

Development of a Formoterol-Loaded PLGA Nanocarrier Targeting Neuroinflammation and Neurodegeneration in an LPS- Induced Rat Model of Parkinson's Disease



Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

Thesis submitted for the degree of Doctor of Philosophy

Rebecca Maher

2026

Supervised by Prof. Andrew Harkin and Associate Prof. Eduardo Ruiz-Hernandez

School of Pharmacy and Pharmaceutical Sciences & Trinity College Institute of
Neuroscience, Trinity College Dublin

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and this body of work is entirely my own. The initial optimisation of nanoparticles was performed by Ms. Almudena Moreno-Borralló and optimisation of the hydrogel was performed alongside Mr. Thomson Santosh Alex, Dr. Elisabet Gomez Gonzalez and Ms. Almudena Moreno-Borralló. In chapter 4, TEM imaging was performed by Dr. Raman Bekarevich and Ms. Almudena Moreno-Borralló. In chapter 5, MSc student Ms. Sreelakshmi Salim assisted with drug release studies under my supervision and Mr. Chris Sheridan provided PBMCs. In chapter 6, Assistant Prof. Oli Gobbo and Dr. Christian Kerskens assisted with MRI scans and Prof. Andrew Harkin and Mr. Chris Sheridan assisted with tissue collection. In chapter 7, Ms. Wandí Zhong assisted with behavioural scoring and Mr. Myles Corrigan assisted with surgical procedures. In appendix I, Dr. Matthew McAuslan assisted with surgical procedures and behavioural testing.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish (EU GDPR May 2018).

Signed: *Rebecca Maher*

Date: 30th October 2025

Disclosures

The author acknowledges the support of the Panoz Pharmaceutical Innovation PhD Scholarship, awarded by the School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin. This scholarship was made possible through a generous bequest from the estate of Dr. Don Panoz.

Acknowledgements

First and foremost I would like to thank my supervisor, Prof. Andrew Harkin for trusting me to undertake this research project. Under his guidance and with his encouragement I have learned so much over the past 4 years. The supportive environment that he has cultivated in his lab has made my experience both enjoyable and enriching. I would also like to thank my co-supervisor, Associate Prof. Eduardo Ruiz-Hernandez, for his advice and support throughout this project.

Thank you to past and present members of the Harkin lab that I am so lucky to have experienced this journey with, especially Dr. Caoimhe Tyndall, Dr. Matthew McAuslan, Myles Corrigan, Daniel McLoone and Andrew Breen who have not just been colleagues but great friends. Thank you also to Wandi Zhong, Christopher Sheridan, Patricia Iusan and Devin Seward, who have all helped me get this project over the line one way or another. Thank you especially to Almudena Moreno-Borllo, for your patience, insights and friendship since the day we started together. Thank you also to other past and present members of the ERH lab, Dr. Amelia Ultimo, Dr. Shubhrima Ghosh, Dr. Binh Mai, Dr. Sara Toseli, Dr. Elisabet Gomez Gonzalez, Thomson Santosh Alex, Ayushi Jain, Sukanya Jana and Gaia Lusoli for their practical help, advice or for just being a friendly face in the lab.

I must also extend my gratitude to members of other labs in TCIN who's generosity of their time and resources has cultivated a community of collaboration on the 4th floor. In particular, thank you to Jessica, Katie and Maddie who began and finished their PhD alongside me. Thank you also to Dr. Hugh Delaney from the Cunningham lab for being a mentor and friend to me since my Master's project.

A special thank you to Assistant Prof. Oli Gobbo for training me in some of the *in vivo* techniques required for this project and assisting with MRI. None of this work would have been possible without CMU staff, especially Dr Rustam Rakhmatullin and Monica Delaney, Dr. Christian Kerskens, who facilitated the MRI work and Brian Talbot, Dr. Pierce Kavanagh and Mark Lyons who provided technical support with HPLC and LC-MS.

Thank you to my friends for being so understanding of my work and encouraging me throughout. There's too many to name, but a special mention to Siofra and Kate for keeping me sane. Thank you to my siblings Eleanor and Stephen for constantly cheering me on. Thank you to my partner Michael, for being my rock throughout.

Finally, thank you to my parents, to whom this thesis is dedicated to. Mum, this thesis would not have been possible without your positivity, encouragement and kindness. Dad, although you never got to see this work, your belief in me motivated me every day and I know you would have been so proud.

Abstract

Therapies for neurodegenerative disorders like Parkinson's disease are limited in efficacy and tolerability, largely due to poor drug penetration across the BBB and off-target peripheral actions, leading to poor side effect profiles. Polymeric nanoparticles are advantageous for brain drug delivery due to their safety profiles, drug-loading capacity, controlled-release properties and potential for drug repurposing. The intranasal route is also emerging as an attractive strategy for bypassing the periphery to deliver therapeutics to the brain. The aim of this work was to develop a polymeric nanoparticle, using the FDA-approved poly(lactic co-glycolic acid) (PLGA) polymer, as a vehicle for intranasal delivery and repurposing of experimental anti-inflammatory therapy formoterol in a preclinical rat model of PD-related neuroinflammation and degeneration following *in vitro* validation of biocompatibility, sustained drug release and anti-inflammatory effects in cell culture.

