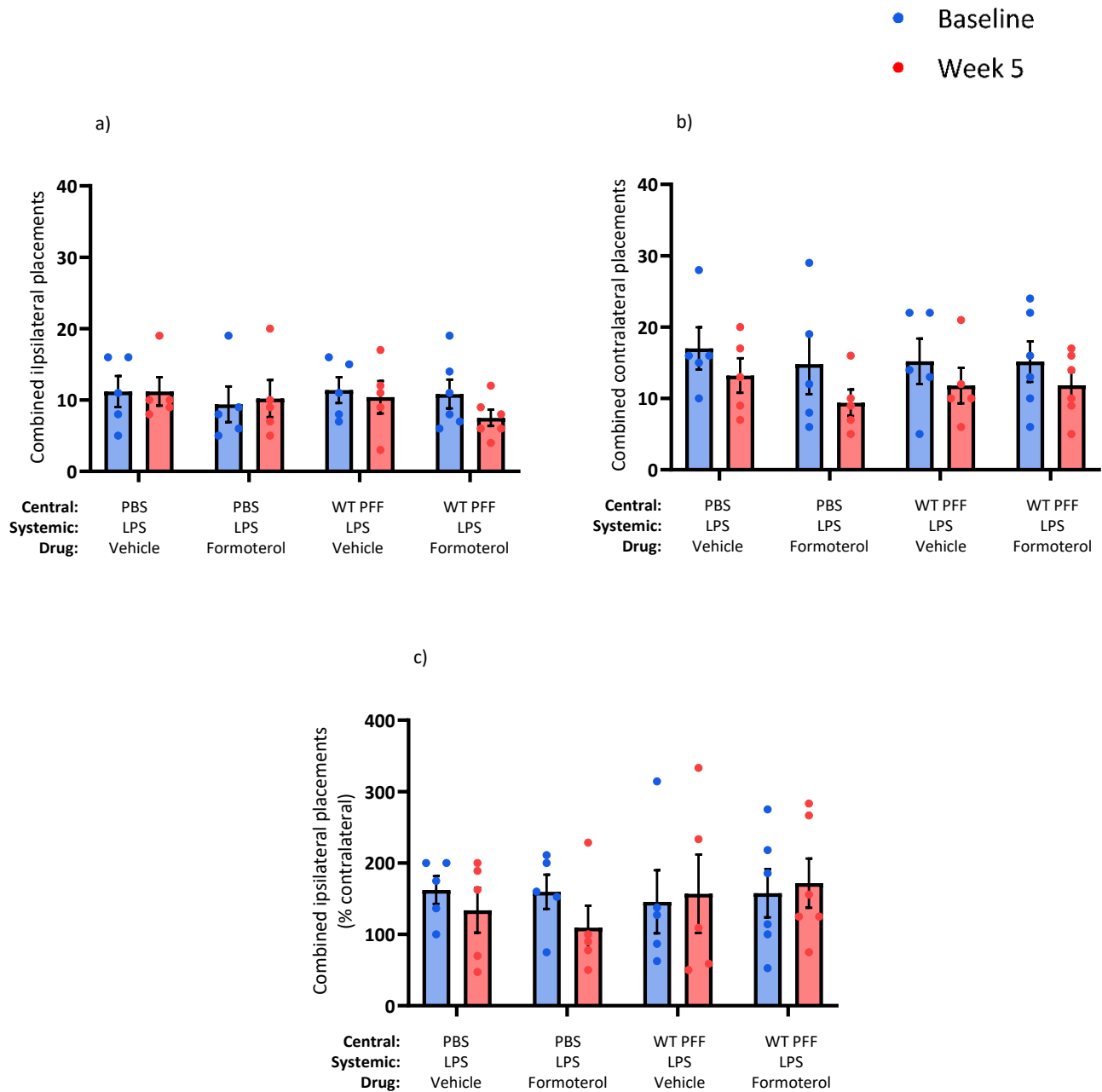


Figure A3.1. Effect of formoterol on combined cylinder test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF. a) Combined placements made with the limb ipsilateral to the injection, b) combined placements made with the limb contralateral to the injection, c) combined contralateral placements expressed as a percentage of combined ipsilateral placements. Test duration of 5 minutes. Data is expressed as mean ± SEM. Three-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. n=5-6.

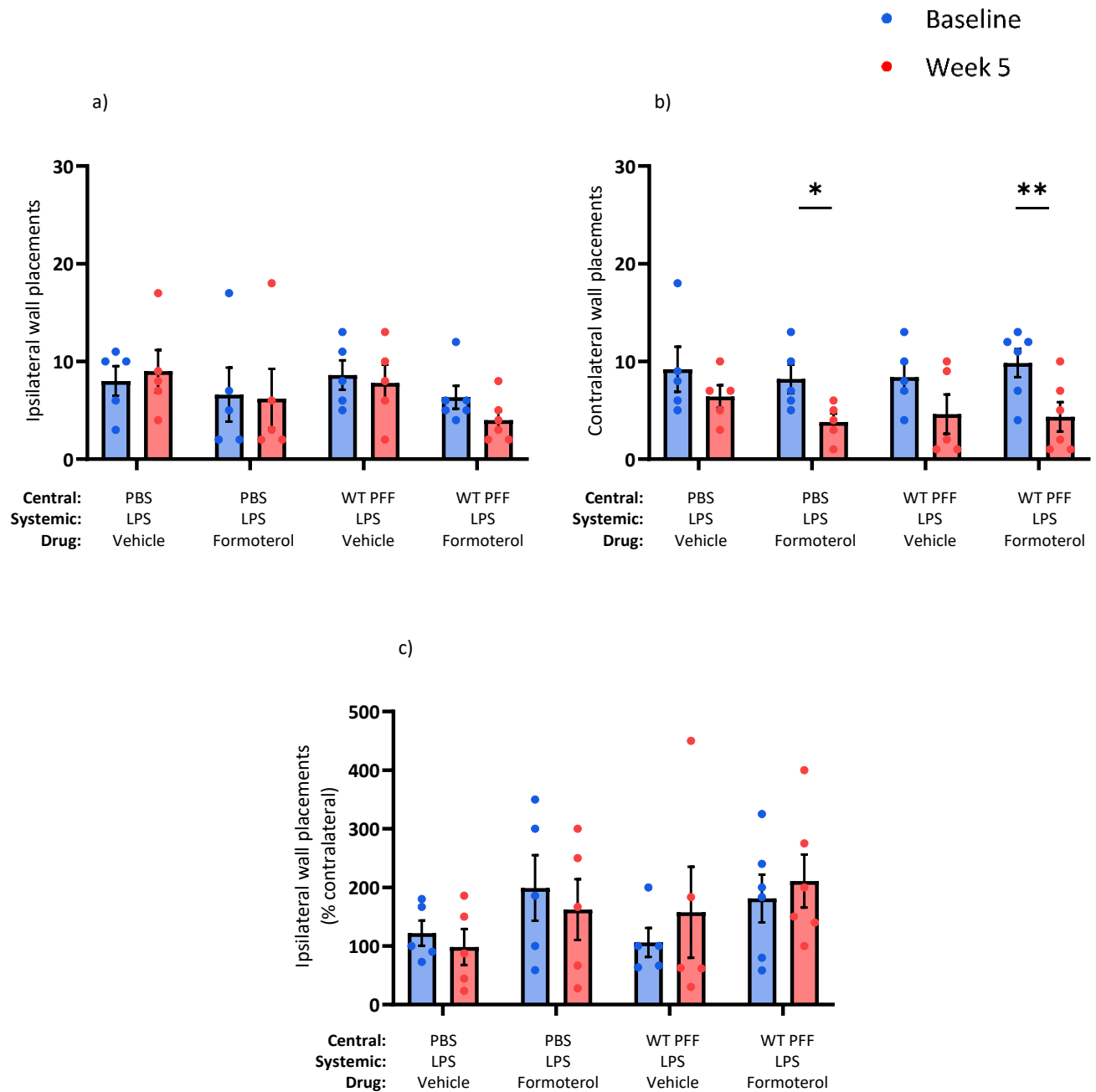


Figure A3.2. Effect of formoterol on wall placement scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF. a) Combined placements made with the limb ipsilateral to the injection, b) combined placements made with the limb contralateral to the injection, c) combined contralateral placements expressed as a percentage of combined ipsilateral placements. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Three-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=5-6$. *= $p \leq 0.05$, **= $p \leq 0.01$.