Nanoparticles were prepared using a polyethylene glycol (PEG)-PLGA copolymer by nanoprecipitation. PEG-PLGA nanoparticles were analysed for size and surface charge, and their biocompatibility was evaluated in primary rat glial and neuronal cultures. PEG-PLGA were loaded with experimental anti-inflammatory drug formoterol (Form) and further characterised. *In vitro* drug release from Form-PEG-PLGA and Form-PEG-PLGA embedded in a mucoadhesive and thermosensitive chitosan hydrogel was quantified by high performance liquid chromatography (HPLC) coupled to UV detection. Form-PEG-PLGA (equivalent to 10 μ M of drug) was applied to primary glial cultures stimulated by interferon γ (IFN γ ; 50 ng/ml) or lipopolysaccharide (LPS; 100 ng/ml) and the expression of inflammation associated genes was assessed. The resulting conditioned media (CM) was transferred to primary rat neurons for assessment of neuronal complexity. Unloaded PEG-PLGA, both alone and when incorporated into a hydrogel, were tested for compatibility with human peripheral blood mononuclear cells (PBMCs). A hydrogel containing the contrast agent manganese (Mn²⁺) was administered intranasally to rats, and MRI was performed to validate the nose-to-brain route of drug delivery. Form-PEG-PLGA-gel were administered intranasally to adult Wistar rats daily for 7 or 3 days, prior to an intraperitoneal (i.p) LPS challenge (0.25 mg/kg) and brain expression of pro-inflammatory markers was analysed. Finally, rats were administered Form-PEG-PLGA intranasally prior to stereotactic unilateral injection of LPS into the substantia nigra. Motor function was assessed using the cylinder, stepping, rotarod and amphetamine-induced rotations tests. Post-mortem analysis of microglia and dopaminergic cell bodies in the substantia nigra was then performed.

100 nm unloaded and formoterol loaded PEG-PLGA were synthesised. PEG-PLGA were compatible with primary rat glia and neurons as well as human PBMCs *in vitro*. Form-PEG-PLGA and Form-PEG-PLGA-gel displayed a sustained drug release profile *in vitro* and the resulting profiles were a good fit to both the Korsmeyer-Peppas and Weibull models of release kinetics. Form-PEG-PLGA reduced the expression of inflammation associated genes

Il1b, *Tnfα*, *Nos2* and *Cd40* in stimulated glia compared to control and free formoterol and protected against the loss of neuronal complexity associated with the transfer of CM from IFNγ and LPS treated microglial cultures. Intranasal administration of the Mn²⁺-gel resulted in brain accumulation of the contrast agent *in vivo*. Intranasal delivery of Form-PEG-PLGA-gel attenuated LPS induced expression of *Il1b*, *Tnfα*, *Nos2* and *Cd40* in both the cortex and striatum of the rat brain. Form-PEG-PLGA-gel administered intranasally reduced motor impairment, neuroinflammation and dopaminergic cell loss in the LPS rat model of Parkinson's disease-related neurodegeneration.

Together, these results indicate that drug encapsulation within polymeric nanoparticles, combined with intranasal drug delivery, represents a promising strategy for targeted brain drug delivery. Form-PEG-PLGA exhibited potent anti-inflammatory and neuroprotective effects both *in vitro* and in a preclinical model of inflammation-associated neurodegeneration, highlighting its potential as a therapeutic approach for neurodegenerative disorders such as Parkinson's disease.

Summary

Current therapies for the treatment of neurodegenerative disorders including Parkinson's disease (PD) are lacking in efficacy and are associated with adverse effects. The high failure rate of clinical trials for new CNS therapies continues to limit the translation of novel treatments to the clinic. As an alternative, drug repurposing leveraging approved compounds with polypharmacological actions, is being actively pursued across multiple brain disorders. Treatment with agonists at the β_2 adrenoreceptor (β_2 AR) has been linked to a decreased risk of developing PD. The β_2 AR agonist formoterol, an FDA approved drug for the treatment of asthma and COPD, has anti-inflammatory and neuroprotective effects in the brain in preclinical disease models. However, formoterol acting on peripheral β_2 ARs in the cardiovascular system can cause tachycardia and arrhythmias, limiting its clinical use in elderly populations. . Drug encapsulation in a nanoparticle can protect against off-target effects by controlling distribution, release and cellular uptake of the compound, preventing premature interaction of the drug with non-target tissues or cells. Furthermore, intranasal delivery of drug-loaded nanoparticles further improves brain targeting, reducing the doses required to achieve therapeutic efficacy while reducing unwanted peripheral drug effects.

In vitro models are valuable for screening biomaterials and assessing drug effects on a cellular level prior to undertaking *in vivo* experiments. Primary glial cultures were established from the neonatal rat brain and enriched for microglia. These cultures showed robust responses to pro-inflammatory stimuli interferon γ (IFN γ) and lipopolysaccharide (LPS). The transfer of IFN γ or LPS glial conditioned media (CM) reduced the complexity of neurons, verifying that these cells were responsive to inflammatory stimuli and were a suitable testbed for assessment of nanoparticle biocompatibility.

Polymeric nanoparticles were synthesised using the FDA approved polyethylene glycol (PEG)-poly(lactic co-glycolic acid) (PLGA) block copolymer and a nanoprecipitation technique. Unloaded PEG-PLGA nanoparticles were under 100 nm in diameter with a slight negative charge. Direct exposure to PEG-PLGA nanoparticles did not affect cell viability, induce pro-inflammatory signalling in microglial cultures, or affect neuronal complexity. Moreover, transferring CM from glial cultures to neurons had no effect on neuronal complexity. Together, these findings confirm the biocompatibility of PEG-PLGA nanoparticles with CNS cell types.

PEG-PLGA were loaded with the experimental drug formoterol (Form) and were approximately 100 nm in size with a slight negative charge. Drug release from Form-PEG-PLGA peaked after 4 hours and was steadily maintained over 24 hours. Form-PEG-PLGA were also incorporated into a mucoadhesive, thermosensitive chitosan-based hydrogel for potential intranasal delivery applications. Form-PEG-PLGA drug release was further decelerated by incorporation into the gel system. Dissolution from Form-PEG-PLGA alone

and in hydrogel were fitted to the Korsmeyer-Peppas and Weibull models of drug release kinetics, which describe an initial burst release from nanoparticles followed by a gradual release as the biomaterials break down. This was also observed in cell culture where Form-PEG-PLGA attenuated IFN γ or LPS mediated pro-inflammatory responses more effectively than free drug, likely due to the slow release of drug from particles. Form-PEG-PLGA also reversed the detrimental effects of IFN γ or LPS CM on neuronal integrity, indicating neuroprotective properties. Neither PEG-PLGA nanoparticles nor hydrogel induced pro-inflammatory gene expression in human peripheral blood mononuclear cells (PBMCs).

The intranasal route for CNS drug delivery was validated by loading the chitosan hydrogel with the contrast agent manganese (Mn^{2+}) and administering it intranasally to rats. Magnetic resonance imaging (MRI) confirmed that Mn^{2+} was released from the hydrogel within the nasal cavity and successfully reached the brain. Intranasal administration of Form-PEG-PLGA-gel (0.1 mg/kg of formoterol in $\sim 30 \mu l$) prior to a systemic LPS challenge (0.25 mg/kg; i.p.) attenuated the expression of pro-inflammatory *Il1b*, *Tnfa*, *Nos2* and *Cd40* mRNA in the rat cortex, striatum and spleen. These findings imply that Form-PEG-PLGA treatment can suppress neuroinflammatory signalling in the brain. Since excessive inflammation in the CNS contributes to many brain disorders, the ability to downregulate these markers may reflect neuroprotective or anti-inflammatory potential.

Motor deficits, extensive microgliosis and loss of dopaminergic neurons in the substantia nigra are well characterised in preclinical rodent models of PD. Intranasal administration of Form-PEG-PLGA to rats prior to stereotactic unilateral injection of LPS to the substantia nigra protected against local microgliosis and dopaminergic cell loss, and rescued motor deficits in the cylinder and amphetamine-induced rotations tests. These results indicate that Form-PEG-PLGA exerts anti-inflammatory and neuroprotective effects accompanied by preserved motor function in the unilateral intranigral LPS rat model of PD-associated neuroinflammation and degeneration.

List of figures

Figure 1.1.	Chronic microglial activation in PD drives neurodegeneration.....	5
Figure 1.2.	Transport across to the CNS is tightly regulated by the BBB.....	10
Figure 1.3.	Noradrenaline acting at the β_2 AR suppresses neuroinflammation and promotes neuronal survival.....	15
Figure 1.4.	PLGA nanoparticle design.....	22
Figure 1.5.	Nose-to-brain drug delivery.....	27
Figure 1.6.	Timeline of approvals of intranasal formulations for CNS disease.....	28
Figure 2.1.	Formoterol hemifumarate detection by HPLC-UV.....	44
Figure 2.2.	Drug release experiments.....	46
Figure 2.3.	Sholl analysis of MAP2+ neurons.....	54
Figure 2.4.	Stereotactic injection of LPS to the substantia nigra.....	58
Figure 2.5.	Analysis of IBA1 ⁺ microglia using ImageJ.....	62
Figure 3.1.	IBA1 ⁺ cells in mixed and enriched microglial cultures.....	67
Figure 3.2.	IFN γ promotes a pro-inflammatory response by enriched microglial cultures.....	69
Figure 3.3.	LPS promotes a pro-inflammatory response by enriched microglial cultures.....	71
Figure 3.4.	CM from enriched microglial cultures exposed to IFN γ or LPS reduces neuronal complexity.....	73
Figure 4.1.	PEG-PLGA characterisation.....	80
Figure 4.2.	Biocompatibility of PEG-PLGA in primary cortical glial cultures.....	82
Figure 4.3.	Biocompatibility of PEG-PLGA in primary cortical neuronal cultures.....	84
Figure 4.4.	CM from glia treated with PEG-PLGA does not affect neuronal complexity.....	85
Figure 4.5.	PEG-PLGA nanoparticle formation	87
Figure 5.1.	Experimental design.....	94