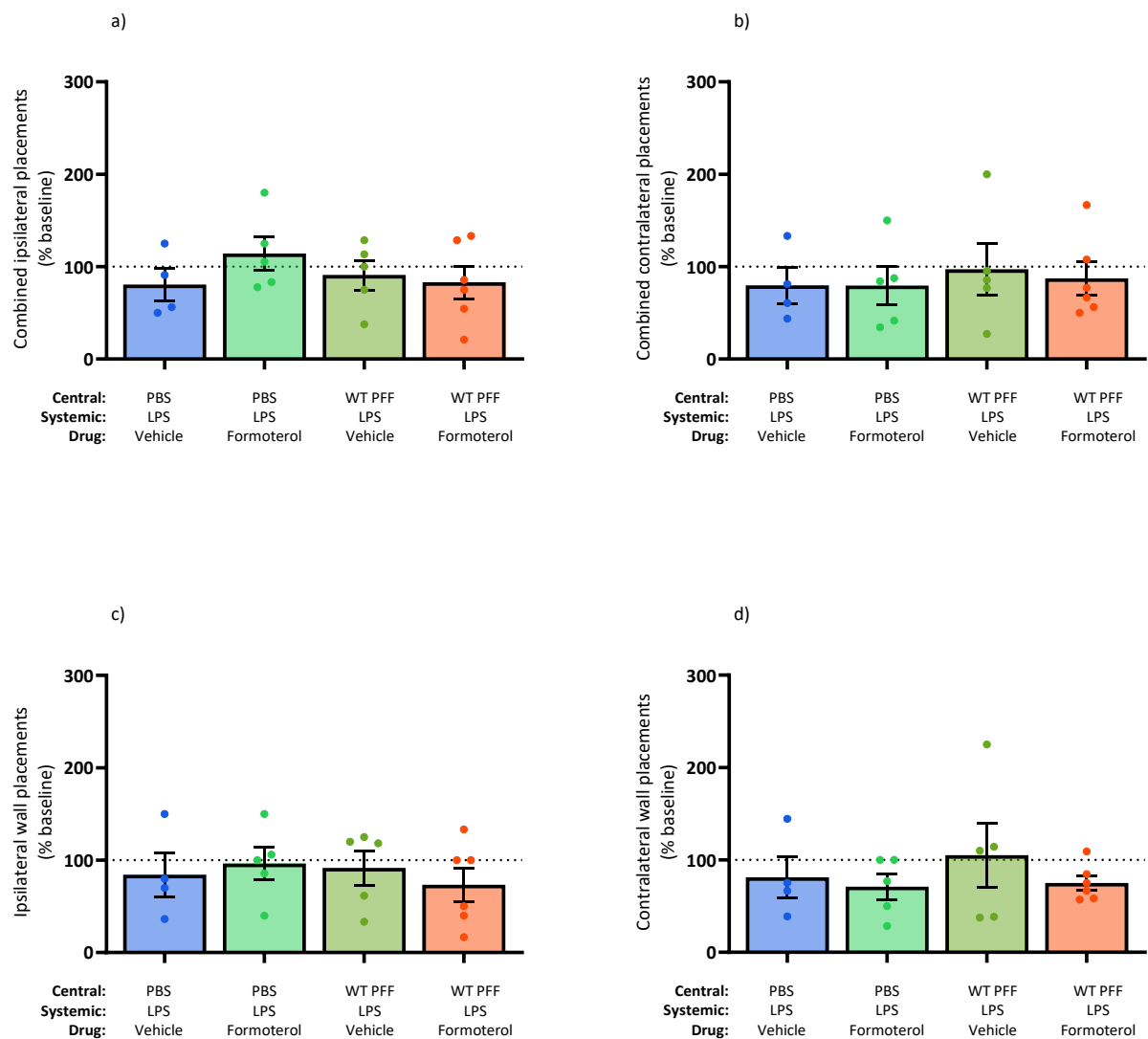


Figure A3.3. Effect of formoterol on cylinder test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF. Scores of a) combined ipsilateral placements, b) combined contralateral placements, c) ipsilateral wall placements, and d) contralateral wall placements, expressed as % of baseline. Test duration of 5 minutes. Data is expressed as mean $\pm$ SEM. Two-way ANOVA. $n=5-6$.

3.2.2. Stepping test

A three-way ANOVA of combined ipsilateral steps shows an effect of time [$F(1, 17)=34.63$, $p<0.0001$], no effect of PFF [$F(1, 17)=1.657$, $p=0.2153$], an effect of formoterol [$F(1, 17)=4.856$, $p=0.0416$], and an interaction between time and formoterol [$F(1, 17)=6.896$, $p=0.0177$]. Post hoc comparisons reveal reduced combined ipsilateral steps at five weeks post-delivery in PBS+vehicle ($p=0.0006$), PFF+vehicle ($p=0.0007$), and PFF+formoterol ($p=0.0018$) groups compared to respective baselines. Uneven baseline scores should be noted when interpreting the data. Post hoc comparisons reveal differences in baseline scores between PBS+formoterol and PBS+vehicle groups ($p=0.0261$), and between PFF+formoterol and PFF+vehicle groups ($p=0.0376$) (Figure A3.4a).

A three-way ANOVA of combined contralateral steps shows an effect of time [$F(1, 17)=37.91$, $p<0.0001$], no effect of PFF [$F(1, 17)=0.03236$, $p=0.8594$], no effect of formoterol [$F(1, 17)=0.002981$, $p=0.9571$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced combined contralateral steps at five weeks post-delivery in PBS+vehicle ($p=0.0146$), PBS+formoterol ($p=0.0066$), PFF+vehicle ($p=0.0077$), and PFF+formoterol ($p=0.0026$) groups compared to respective baselines (Figure A3.4b).

A three-way ANOVA of % combined ipsilateral steps shows no effect of time [$F(1, 17)=0.2626$, $p=0.6149$], PFF [$F(1, 17)=0.2059$, $p=0.6557$], formoterol [$F(1, 17)=0.2196$, $p=0.6453$], or interaction between time, PFF and/or formoterol (Figure A3.4c).

A three-way ANOVA of ipsilateral forward steps shows an effect of time [$F(1, 17)=49.36$, $p<0.0001$], no effect of PFF [$F(1, 17)=0.1944$, $p=0.6648$], no effect of formoterol [$F(1, 17)=2.020$, $p=0.1734$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced ipsilateral forward steps five weeks post-delivery in PBS+vehicle ($p=0.0006$), PFF+vehicle ($p=0.0016$), and PFF+formoterol ($p<0.0001$) groups compared to respective baselines. Forward steps are also reduced in PFF+formoterol-treated animals compared to PFF+vehicle treated animals at five weeks post-delivery ($p=0.0410$) (Figure A3.5a).

A three-way ANOVA of contralateral forward steps shows an effect of time [$F(1, 17)=56.67$, $p<0.0001$], no effect of PFF [$F(1, 17)=0.02792$, $p=0.8693$], no effect of formoterol [$F(1, 17)=1.824$, $p=0.1946$], and no interactions between time, PFF, and/or formoterol. Post hoc comparisons reveal reduced contralateral forward steps five weeks post-delivery in PBS+vehicle ($p=0.0006$), PFF+vehicle ($p=0.0002$), and PFF+formoterol ($p=0.0003$) groups compared to respective baselines (Figure A3.5b).

A three-way ANOVA of % ipsilateral forward steps shows no effect of time [$F(1, 17)=0.9099$, $p=0.3535$], PFF [$F(1, 17)=0.2621$, $p=0.6153$], formoterol [$F(1, 17)=0.1638$, $p=0.6907$], or interactions between time, PFF, and/or formoterol (Figure A3.5c).

Two-way ANOVA of combined ipsilateral steps expressed as a percentage of baseline shows no effect PFF [$F(1, 17)=0.11$, $p=0.7458$], no effect of formoterol [$F(1, 17)=0.75$, $p=0.3981$], and an interaction between PFF and formoterol [$F(1, 17)=2.5$, $p=0.1354$] (Figure A3.6a). Two-way ANOVA of combined contralateral steps expressed as a percentage of baseline shows no effect PFF [$F(1, 17)=0.016$, $p=0.9016$], formoterol [$F(1, 17)=0.012$, $p=0.9129$], or interaction between PFF and formoterol [$F(1, 17)=5.0e-005$, $p=0.9944$]. Post hoc comparisons reveal reduced steps in the PBS+vehicle ($p=0.0220$) and PFF+formoterol ($p=0.0105$) groups compared to the PBS+formoterol group (Figure A3.6b). Two-way ANOVA of ipsilateral steps expressed as a percentage of baseline shows no effect PFF [$F(1, 17)=1.8$, $p=0.1974$], formoterol [$F(1, 17)=1.0$, $p=0.3281$], or interaction between PFF and formoterol [$F(1, 17)=6.9$, $p=0.0174$] (Figure A3.6c). Two-way ANOVA of contralateral steps expressed as a

percentage of baseline shows no effect PFF [$F(1, 17)=0.45$, $p=0.5091$], formoterol [$F(1, 17)=0.63$, $p=0.4372$], or interaction between PFF and formoterol [$F(1, 17)=0.43$, $p=0.5193$] (Figure A3.6d)

● Baseline
● Week 5

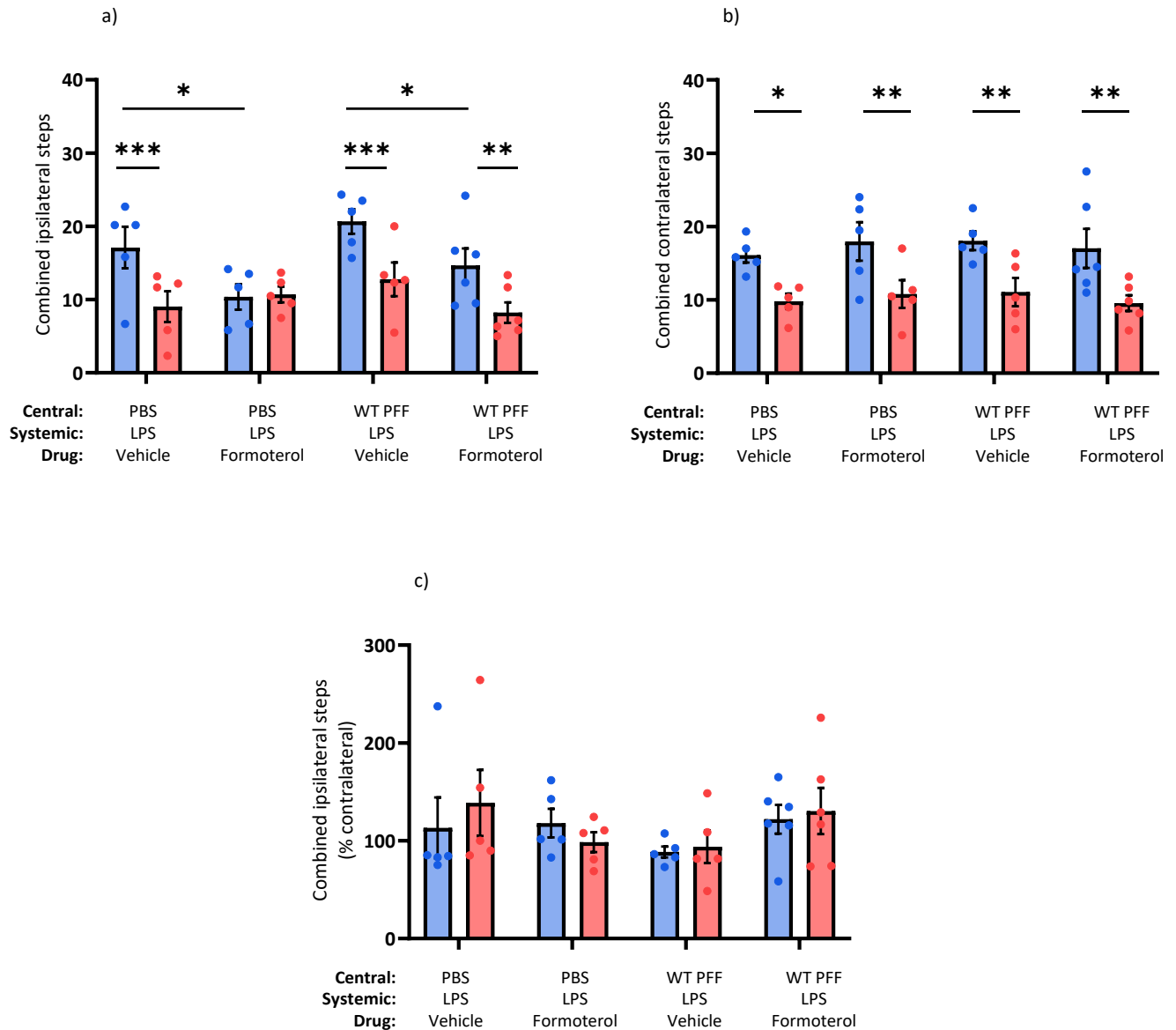


Figure A3.4. Effect of formoterol on combined stepping test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF. a) Combined steps made with the limb ipsilateral to the injection, b) combined steps made with the limb contralateral to the injection, c) combined contralateral steps expressed as a percentage of combined ipsilateral steps. Data is expressed as mean $\pm$ SEM. Three-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=5-6$. *= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$.