Figure 5.2.	Form-PEG-PLGA characterisation.....	95
Figure 5.3.	Sustained drug release from Form-PEG-PLGA over 24 hours <i>in vitro</i>	97
Figure 5.4.	Sustained drug release from Form-PEG-PLGA-gel over 72 hours <i>in vitro</i>	99
Figure 5.5.	Form-PEG-PLGA attenuates IFN γ mediated pro-inflammatory responses <i>in vitro</i>	101
Figure 5.6.	Form-PEG-PLGA rescues CM from IFN γ treated glia mediated reductions in neuronal complexity.....	103
Figure 5.7.	Form-PEG-PLGA attenuates <i>Nos2</i> and <i>Cd40</i> mRNA expression 6 hours post LPS stimulation <i>in vitro</i>	106
Figure 5.8.	Form-PEG-PLGA attenuates LPS mediated pro-inflammatory responses after 24 hours <i>in vitro</i>	108
Figure 5.9.	Form-PEG-PLGA rescues LPS CM mediated changes in neuronal complexity.....	110
Figure 5.10.	Unloaded PEG-PLGA do not attenuate IFN γ mediated pro-inflammatory responses <i>in vitro</i>	113
Figure 5.11.	Form-PEG-PLGA somewhat attenuates LPS mediated pro-inflammatory responses after 6 hours <i>in vitro</i>	114
Figure 5.12.	PEG-PLGA and hydrogel do not affect the viability or increase <i>IL1B</i> , <i>TNFα</i> or <i>IL6</i> mRNA in human PBMCs <i>in vitro</i>	116
Figure 6.1.	Chapter 6 experiment 2 timeline.....	130
Figure 6.2.	Slow release of contrast agent Mn ²⁺ from chitosan-hydrogel <i>in vivo</i>	132
Figure 6.3.	Daily intranasal administration of Form-PEG-PLGA does not affect bodyweight.....	133
Figure 6.4.	Daily intranasal administration of Form-PEG-PLGA does not affect LPS induced increase in body temperature.....	134
Figure 6.5.	Intranasal Form-PEG-PLGA attenuates LPS-induced pro-inflammatory gene expression in the rat cortex.....	136
Figure 6.6.	Intranasal Form-PEG-PLGA attenuates LPS-induced pro-inflammatory gene expression in the rat striatum.....	138
Figure 6.7.	Intranasal Form-PEG-PLGA attenuates LPS-induced pro-inflammatory gene expression in the rat spleen.....	140
Figure 6.8.	Drug placement in the rat nasal cavity affects brain uptake.....	147
Figure 7.1	Experimental timeline	154

Figure 7.2.	Unilateral intranigral LPS induced forelimb asymmetry in the cylinder test is attenuated by intranasal Form-PEG-PLGA	156
Figure 7.3.	Sex differences in forelimb asymmetry in the cylinder test following unilateral intranigral LPS lesion	158
Figure 7.4.	Unilateral intranigral LPS did not induce forelimb akinesia in the stepping test	160
Figure 7.5.	Sex did not affect performance in the stepping test following unilateral intranigral LPS lesion.....	162
Figure 7.6.	Unilateral intranigral LPS did not affect rotarod performance	163
Figure 7.7.	Sex did not affect performance in the rotarod test following unilateral intranigral LPS lesion.....	164
Figure 7.8.	Unilateral intranigral LPS mediated increases in ipsilateral rotations in the D-amphetamine rotations test is attenuated by intranasal Form-PEG-PLGA.....	166
Figure 7.9.	Sex differences in rotational behaviour in the D-amphetamine induced rotations test following unilateral intranigral LPS.....	168
Figure 7.10.	Unilateral intranigral LPS induced increases in microglial cell density is attenuated by intranasal Form-PEG-PLGA	170
Figure 7.11.	Unilateral intranigral LPS induced microgliosis is attenuated by intranasal Form-PEG-PLGA	171
Figure 7.12.	Unilateral intranigral LPS does not increase microglial cell density in the uninjected hemisphere.....	172
Figure 7.13.	Sex differences following unilateral intranigral LPS induced microgliosis	173
Figure 7.14.	Unilateral intranigral LPS mediated TH ⁺ cell loss is rescued by intranasal Form-PEG-PLGA.....	175
Figure 7.15.	Unilateral intranigral LPS mediated TH ⁺ cell loss is rescued by intranasal Form-PEG-PLGA.....	176
Figure 7.16.	Sex did not affect TH ⁺ cell loss following unilateral intranigral LPS.....	177
Figure A1.1	Experimental timeline.....	200
Figure A1.2	Increases in ipsilateral rotations induced by unilateral lesion of the substantia nigra in the D-amphetamine rotations test is not attenuated by intranasal Form-PEG-PLGA.....	202
Figure A1.3	Unilateral intranigral LPS induced increases in microglial cell density is not attenuated by intranasal Form-PEG-PLGA.....	203

Figure A1.4	Unilateral intranigral LPS mediated TH ⁺ cell loss is not rescued by intranasal Form-PEG-PLGA.....	204
-------------	---	-----

List of tables

Table 1.1.	PLGA nanoparticle-mediated drug delivery in preclinical rodent PD model.....	25
Table 1.2.	Biomaterials for intranasal drug delivery in preclinical rodent PD models....	31
Table 2.1.	aCSF ingredients	45
Table 2.2.	Cell culture media ingredients.....	48
Table 2.3.	Human and rat PCR primers.....	52
Table 2.4.	10X PBS recipe for tissue work.....	60
Table 2.5.	0.2M PBS recipe for tissue work.....	61
Table 5.1.	Formoterol-loaded PEG-PLGA for renal targeting.....	119
Table 6.2.	Experiment 2 treatment groups.....	130

List of abbreviations

AAV	adeno-associated viral vector
aCSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
ADHD	attention deficit hyperactivity disorder
AED	anti-epileptic drug
AI	artificial intelligence
AICc	Akaike's information criterion corrected
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
AP	anterior-posterior
AR	adrenoreceptor
<i>Adrb2</i>	β_2 -adrenoreceptor (rodent gene)
ATP	adenosine triphosphate
AUC	area under the curve
Aβ	amyloid β
BBB	blood-brain barrier
<i>Bdnf</i>	brain derived neurotrophic factor (rodent gene)
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
β_2AR	β_2 -adrenoreceptor
cAMP	cyclic adenosine monophosphate
CCL	C-C motif chemokine ligand
<i>Ccl5</i>	C-C motif chemokine ligand 5 (rodent gene)
CD	cluster of differentiation molecule
<i>Cd11b</i>	cluster of differentiation 11b (rodent gene)
<i>Cd40</i>	cluster of differentiation 40 (rodent gene)
<i>Cd68</i>	cluster of differentiation 68 (rodent gene)
cDNA	complimentary deoxyribonucleic acid
CM	conditioned media
CNS	central nervous system
CPP	cell-penetrating peptides
COMT	catechol-O-methyl-transferase
COPD	chronic obstructive pulmonary disorder
COX2	cyclo-oxygenase 2
CR	complement receptor
CREB	cAMP-responsive element-binding protein
<i>Cxcl10</i>	C-X-C motif chemokine ligand 10 (rodent gene)
CXCL10	C-X-C motif chemokine ligand 10
DAB	3,3'-diaminobenzidine
DAMPs	damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DAT	dopamine transporter
DBS	deep brain stimulation