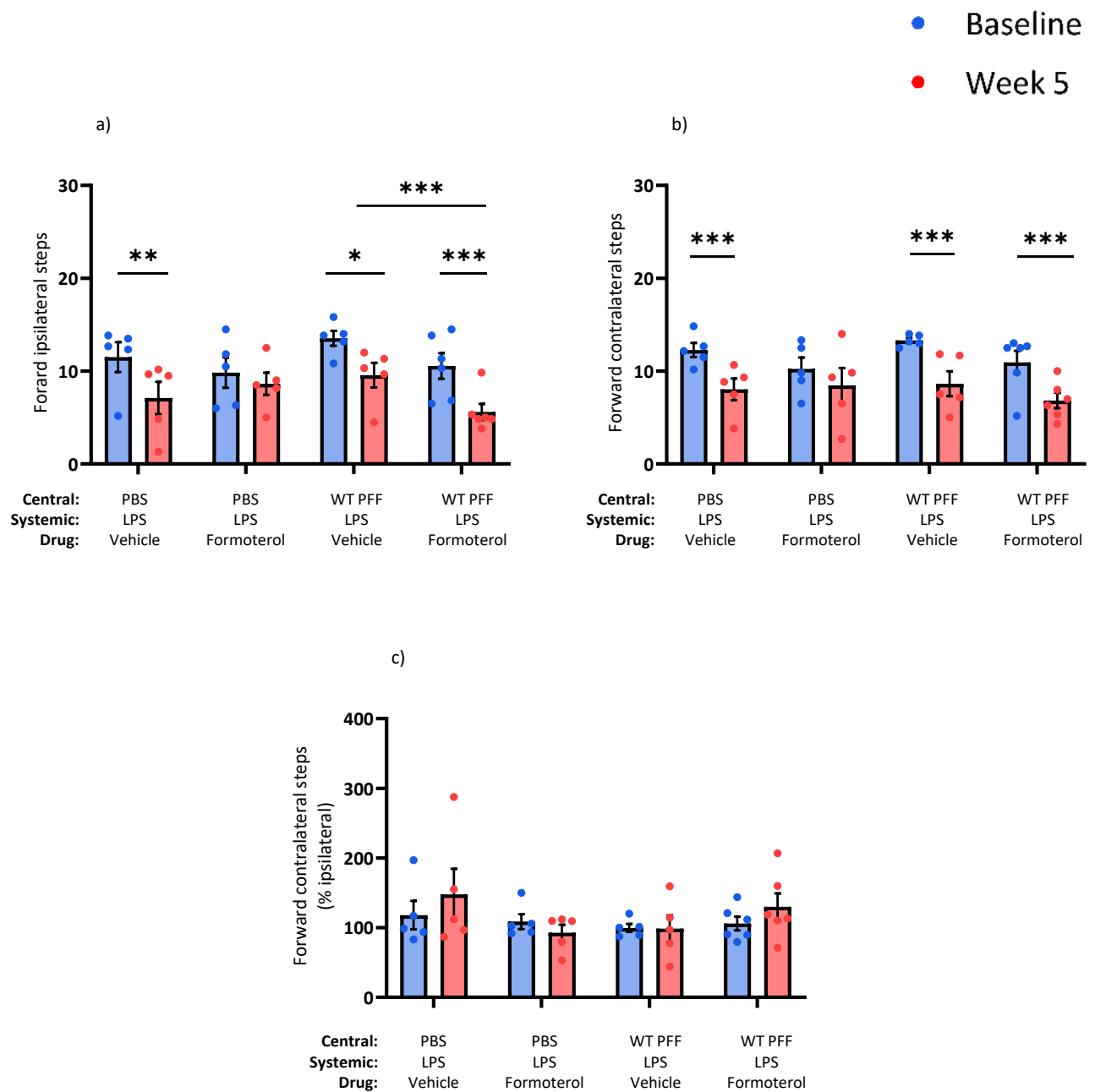


Figure A3.5. Effect of formoterol on forward stepping test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF. a) Forward steps made with the limb ipsilateral to the injection, b) forward steps made with the limb contralateral to the injection, c) forward contralateral steps expressed as a percentage of forward ipsilateral steps. Data is expressed as mean $\pm$ SEM. Three-way repeated measures ANOVA followed by Fisher's test for multiple comparisons. $n=5-6$. $*$ = $p \leq 0.05$, $**$ = $p \leq 0.01$, $***$ = $p \leq 0.001$.

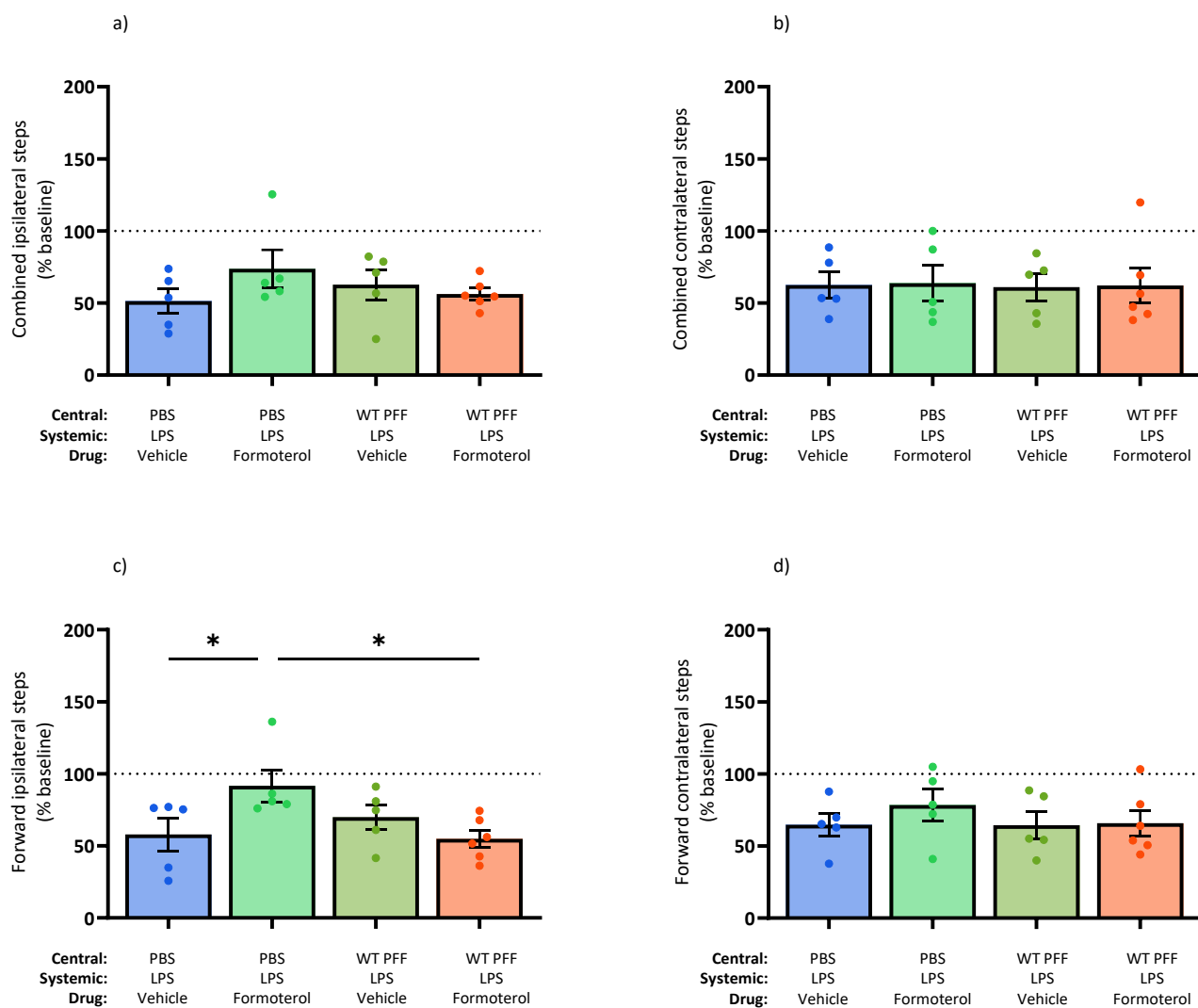


Figure A3.6. Effects of formoterol on stepping test scores of LPS-challenged animals five weeks-post intranigral delivery of WT PFF. Scores of a) combined ipsilateral steps, b) combined contralateral steps, c) forward ipsilateral steps, and d) forward contralateral steps, expressed as % of baseline. Data is expressed as mean $\pm$ SEM. Two-way ANOVA followed by Fisher's test for multiple comparisons $n=5-6$.

3.2.3. Rotarod test

A three-way ANOVA of rotarod scores shows no effect of time [$F(1, 16)=0.5822$, $p=0.4566$], PFF [$F(1, 16)=2.750$, $p=0.1167$], or formoterol [$F(1, 16)=4.229$, $p=0.0564$], but shows an interaction between time and formoterol [$F(1, 16)=13.32$, $p=0.0022$]. Post hoc comparisons reveal reduced rotarod scores at six weeks post-delivery in PFF+vehicle ($p=0.0284$) and PFF+formoterol ($p=0.0033$) groups compared to respective baselines. Reduced latency to fall is also apparent in the PFF+formoterol-treated group compared to the PFF+vehicle-treated group at six weeks post-delivery ($p=0.0081$) (Figure A3.7a). Two-way ANOVA of rotarod scores expressed as a percentage of baseline shows no effect of PFF [$F(1, 16)=0.17$, $p=0.6886$], an effect of formoterol [$F(1, 16)=9.1$, $p=0.0081$], and no interaction between PFF and formoterol [$F(1, 16)=2.1$, $p=0.1689$]. Post hoc comparisons reveal reduced % latency to fall in PFF+formoterol-treated animals compared to PFF+vehicle-treated animals ($p=0.0042$) (Figure A3.7b).

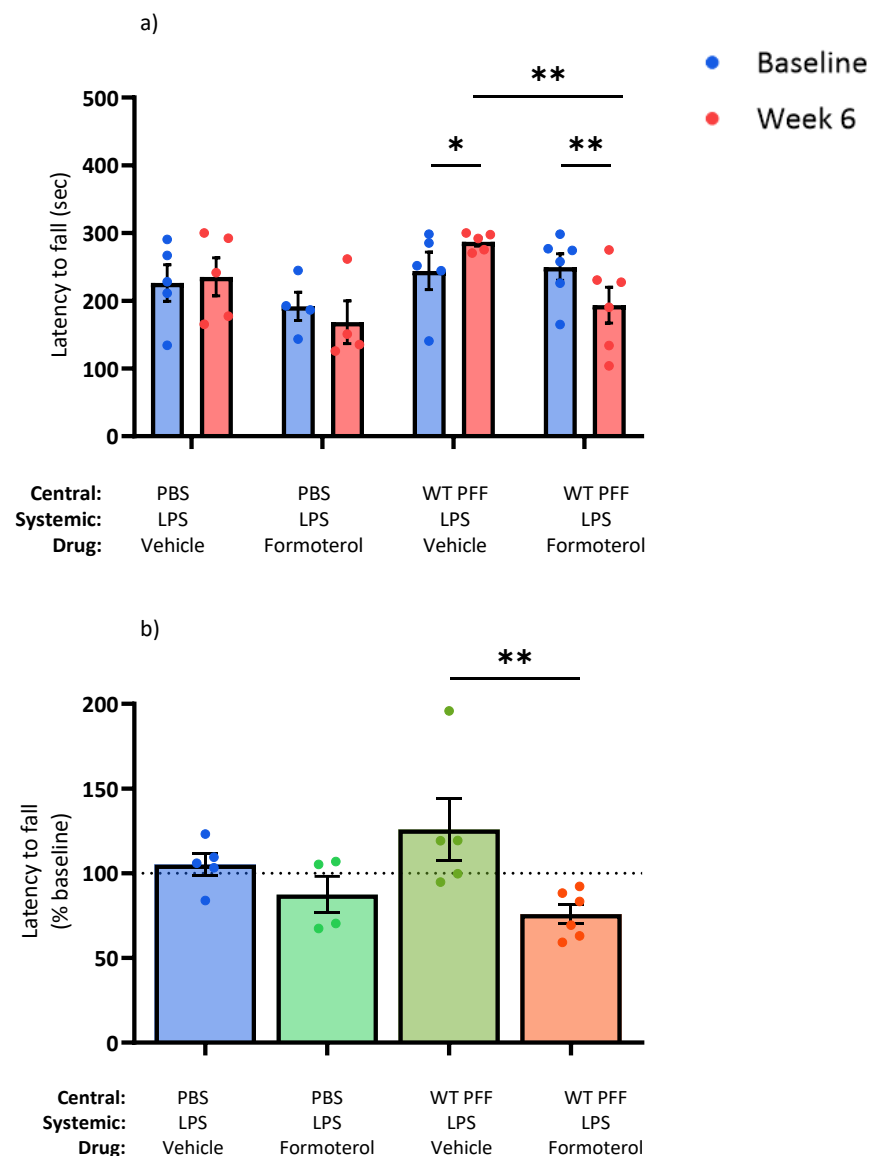


Figure A3.7. Effect of formoterol on rotarod scores of LPS-challenged animals six weeks-post intranigral delivery of WT PFF. a) Latency to fall, b) latency to fall expressed as a percentage of baseline. Test duration of 300 seconds. Data is expressed as mean $\pm$ SEM. a) Three-way repeated measures ANOVA with Fisher's test for multiple comparisons, b) two-way ANOVA followed by Fisher's test for multiple comparisons. $n=5-6$.

3.2.4. pSer129 immunofluorescence

A three-way ANOVA of Iba1pSer129 immunofluorescence shows no effect of laterality [$F(1, 16)=1.562$, $p=0.2293$], PFF [$F(1, 16)=0.005751$, $p=0.9405$], or formoterol [$F(1, 16)=1.045$, $p=0.3218$], and no interactions between laterality, PFF, and/or formoterol (Figure A3.8a). A two-way ANOVA of pSer129 immunofluorescence expressed as a percentage of the contralateral hemisphere shows no effect of PFF [$F(1, 16)=0.70$, $p=0.4162$], formoterol [$F(1, 16)=0.0020$, $p=0.9653$], or interaction between PFF and formoterol [$F(1, 16)=0.36$, $p=0.5553$] (Figure A3.8b).

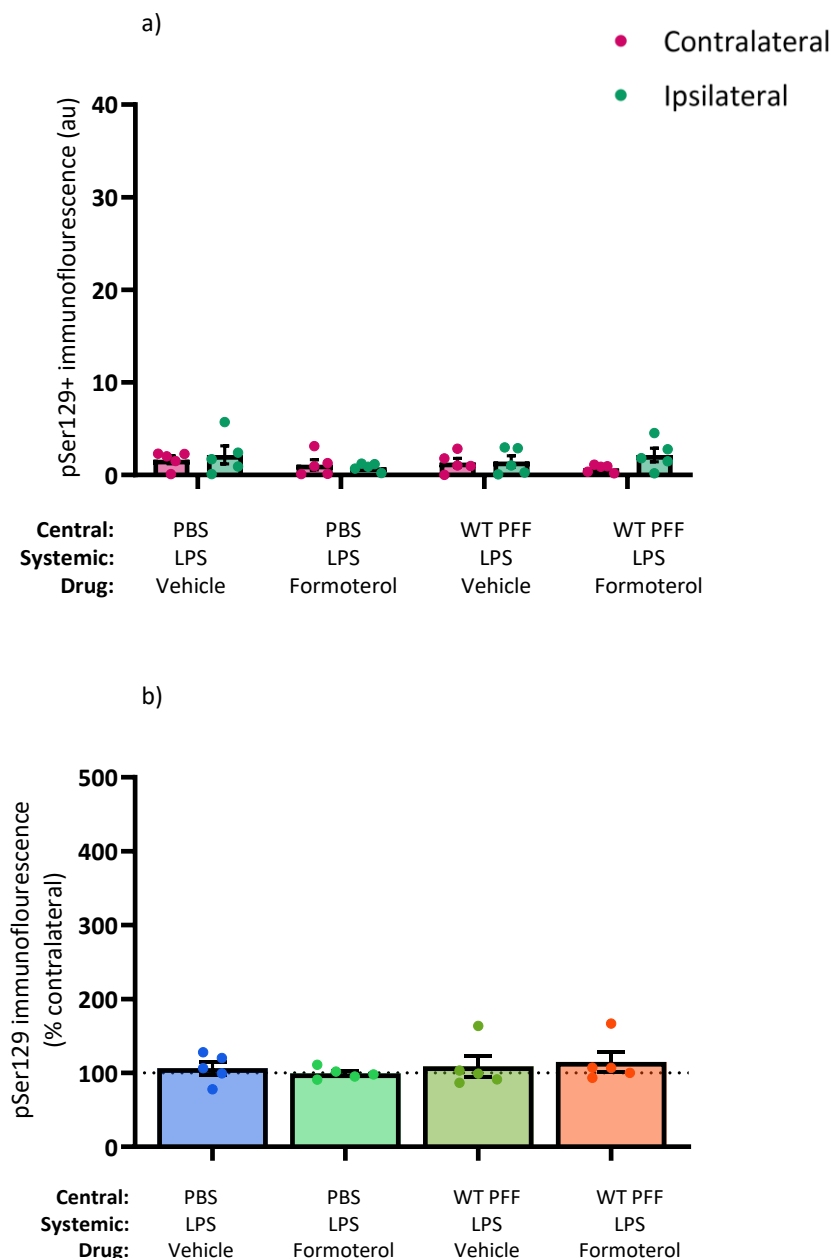


Figure A3.8. pSer129+ α Syn is undetected in the ipsilateral substantia nigra six weeks post intranigral delivery of WT PFF. a) Mean grey value of pSer129 immunofluorescence in the ipsilateral and contralateral substantia nigra, b) mean grey value of pSer129 immunofluorescence in the ipsilateral hemisphere expressed as a percentage of the contralateral hemisphere. Data is expressed as mean $\pm$ SEM. a) Three-way ANOVA, b) two-way ANOVA. $n=5$.

3.2.5. Iba1 immunohistochemistry

A three-way ANOVA of Iba1+ cell counts shows an effect of laterality [$F(1, 30)=9.753$, $p=0.0039$], no effect of PFF [$F(1, 30)=0.03417$, $p=0.8546$], no effect of formoterol [$F(1, 30)=2.904$, $p=0.0987$], and no interactions between laterality, PFF, and/or formoterol. Post hoc comparisons reveal increased Iba1+ cell counts in the ipsilateral hemisphere of WT+formoterol-treated animals compared to the contralateral hemisphere ($p=0.0066$) (Figure A3.9a). A two-way ANOVA of Iba1+ cell counts expressed as a percentage of the contralateral hemisphere shows no effect of PFF [$F(1, 15)=1.8$, $p=0.1948$], formoterol [$F(1, 15)=1.2$, $p=0.2924$] or interaction between PFF and formoterol [$F(1, 15)=2.4$, $p=0.1455$] (Figure A3.9b).