dH₂O	deionised water
DLS	dynamic light scattering
DMEM	dulbecco's modified eagle media
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSP4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
DV	dorsal-ventral
DβH	dopamine β hydroxylase
EDTA	ethylenediaminetetraacetic acid
EE	encapsulation efficiency
ELS	electrophoretic light scattering
EMA	European Medical Agency
EMG	enriched microglial cultures
EtOH	ethanol
FDA	Food and Drug Administration
Form	formoterol
<i>Gapdh</i>	glyceraldehyde 3-phosphate dehydrogenase (rodent gene)
<i>GAPDH</i>	glyceraldehyde 3-phosphate dehydrogenase (human gene)
<i>GBA1</i>	glucosylceramidase β1 (human gene)
GNDF	glial derived nerve growth factor
GFAP	glial fibrillary associated protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GWAS	genome-wide association studies
HDAC6	histone deacetylase inhibitor 6
HPA	hypothalamic-pituitary-adrenal
HPLC	high-performance liquid chromatography
HRP	horse radish peroxidase
HTS	high-throughput screening
IBA1	ionised calcium binding adaptor molecule 1
ICAM	intercellular adhesion proteins
<i>Icam1</i>	intercellular adhesion protein 1 (rodent gene)
IFN	interferon
IκB	inhibitor of nuclear factor κB
<i>Il1b</i>	interleukin 1β (rodent gene)
<i>IL1B</i>	interleukin 1β (human gene)
<i>Il1ra</i>	interleukin 1 receptor antagonist (rodent gene)
<i>Il1r2</i>	interleukin 1 type II receptor (rodent gene)
<i>Il6</i>	interleukin 6 (rodent gene)
<i>Il10</i>	interleukin 10 (rodent gene)
IL-1β	interleukin 1β (cytokine)
IL-1RA	interleukin 1 receptor antagonist
IL-1RII	interleukin 1 type II receptor
<i>IL6</i>	interleukin 6 (human gene)
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal

iPSC	induced pluripotent stem cell
IP-10	interferon-inducible protein 10
i.v.	intravenous
JAK-STAT	Janus kinase-signal transducer and activator of transcription
LAT	large neutral amino acid transporter
LC	loading capacity
LC-MS	liquid chromatography coupled to mass spectrometry
LDH	lactate dehydrogenase
L-DOPA	levodopa
LFE	laminar fluid ejection
LPS	lipopolysaccharide
LRRK	dardarin (human gene)
MAO	monoamine oxidase
MAP2	microtubule associated protein 2
MEMRI	manganese-enhanced magnetic resonance imaging
MeOH	methanol
MHC	major histocompatibility complex
ML	mediolateral
Mn²⁺	manganese
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MxG	mixed glial cultures
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NBM	neurobasal media
NET	noradrenaline transporter
NFκB	nuclear Factor κ-light-chain-enhancer of activated B cells
<i>Ngf</i>	nerve growth factor (rodent gene)
NGF	nerve growth factor
NGS	normal goat serum
NG2	nerve/glial antigen 4
NLR	NOD-like receptors
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain receptor
<i>Nos2</i>	nitric oxide synthase 2 (rodent gene)
NOX2	NADPH oxidase 2
NRI	noradrenaline reuptake inhibitor
OCT	optimal cutting temperature media
O4	oligodendrocyte marker 4
PAG	periaqueductal grey area
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells

PBS	phosphate buffered saline
PCL	poly(ϵ -caprolactone)
PCR	polymerase chain reaction
PD	Parkinson's disease
PdI	polydispersity index
PEG	poly(ethylene glycol)
PFA	paraformaldehyde
PGA	polyglycolic acid
P-gp	P-glycoprotein
<i>PINK1</i>	phosphatase and tensin homolog-induced kinase 1 (human gene)
PLA	polylactic acid
PLGA	poly(lactic-co-glycolic acid)
<i>PRKN</i>	parkin (human gene)
PRR	pathogen recognition receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
s.c.	subcutaneous
<i>Snca</i>	α -synuclein (rodent gene)
<i>SNCA</i>	α -synuclein (human gene)
SEM	standard error of the mean
SLN	solid lipid nanoparticle
<i>Socs3</i>	suppressor of cytokine signaling-3 (rodent gene)
SOCS-3	suppressor of cytokine signaling-3
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TGF	transforming growth factor
TH	tyrosine hydroxylase
THF	tetrahydrofuran
TLR	toll-like receptor
TNFα	tumour necrosis factor alpha (cytokine)
<i>Tnfa</i>	tumour necrosis factor alpha (rodent gene)
<i>TNFA</i>	tumour necrosis factor alpha (human gene)
T1	longitudinal relaxation time
UDP	uridine diphosphate
UGT	UDP-glucuronosyltransferases
UV	ultraviolet
VCAM	vascular adhesion proteins
<i>Vcam1</i>	vascular adhesion protein 1 (rodent gene)
WGA	wheat germ agglutinine
ZP	zeta-potential
βGP	β -glycerophosphate disodium salt
3-OMD	3-O-methyldopa
6-OHDA	6-hydroxydopamine

Table of Contents

Declaration.....	i
Disclosures	ii
Acknowledgements	iii
Abstract	iv
Summary.....	vi
List of figures.....	viii
List of tables.....	xii
List of abbreviations.....	xiii
Chapter 1: Introduction	1
1.1. Parkinson's disease	1
1.1.1. Parkinson's disease aetiology	1
1.1.2. Parkinson's disease pathology	1
1.1.3. The role of neuroinflammation in Parkinson's disease.....	3
1.1.4. Management of Parkinson's disease	6
1.1.5. Preclinical models of Parkinson's disease	6
1.2. Challenges in CNS drug delivery	8
1.2.1. The blood-brain barrier	8
1.2.2. Peripheral drug metabolism	9
1.2.3. Limitations of Parkinson's disease therapies	11
1.3. Drug repurposing for CNS disease.....	11
1.3.1. Advantages of drug repurposing	11
1.3.2. Identifying candidates for repurposing	12
1.3.3. Successful drug repurposing for CNS disease.....	13
1.3.4. Challenges in drug repurposing	13
1.4. Breathing new life into brain therapies: anti-inflammatory effects of β_2 -adrenoceptor agonists in the CNS	14
1.4.1. Noradrenaline as an endogenous regulator of neuroinflammation	14
1.4.2. Pharmacological manipulation of noradrenaline alters inflammatory responses	17
1.4.3. β_2 AR agonists are anti-inflammatory and neuroprotective in preclinical Parkinson's disease models	18

1.4.4. Barriers to clinical use of β_2 AR agonists for treatment of Parkinson's disease	19
1.5. Nanomedicine for targeted brain therapies	19
1.5.1. Overview of nanomedicine	19
1.5.2. Polymeric nanoparticles	20
1.5.3. Surface modifications	21
1.5.4. Biocompatibility and brain distribution of PLGA nanoparticles <i>in vivo</i>	23
1.5.5. PLGA nanoparticles for drug delivery in Parkinson's disease: preclinical evidence	23
1.6. Intranasal drug delivery	26
1.6.1. Nose-to-brain pathways	26
1.6.2. Intranasal therapies used in the clinic for CNS disorders	26
1.6.3. Limitations of intranasal drug administration	28
1.6.4. Biomaterials for intranasal drug delivery	29
1.6.5. Intranasal biomaterial-mediated drug delivery in preclinical Parkinson's models	29
1.7. Thesis aims	33
Chapter 2: Materials & Methods	34
2.1. Materials	34
2.2. Pharmaceutical methods	42
2.2.1. Nanoparticle synthesis	42
2.2.2. Nanoparticle characterisation	42
2.2.3. Encapsulation efficiency and High-Performance Liquid Chromatography (HPLC) coupled to UV detection	43
2.2.4. Hydrogel preparation	43
2.2.5. Drug release	45
2.2.6. Mathematical models of drug release	47
2.3. Cell culture methods	48
2.3.1. Aseptic technique	48
2.3.2. Preparation of cell culture media	48
2.3.3. Preparation of primary cortical rat neuronal cultures	48
2.3.4. Preparation of primary cortical rat glial cultures enriched for microglia	49
2.3.5. Cell culture treatments	49

2.3.6. Peripheral blood mononuclear cell (PBMC) cell treatments	50
2.3.7. Lactate dehydrogenase (LDH) assay	51
2.3.8. Cell lysis, RNA isolation, cDNA synthesis and qRT-PCR.....	51
2.3.9. Immunocytochemistry	53
2.3.10. Imaging of MAP2 ⁺ neurons and Sholl analysis.....	53
2.3.11. Imaging of IBA1 ⁺ microglia and cell counts	54
2.4. <i>In vivo</i> methods.....	54
2.4.1. Animals	54
2.4.2. Power analysis and randomisation	55
2.4.3. Drug administration.....	55
2.4.4. MRI.....	56
2.4.5. Tissue harvesting and preparation for RNA isolation	56
2.4.6. Stereotactic surgery	57
2.4.7. Cylinder test.....	58
2.4.8. Stepping test	59
2.4.9. Rotarod test	59
2.4.10. Amphetamine-induced rotations test	60
2.4.11. Transcardial perfusion.....	60
2.4.12. Tissue processing for immunohistochemistry	61
2.4.13. Immunohistochemistry.....	61
2.4.14. Imaging and analysis of IBA1 ⁺ microglia and TH ⁺ dopaminergic neurons	62
2.5. Data analysis	63
Chapter 3: Establishing rat primary glial cultures that respond to inflammatory stimuli ...	64
3.1. Introduction	64
3.2. Aims	66
3.3. Experimental design	66
3.4. Results	67
3.4.1. Enriched microglial cultures	67
3.4.2. IFN γ stimulation of enriched microglial cultures increased mRNA levels of pro-inflammatory markers after 24 hours.....	68
3.4.3. LPS stimulation of enriched microglial cultures increased mRNA levels of pro-inflammatory markers after 6 hours.....	70