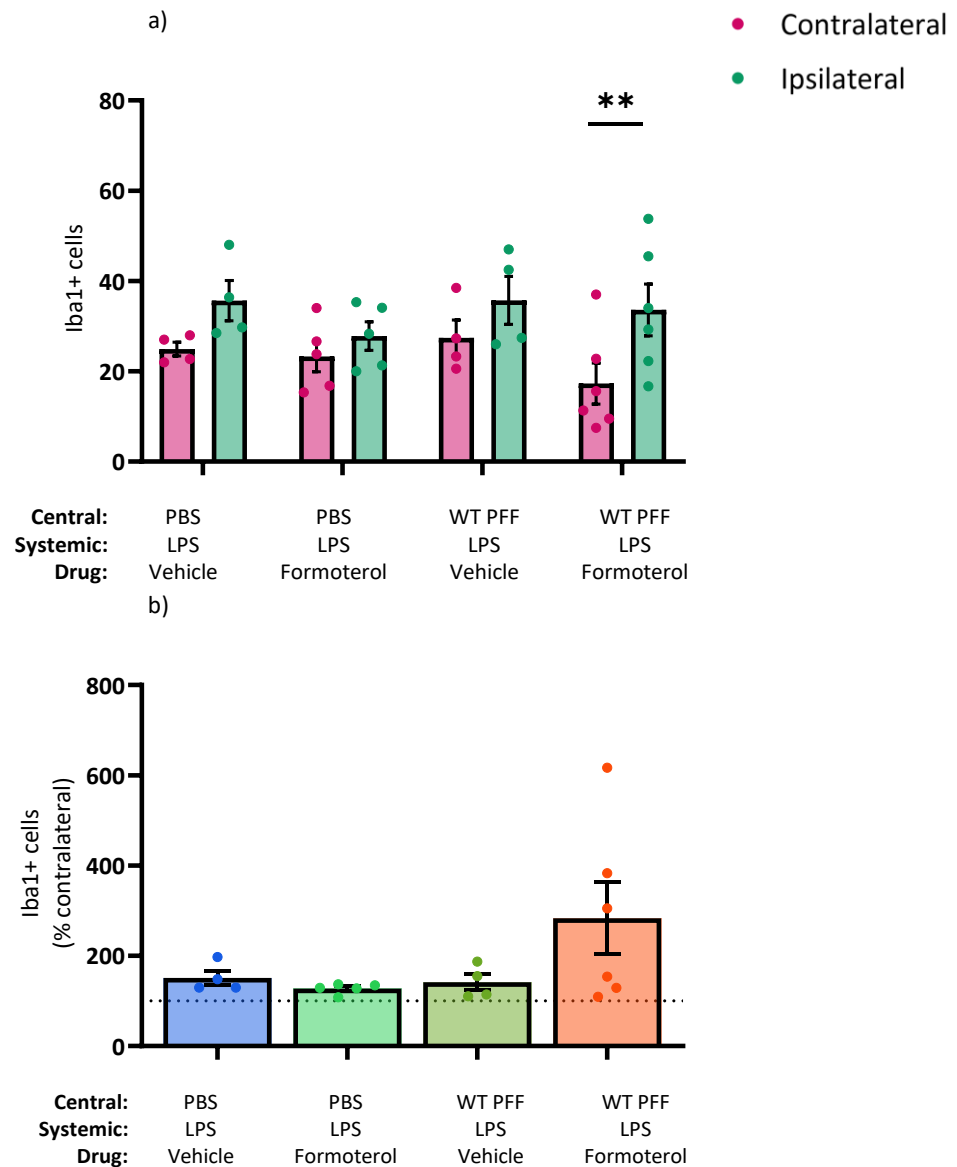


Figure A3.9. Effect of formoterol on Iba1+ microglial cell counts in LPS-challenged animals six weeks post intranigral delivery of WT PFF. a) Number of Iba1+ cells in the ipsilateral and contralateral substantia nigra, b) number of Iba1+ cells in the ipsilateral hemisphere expressed as a percentage the contralateral hemisphere. a) Three-way ANOVA followed by Fisher's test for multiple comparisons, b) two-way ANOVA. $n=5-6$. **= $p \leq 0.01$.

3.2.6. TH immunohistochemistry

A three-way ANOVA of TH+ cell counts shows no effect of laterality [$F(1, 13)=0.2331$, $p=0.6372$], no effect of PFF [$F(1, 13)=1.334$, $p=0.2689$], an effect of formoterol [$F(1, 13)=5.040$, $p=0.0428$], and no interactions between laterality, PFF, and/or formoterol. Post hoc comparisons reveal lower numbers of TH+ cell numbers in the ipsilateral hemisphere of the PFF+vehicle group compared to PFF+formoterol group ($p=0.0168$), and in the contralateral hemisphere of the PFF+vehicle group compared to the PFF+formoterol group ($p=0.0094$) (Figure A3.10a). A two-way ANOVA of TH+ cell counts expressed as a percentage of the contralateral hemisphere shows no effect of PFF [$F(1, 11)=0.0098$, $p=0.9228$], formoterol [$F(1, 11)=0.98$, $p=0.3432$] or interaction between PFF and formoterol [$F(1, 11)=0.098$, $p=0.7603$] (Figure A2.10b).

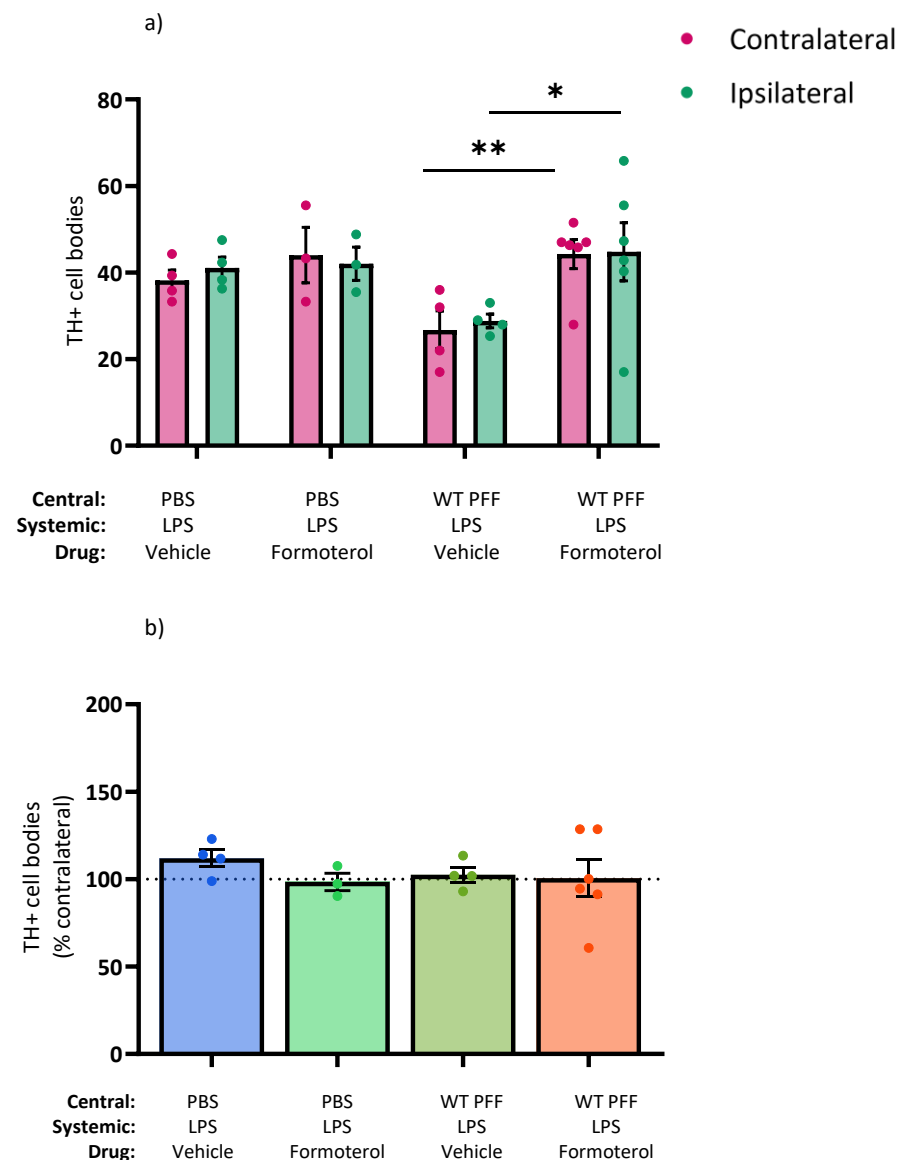


Figure A3.10. Effect of formoterol on TH+ dopaminergic neuronal cell counts in the substantia nigra of LPS-challenged animals six weeks post intranigral delivery of WT PFF. a) Number of nigral TH+ cells in the ipsilateral and contralateral substantia nigra, b) number of TH+ cells in the ipsilateral hemisphere expressed as a percentage the contralateral hemisphere. a) Three-way ANOVA followed by Fisher's test for multiple comparisons, b) two-way ANOVA. $n=5-6$. *= $p \leq 0.05$, **= $p \leq 0.01$.

3.3. Brief conclusion

pSer129+ immunofluorescence was not detected in brain sections of WT PFF-treated animals from this investigation. As the presence of pSer129+ α Syn could not be confirmed, the results from this study are limited by the lack of evidence confirming an α Syn burden driven by PFF delivery in the substantia nigra. Various histological approaches were employed to unveil the presence of α Syn in the substantia nigra, but no approach provided confirmatory results. Further work is warranted to explore the cellular interactions between primed microglia and dopaminergic neurons in the substantia nigra following a systemic challenge of LPS.

Appendix IV: Immunofluorescence and histochemical approaches to confirm the presence of alpha-synuclein in WT PFF-treated animals

4.1. Fluorescence immunohistochemistry

Fluorescence immunohistochemistry was first employed to identify and confirm the presence of α Syn expression in the substantia nigra six weeks following intranigral WT PFF delivery. Brain tissue from animals included in chapter 3 investigations were used as positive controls for both pSer129+ α Syn expression and α Syn(221)+ expression, six weeks following intranigral delivery of WT PFF. Tissue differed in thickness, but was otherwise collected via comparable methodologies.

Slide mounted tissue sections were thawed at room temperature. Sections were outlined with a hydrophobic pen to allow for even dispersal of reagents throughout the staining process. Sections were rehydrated by washing 3 times in PBS with 0.1% Triton-X (PBS-T) for 10 minutes per wash with gentle agitation. Non-specific binding sites were blocked with buffer for 2 hours at room temperature. Blocking buffers tested include 0.1% NGS, 1% NGS, 5% NGS, 0.1% NGS + 0.1% NHS, 1% NGS + 1% NHS, and 5% NGS + 5% NHS, all tested in either 0.01%, 0.1% or 1% PBS-T.

Primary antibody was applied in blocking buffer overnight in a light protected incubation chamber. Primary antibodies tested include rabbit anti-pSer129 α Syn (Stressmarq Biosciences, SMC-600) (Figure A4.1) and mouse anti- α Syn clone Syn211 (Merck Millipore, 36-008) (Figure A4.2). Optimal antibody concentration was tested at dilutions of 1:250, 1:500, 1:1000, and 1:2000. After 24 hours, sections were washed 3 times in 0.1% PBS-T for 10 minutes per wash with gentle agitation, and secondary antibody was applied in PBS for 2 hours in a light protected incubation chamber.

Secondary antibodies tested included Alexa Fluor 488 goat anti-rabbit (Invitrogen), Alexa Fluor 546 goat anti-rabbit (Invitrogen), Alexa Fluor 647 goat anti-rabbit (Invitrogen), Alexa Fluor 488 mouse anti-rabbit (Invitrogen), Alexa Fluor 546 mouse anti-rabbit (Invitrogen), Alexa Fluor 546 mouse anti-rabbit (Invitrogen), Alexa Fluor 488 horse anti-rabbit (Invitrogen), Alexa Fluor 546 horse anti-rabbit (Invitrogen), and Alexa Fluor 546 horse anti-rabbit (Invitrogen). Optimal antibody concentrations were trialled at dilutions of 1:250, 1:500, 1:1000, and 1:2000.