3.4.4. Transfer of conditioned media from IFN γ and LPS-treated enriched microglia for 24 hours reduces neuronal complexity	72
3.5. Discussion.....	74
Chapter 4: Biomaterial preparation and biocompatibility <i>in vitro</i>	77
4.1. Introduction	77
4.2. Aims.....	79
4.3. Experimental design.....	79
4.4. Results	80
4.4.1. Characterisation of PEG-PLGA.....	80
4.4.2. PEG-PLGA are biocompatible with primary rat enriched microglial cultures	81
4.4.3. PEG-PLGA are biocompatible with primary rat neuronal cultures	83
4.4.4. Conditioned media from glia exposed to PEG-PLGA transferred to primary rat neuronal cultures does not affect neuronal complexity.....	83
4.5. Discussion.....	86
4.5.1. Considerations for PLGA nanoparticle synthesis	86
4.5.2. Biocompatibility of PEG-PLGA nanoparticles in neuronal and glial cultures	88
4.5.3. Future directions	89
Chapter 5: PEG-PLGA drug loading, release and <i>in vitro</i> anti-inflammatory effects	90
5.1. Introduction	90
5.2. Aims.....	93
5.3. Experimental design.....	93
5.4. Results	95
5.4.1. Characterisation of Form-PEG-PLGA	95
5.4.2. Formoterol release from Form-PEG-PLGA is sustained over 24 hours	96
5.4.3. Formoterol release from Form-PEG-PLGA-gel is sustained over 72 hours	98
5.4.4. Form-PEG-PLGA has anti-inflammatory effects in IFN γ stimulated enriched microglia.....	100
5.4.5. Form-PEG-PLGA CM protects against IFN γ CM-mediated reductions in neuronal complexity.....	102
5.4.6. Form-PEG-PLGA has anti-inflammatory effects in LPS stimulated enriched microglia after 6 hours	105
5.4.7. Form-PEG-PLGA has anti-inflammatory effects in LPS stimulated enriched microglia after 24 hours	107

5.4.8. Form-PEG-PLGA CM protects against LPS CM (24 hour)-mediated reductions in neuronal complexity	109
5.4.9. Unloaded PEG-PLGA are not anti-inflammatory in IFN γ stimulated enriched microglial cultures.....	112
5.4.10. Unloaded PEG-PLGA are not anti-inflammatory in LPS stimulated enriched microglial cultures.....	112
5.4.11. Biomaterials do not induce pro-inflammatory gene expression in human PBMCs.....	115
5.5. Discussion	117
5.5.1. PEG-PLGA drug loading	117
5.5.2. Controlled formoterol release from Form-PEG-PLGA and Form-PEG-PLGA-gel	119
5.5.3. Anti-inflammatory and neuroprotective effects of formoterol released from Form-PEG-PLGA in an <i>in vitro</i> model of neuroinflammation.....	123
5.5.4. Compatibility of unloaded PEG-PLGA and gel with human PBMCs.....	125
5.5.5. Future directions.....	125
Chapter 6: Anti-inflammatory effects of intranasal Form-PEG-PLGA in the rat brain following systemic LPS challenge.....	126
6.1. Introduction	126
6.2. Aims	129
6.3. Experimental design	129
6.4. Results	131
6.4.1. Hydrogel displays slow-release properties <i>in vivo</i>	131
6.4.2. Daily intranasal administration of Form-PEG-PLGA does not affect bodyweight	131
6.4.3. Daily intranasal administration of Form-PEG-PLGA does not affect the LPS-mediated increase in body temperature	131
6.4.4. Daily intranasal administration of Form-PEG-PLGA attenuates pro-inflammatory gene expression in the rat cortex following systemic LPS challenge ..	135
6.4.5. Daily intranasal administration of Form-PEG-PLGA attenuates pro-inflammatory gene expression in the rat striatum following systemic LPS challenge	137
6.4.6. Daily intranasal administration of Form-PEG-PLGA attenuates pro-inflammatory gene expression in the rat spleen following systemic LPS challenge..	139
6.5. Discussion	141

6.5.1. Mn ²⁺ reaches the brain via intranasal hydrogel delivery	141
6.5.2. Intranasal Form-PEG-PLGA does not affect bodyweight or temperature	142
6.5.3. Form-PEG-PLGA attenuates LPS-mediated neuroinflammatory responses	143
6.5.4. Anti-inflammatory effects of Form-PEG-PLGA in the spleen	144
6.5.5. CNS bioavailability following intranasal PLGA-mediated drug delivery.....	145
6.5.6. Considerations for intranasal delivery to rats	146
6.5.7. Future directions	148
Chapter 7: Anti-inflammatory and neuroprotective effects of intranasal Form-PEG-PLGA in the LPS inflammatory rat model of Parkinson's disease	149
7.1. Introduction	149
7.2. Aims.....	153
7.3. Experimental design.....	153
7.4. Results	155
7.4.1. Form-PEG-PLGA attenuates LPS-mediated forelimb asymmetry in the cylinder test	155
7.4.2. LPS did not induce forelimb akinesia in the stepping test.....	159
7.4.3. LPS did not affect latency to fall in the rotarod test	163
7.4.4. Form-PEG-PLGA attenuates LPS-mediated ipsilateral turning behaviour in the D-amphetamine induced rotations test.....	165
7.4.5. LPS induced microgliosis in the substantia nigra is attenuated by Form-PEG-PLGA	169
7.4.6. LPS mediated TH ⁺ cell loss is rescued by Form-PEG-PLGA.....	174
7.5. Discussion.....	178
7.5.1. Motor dysfunction in the intranigral LPS model	178
7.5.2. Sex differences in PD and in preclinical models.....	180
7.5.3. Form-PEG-PLGA attenuated dopaminergic cell loss in the substantia nigra ...	182
7.5.4. Pre-dosing with Form-PEG-PLGA is necessary to confer immunomodulatory and neuroprotective effects against intranigral LPS	184
7.5.5. Future directions	185
Chapter 8: Concluding remarks.....	186
8.1. Summary of main findings	186
8.2. Considerations for PLGA nanoparticle design.....	187
8.3. Intranasal Form-PEG-PLGA dosing schedules	190