Sections were washed twice in PBS for 10 minutes per wash with gentle agitation. Nuclei were counter stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, 1:20,000) in PBS for 10 minutes. Sections were washed in a final set of 3 x 10-minute washes in PBS before mounting under glass coverslips with anti-fade mounting medium. Mounting media tested include VECTASHIELD® Antifade Mounting Medium (Vector Labs, H-1000), VECTASHIELD® PLUS Antifade Mounting Medium (Vector Labs, H-1900), Fluoromount™ Aqueous Mounting Medium (Merck Millipore, F4680), Aqueous Mounting Medium (DAKO, S302380-2), FluoroQuest™ Anti-fading Mounting Medium (FluoroQuest, AAT-20005), and Hydromount™ Mounting Medium (National Diagnostics, NAT1324). Sections were stored at 4°C in a light protected environment. These staining protocols proved ineffective in confirming the presence of α Syn PFFs in the substantia nigra six weeks following intranigral WT PFF delivery.

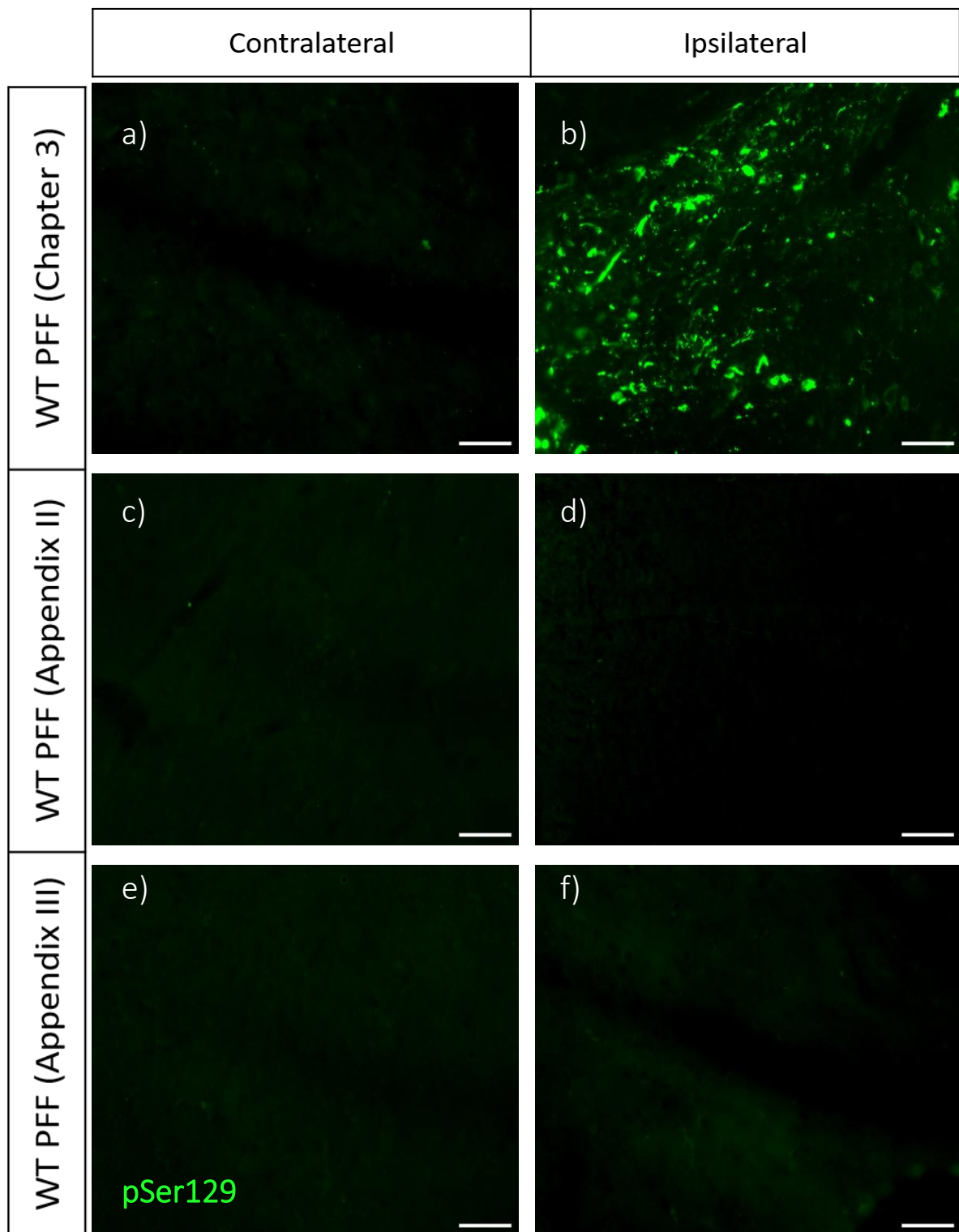


Figure A4.1. pSer129+ α Syn immunofluorescence in midbrain tissue six weeks following unilateral intranigral delivery of WT PFF. Brain tissue was cryosectioned and fluorescently stained for pSer129+ α Syn. Representative 40x images show pSer129+ α Syn immunofluorescence (primary antibody 1:500 dilution, secondary antibody 1:500 dilution) in the a) contralateral hemisphere of WT PFF-injected animals from chapter 3 investigations, b) ipsilateral hemisphere of WT PFF-injected animals from chapter 3 investigations, c) contralateral hemisphere of WT PFF-injected animals from Appendix II investigations, d) ipsilateral hemisphere of WT PFF-injected animals from Appendix II investigations, e) contralateral hemisphere of WT PFF-injected animals from Appendix III investigations, f) ipsilateral hemisphere of WT PFF-injected animals from Appendix III investigations. Scale bar=50 μ m.

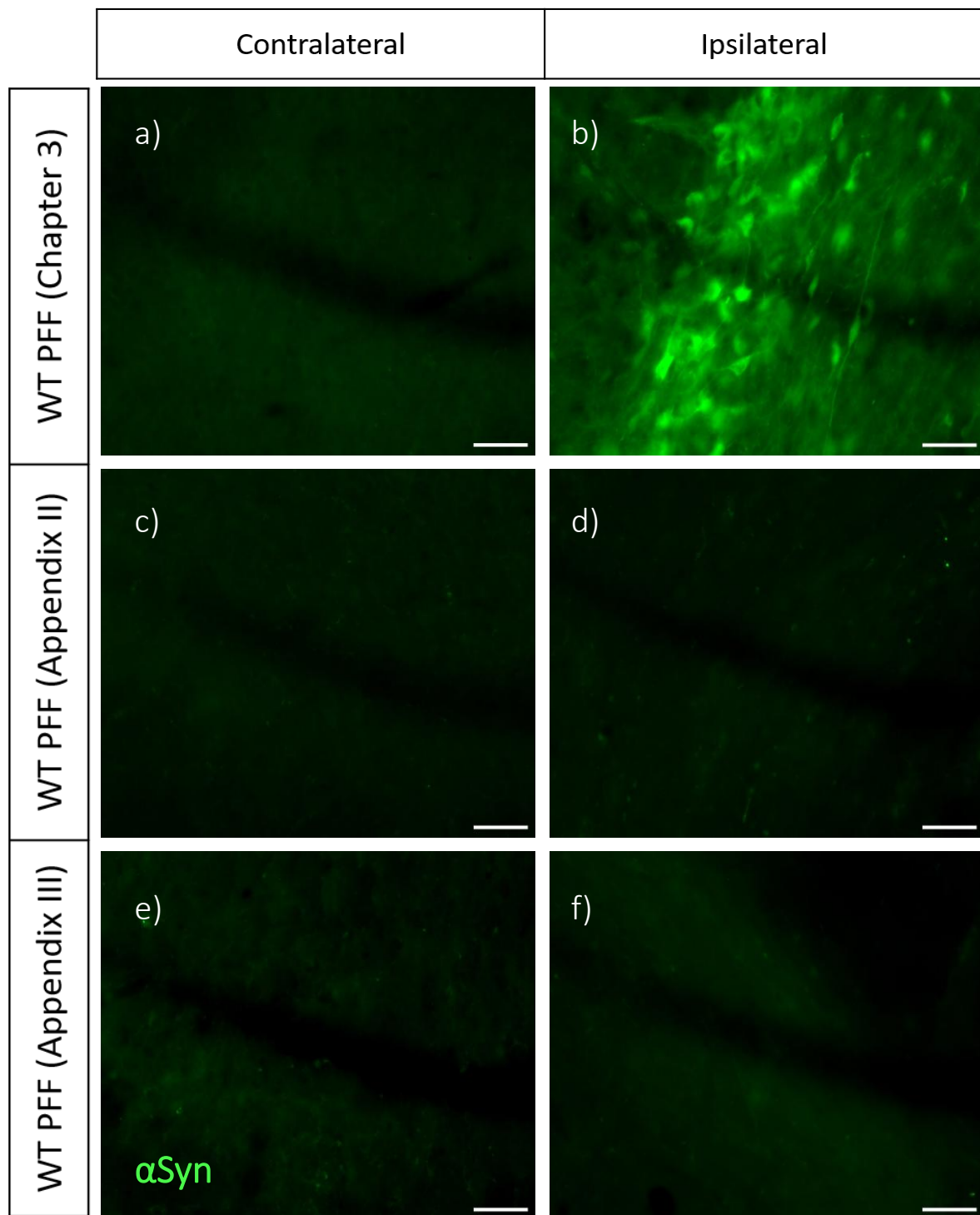


Figure A4.2. α Syn⁺ immunofluorescence in midbrain tissue six weeks following unilateral intranigral delivery of WT PFF. Brain tissue was cryosectioned and fluorescently stained for α Syn. Representative 40x images show α Syn⁺ immunofluorescence (primary antibody 1:500 dilution, secondary antibody 1:500 dilution) in the a) contralateral hemisphere of WT PFF-injected animals from chapter 3 investigations, b) ipsilateral hemisphere of WT PFF-injected animals from chapter 3 investigations, c) contralateral hemisphere of WT PFF-injected animals from Appendix II investigations, d) ipsilateral hemisphere of WT PFF-injected animals from Appendix II investigations, e) contralateral hemisphere of WT PFF-injected animals from Appendix III investigations, f) ipsilateral hemisphere of WT PFF-injected animals from Appendix III investigations. Scale bar=50 μ m.

4.2. DAB immunohistochemistry

DAB immunohistochemistry was employed to unveil evidence of α Syn or pSer129+ α Syn expression in the substantia nigra six weeks following intranigral WT PFF delivery. The ABC method was used to visualise staining using peroxidase as enzyme, hydrogen peroxide as substrate, and DAB as chromogen to visualise the antibody-bound peroxidase complex. Brain tissue from animals included in chapter 3 investigations were used as positive controls for both pSer129+ α Syn expression and α Syn(221)+ expression, six weeks following intranigral delivery of WT PFF. Tissue differed in thickness, but was otherwise collected via comparable methodologies.

Slide mounted tissue sections were thawed at room temperature and rehydrated in PBS-T for three 10-minute washes with gentle agitation. Endogenous peroxidase activity was quenched in various solutions including 0.1% H_2O_2 in 20% MeOH, 1% H_2O_2 in 20% MeOH, 3% H_2O_2 in 20% MeOH, 10% H_2O_2 in 20% MeOH, 0.1% H_2O_2 in 70% MeOH, 1% H_2O_2 in 70% MeOH, 3% H_2O_2 in 70% MeOH, or 10% H_2O_2 in 70% MeOH in dH_2O for 20 minutes at room temperature. Sections were washed 3 times in PBS-T for 10 minutes per wash with gentle agitation. Sections were outlined with a hydrophobic pen to allow for even dispersal of reagents throughout the staining process. Non-specific bindings sites were blocked with buffer for 2 hours at room temperature. Buffers tested included 1% NGS, 3% NGS, 5% NGS, and 10% NGS in PBS-T.

Primary antibody was applied in blocking buffer overnight in a light protected incubation chamber. Primary antibodies tested include rabbit anti-pSer129 α Syn (Stressmarq Biosciences, SMC-600) (Figure A4.3) and mouse anti- α Syn clone Syn211 (Merck Millipore, 36-008) (Figure A4.4). Optimal antibody concentration was tested at dilutions of 1:250, 1:500, 1:1000, and 1:2000. The next day, sections were washed 3 times in PBS-T for 10 minutes per wash with gentle agitation, and incubated with HRP conjugated goat anti-rabbit secondary antibody for 2 hours at room temperature in the incubation chamber.

Secondary antibody was tested at a variety of different concentrations, including 1:250, 1:500, 1:1000, and 1:2000. Sections were again washed 3 times in PBS for 10 minutes per wash with gentle agitation, and incubated with ABC for 2 hours at room temperature. Sections were washed in a final set 3 x 10-minute washes in PBS before incubated in DAB for 2, 5, 10, or 20 minutes.

Sections were submerged in dH_2O to stop the reaction, and excess chromogen was removed by washing in a series of dH_2O baths. Finally, sections were dehydrated in a series of ascending EtOH concentrations (70% EtOH, 90% EtOH, 95% EtOH, 100% EtOH) for 2 minutes each, and cleared with 2 x 2-minute rounds of in xylene incubation. DPX mounting medium was used to apply glass coverslips and sections are left to dry overnight. These staining protocols proved ineffective in confirming the presence of α Syn PFFs in the substantia nigra six weeks following intranigral WT PFF delivery.

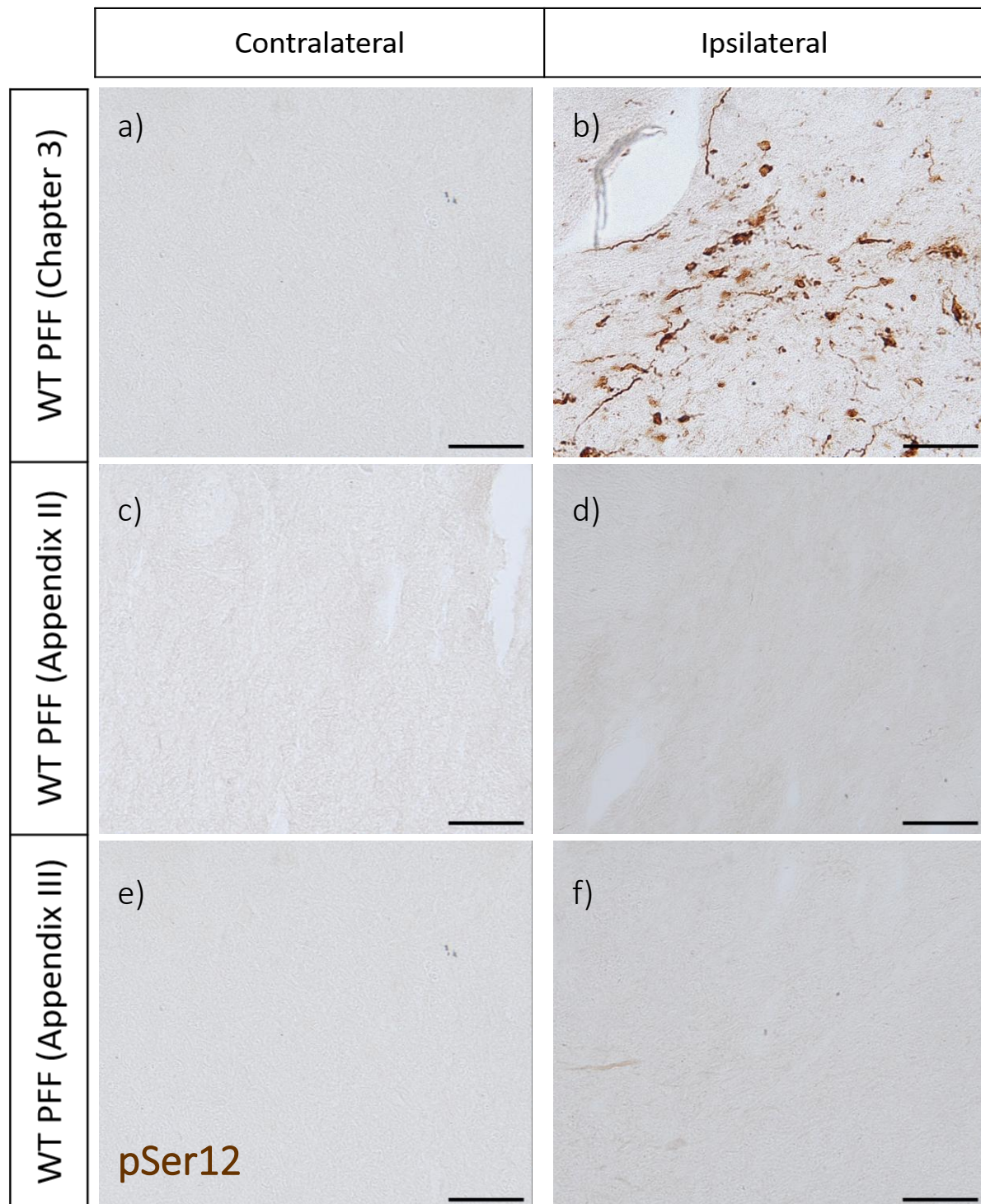


Figure A4.3. pSer129+ αSyn immunohistochemistry in midbrain tissue six weeks following unilateral intranigral delivery of WT PFF. Brain tissue was cryosectioned and immunohistochemically stained for pSer129+ αSyn. Representative 40x images show pSer129+ αSyn immunostaining (primary antibody 1:500 dilution, secondary antibody 1:500 dilution) in the a) contralateral hemisphere of WT PFF-injected animals from chapter 3 investigations, b) ipsilateral hemisphere of WT PFF-injected animals from chapter 3 investigations, c) contralateral hemisphere of WT PFF-injected animals from Appendix II investigations, d) ipsilateral hemisphere of WT PFF-injected animals from Appendix II investigations, e) contralateral hemisphere of WT PFF-injected animals from Appendix III investigations, f) ipsilateral hemisphere of WT PFF-injected animals from Appendix III investigations. Scale bar=50μm.

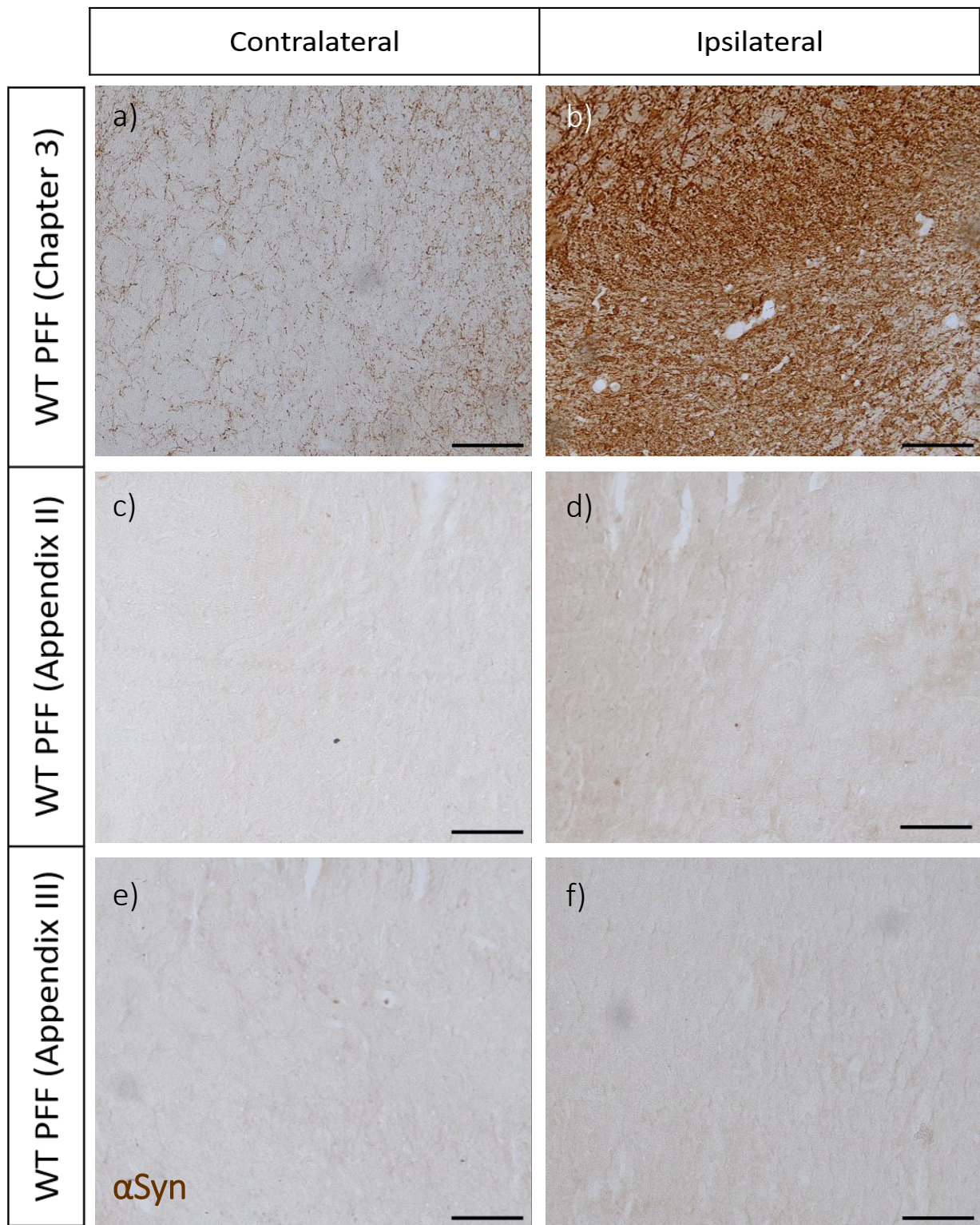


Figure A4.4. α Syn+ immunohistochemistry in midbrain tissue six weeks following unilateral intranigral delivery of WT PFF. Brain tissue was cryosectioned and immunohistochemically stained for α Syn. Representative 40x images show α Syn+ immunostaining (primary antibody 1:500 dilution, secondary antibody 1:500 dilution) in the a) contralateral hemisphere of WT PFF-injected animals from chapter 3 investigations, b) ipsilateral hemisphere of WT PFF-injected animals from chapter 3 investigations, c) contralateral hemisphere of WT PFF-injected animals from Appendix II investigations, d) ipsilateral hemisphere of WT PFF-injected animals from Appendix II investigations, e) contralateral hemisphere of WT PFF-injected animals from Appendix III investigations, f) ipsilateral hemisphere of WT PFF-injected animals from Appendix III investigations. Scale bar=50 μ m.

Appendix V: Dissemination

Publications

C. Tyndall, M. McAuslan, R. Maher, T. Burke-Mullaly, D. Kozareva, J. Prenderville, A. Harkin (in preparation, 2024). Targeting beta2 adrenergic receptors to influence motor behaviour, alpha-synuclein phosphorylation, microglial reactivity, and dopaminergic neuronal cell loss in the rat substantia nigra following unilateral intranigral delivery of alpha-synuclein preformed fibrils in combination with alpha-synuclein-expressing adeno-associated viral vector. Targeted journal: British Journal of Neuropharmacology.

C. Tyndall, J. Prenderville, A. Harkin (in preparation, 2024). Experimental challenges associated with the use of recombinant alpha-synuclein preformed fibrils in the development of *in vitro* and *in vivo* experimental models of synucleinopathic disease. Targeted journal: Frontiers Neuroscience.

Poster Presentations

March 2022, Alzheimer's Disease & Parkinson's Disease Conference (ADPD), Virtual

C. Tyndall, A. Netter-Glangeaud, A. Louwier, J. Prenderville, A. Harkin (2022). Assessing the immunogenicity of alpha-synuclein oligomers, filaments, and fibrils in a clinically relevant model of synucleinopathy in vitro.

September 2022, Young Investigator Symposium, Neuroscience Ireland, Dublin, Ireland

C. Tyndall, A. Netter-Glangeaud, A. Louwier, J. Prenderville, A. Harkin (2022). Assessing the immunogenicity of alpha-synuclein fibrils in a clinically relevant model of synucleinopathy in vitro.

March 2021, Alzheimer's Disease & Parkinson's Disease Conference (ADPD), Virtual

C. Tyndall, T. Burke-Mullaly, D. Kozareva, A. Netter-Glangeaud, A. Louwier, E. Sokolowska, J. Prenderville, A. Harkin (2021). Glial-neuronal interaction prompted by recombinant fibrillar protein constructs of alpha-synuclein – an in vitro characterisation.

March 2021, Alzheimer's Disease & Parkinson's Disease Conference (ADPD), Virtual

T. Burke-Mullaly, **C. Tyndall**, D. Kozareva, A. Netter-Glangeaud, A. Louwier, E. Sokolowska, J. Prenderville, A. Harkin (2021). Development and characterisation of a humanised model of Parkinson's disease associated with delivery of adeno-associated virus expressing alpha-synuclein and pre-formed alpha-synuclein fibrils unilaterally into the rat substantia nigra.

September 2020, Young Investigator Symposium, Neuroscience Ireland, Virtual

C. Tyndall, J. Prenderville, A. Harkin (2020). Glial-neuronal interaction prompted by recombinant fibrillar protein constructs of alpha-synuclein – an in vitro characterisation

Oral Presentations

2024, NeuroConnect, Early Career Researchers Network (ECRN), Neuroscience Ireland

C. Tyndall, J. Prenderville, A. Harkin (2024). “A new cell culture model to study Lewy body disorders.”

2023, Lewy Body Ireland Meeting, Virtual

C. Tyndall, J. Prenderville, A. Harkin (2023). “Breathing new life into PD therapeutics: assessing the neuroprotective role of noradrenergic drugs in combating alpha-synuclein-related neuroinflammation”

2022, Terry Pratchett at the Unseen University, Dublin, Ireland

C. Tyndall, J. Prenderville, A. Harkin (2022). “Current research in the field of Lewy Body Dementia: Novel methods for deducing new treatments for Lewy Body Dementia and other synuclein-related diseases”

2021, International Dementia Conference, Engaging Dementia

C. Tyndall, J. Prenderville, A. Harkin (2021). “Breathing new life into PD therapeutics: assessing the neuroprotective role of noradrenergic drugs in combating alpha-synuclein-related neuroinflammation”

2021, NeuroConnect, Early Career Researchers Network (ECRN), Neuroscience Ireland

C. Tyndall, J. Prenderville, A. Harkin (2021). “Glial-neuronal interaction prompted by recombinant fibrillar protein constructs of alpha-synuclein – an in vitro characterisation”

Outreach

2024, Creative Brain Week “Living Labs” Exhibition, Trinity College Dublin, Ireland

Creative Brain Week was hosted in Trinity College in March 2024. In partnership with Stockton’s Wing’s guitarist Mike Hanrahan, I aided in the development of a “Living Lab” exhibition, which showcased music’s ability to communicate the dementia experience. This interactive display informed by research in music perception and emotion-related neural networks, allowed participants to articulate the innerworkings of the brain through music.

2022, Abbey Theatre Dementia Consultant, Dublin, Ireland

I served as a dementia consultant on the development and production of *An Old Song Half Forgotten*, a new play written by award-winning Irish playwright, Deirdre Kinahan, and directed by Louise Lowe. *An Old Song, Half Forgotten* was inspired by, written for, and performed in the Abbey Theatre by Ireland’s acclaimed actor, Bryan Murray, who himself was diagnosed with Alzheimer’s disease. The play presented an example of the lived experience of one person living with Alzheimer’s disease, and

personified the Alzheimer's brain live on stage. My involvement included 'translating' Alzheimer's disease and dementia research into digestible language for theatre artists, patrons, and other non-scientists involved in the project, while sharing advances in the research world with the general public. I contributed to the development a guide for environmental, social, and infrastructural changes which may be implemented into rehearsal spaces and theatres in order to accommodate theatre workers and patrons with dementia, and their carer's. This protocol was used to create, for the first time, a dementia-safe and dementia-friendly environment within the national theatre of Ireland. Dierdre Kinahan and Bryan Murray were subsequently awarded the inaugural Pratchett Project Award during 2023 Creative Brain Week for their contribution to dementia awareness.

2022; 2023, Terry Pratchett Culture Night, Dublin Ireland

The Trinity College Dublin Pratchett Project is an interdisciplinary platform of research surrounding the life and works of Sir Terry Pratchett. In September 2022 and 2023, the Harkin lab organised a Culture Night event, inspired by Sir Terry Pratchett, the world acclaimed fantasy writer, who himself suffered from neurodegenerative disease. This public outreach event was co-hosted by Trinity College Institute for Neuroscience, Trinity Centre for Literary and Cultural Translation, and Global Brain Health Institute. This event aimed to raise awareness about Alzheimer's and dementia with the general public, 'translate' science, demystify research, and share advances in the field with those who attended. The event showcased the mutualistic relationship between science and humanities - showing how the arts & humanities are crucial for spreading the word of science, and how science can better inform the humanities. The event was led by a team of scientists, clinicians, authors, poets, people living with dementia, carers, and dementia advocates as they conducted a Pratchett-inspired discussion. Of particular note Rob Wilkins, Terry Pratchett's personal assistant, attended and participated at the event. Terry was a public advocate for Alzheimer's research, following his diagnosis with posterior cortical atrophy. During this event, I shared recent advocacy and outreach initiatives developed with Lewy Body Ireland.

2020-2023, co-founder of Neuroscience Ireland's Early Career Researchers Network (ECRN)

ECRN is an academic organisation of early career neuroscience researchers in Ireland. Since its founding in 2020, it has become the largest national forum for postgraduate and post-doctoral researchers in neuroscience to discuss research, share technical expertise, foster collaboration, and grow the emerging neuroscience community on the island of Ireland. ECRN organises a monthly academic seminar series called "NeuroConnect", where members present research, share recent publications, discuss findings, and plan future experiments with the help of peers. ECRN also hosts guest seminars from experts on career development, equality in science, thesis and grant writing, and sustainability in the laboratory. ECRN hosts dedicated communication channels where members can post research, ask technical questions, promote new job opportunities, and share recent advances in the field, as well as shared information on relevant scientific conferences, outreach events, grant applications, and available stipends. The committee of ECRN co-founders organised and hosted Neuroscience Ireland's Young Investigator Symposium in 2021 (virtual) and 2023 (Trinity Biomedical Sciences Institute, Dublin, Ireland). After just its second year in development, the Young Investigator Symposium was attended by over 280 delegates from all four corners of Ireland. Over the course of two days, the conference showcased 70 poster presentations, 22 oral presentations,

keynote speakers from esteemed researchers around Ireland, an industry guided session, and a public patient involvement (PPI) panel discussion. As a committee, we raised €6,000 in sponsorship from 16 industry sponsors. We organised advertising, managed social media steaming, secured sponsorship, and aligned all details of the symposium.

2020-2024, Active member of Lewy Body Ireland

Lewy Body Ireland is Ireland's first and only charity which aims specifically to spread awareness of Lewy Body Dementia, and support those who are affected by the disease. Lewy Body Ireland was founded in 2020 by Karen Meenan and Dr. Kevin Quaid. Since its founding, I have been an active member of the team, leading monthly virtual communication and information sessions (Teatime with Lewy, Lewy Buddies UK), attending conferences (Lewy Body Meetings, Dementia Research Network Ireland), and speaking publicly about ongoing Lewy body research and ways to support and spread knowledge (newsletters with Neurology Academy, radio segments with FM community radio).

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