8.4. Clinical relevance of these findings	190
8.5. Translating rodent studies to the human condition	191
8.6. Scale-up of biomaterial formulations	194
8.7. Future directions.....	195
8.7.1. Efficiency of intranasal PLGA-mediated CNS transport	195
8.7.2. A role for astrocytes in β_2 AR-mediated neuroprotection.....	196
8.7.3. Formoterol and protection of mitochondrial function.....	196
8.7.4. Efficacy of Form-PEG-PLGA against subsequent immune challenges.....	197
8.7.5. Progressing to human iPSC models	197
Appendix I: Intranasal administration of Form-PEG-PLGA post-lesion does not rescue pathology or motor deficits in the intranigral LPS rat model of PD	199
A1.1. Aims and objectives.....	199
A1.2. Experimental design.....	199
A1.3. Results	201
A1.4. Brief conclusion	205
Appendix II: Dissemination	206
List of publications	206
Dissemination plan	206
Poster presentations	206
Oral presentations	207
Public and Patient Involvement (PPI)/outreach.....	207
Bibliography.....	208

Chapter 1: Introduction

*Sections of this chapter are adapted from Maher, R., Moreno-Borralló, A., Jindal, D., Mai, B. T., Ruiz-Hernandez, E., & Harkin, A. (2023). Intranasal Polymeric and Lipid-Based Nanocarriers for CNS Drug Delivery. *Pharmaceutics*, 15(3), 746.*

1.1. Parkinson's disease

1.1.1. Parkinson's disease aetiology

Parkinson's disease (PD) is a chronic and progressive age-related neurodegenerative disease affecting motor function typically in individuals of over 60 years. Recent global estimates indicate that over 10 million individuals are currently living with PD, which is projected to increase by over 1.5-fold by the year 2035 (Dorsey et al., 2018; Luo et al., 2025b). Patients present with motor symptoms such as bradykinesia, resting tremor, rigidity and postural instability but non-motor symptoms including sleep disturbances, depression and cognitive impairment are also common (Bloem et al., 2021; Meissner, 2012). Diagnosis is made primarily through a neurological exam and physical assessment. Imaging is largely conducted to rule out other conditions, but a dopamine transporter (DAT) scan may be used to support a PD diagnosis in complex cases (Marsili et al., 2018; Suwijn et al., 2015).

A higher incidence of PD is reported in Europe, North America and South America than African, Asian and Arabic countries and there is a higher disease rate in males than females (Kalia & Lang, 2015). There are genetic and environmental risk factors for PD, however, most PD cases are idiopathic with complex multifaceted pathogenesis. Familial PD has been linked to over 20 genes, including autosomal dominant genes *SNCA* and *LRRK2*, and autosomal recessive genes *PRKN*, *PINK1* and *GBA*, with a further 90 genes implicated in an increased risk of PD (Bloem et al., 2021). Exposure to specific environmental toxins have also been linked to PD cases. These include pesticides like rotenone and paraquat, and chemicals trichloroethylene and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Bloem et al., 2021; Konnova & Swanberg, 2018). Prior head injury, agricultural occupation, the use of β -blockers and rural living have all been identified as risk factors for PD, while smoking, coffee consumption and use of medications like non-steroidal anti-inflammatory drugs and calcium channel blockers are associated with a decreased risk of PD (Iranzo et al., 2013).

1.1.2. Parkinson's disease pathology

PD is characterised by the degeneration of dopaminergic neurons in the substantia nigra, resulting in reduced dopamine input to the striatum and disruption of the basal ganglia

circuits that are responsible for producing co-ordinated movements. It is estimated that over 60% of neurons in the substantia nigra are degenerated at the time of symptom manifestation (Greffard et al., 2006; Ma et al., 1997) and dopaminergic cell loss is thought to begin 5-10 years prior to the onset of motor features (Greffard et al., 2006; Meissner, 2012). However, there is also evidence that upon PD diagnosis, striatal axon terminal loss is greater than cell body degeneration in the substantia nigra. This pattern is reported to continue throughout the disease and corresponds to a worsening of motor features, suggesting early degeneration of distal axons that progresses in a retrograde manner (Tagliaferro & Burke, 2016).

Abnormal intracellular plaques called Lewy bodies are found widespread throughout the brains of PD patients. A major component of Lewy bodies is the misfolded α -synuclein protein, which is responsible for controlling neurotransmitter release at synapses in the healthy brain (Calabresi et al., 2023). The mechanisms that underly abnormal α -synuclein production and how this specifically leads to midbrain dopaminergic cell loss have not been fully elucidated. However, dopaminergic neurons in the substantia nigra are thought to be vulnerable to PD pathology. These neurons have extensive branching with many sites for neurotransmitter release and therefore, have a high energy demand and baseline mitochondrial oxidative stress (Pacelli et al., 2015). Dopaminergic neurons in the substantia nigra also have distinctive action potential firing patterns, that are much slower than other populations of dopaminergic neurons in the brain (Foehring et al., 2009). This slow rhythmic activity means that calcium channels remain open for longer. Increases in intracellular calcium levels can interfere with mitochondrial homeostasis, exacerbate the production of reactive oxygen species, disrupt lysosomal functioning and increase α -synuclein aggregation (Surmeier, 2018; Viridi et al., 2022). Mitochondrial damage also reduces the autophagic capacity of these cells, allowing damaged proteins and dysfunctional organelles to accumulate inside cells (Surmeier, 2018). Furthermore, as α -synuclein is primarily located in synapses where these phenomena are occurring, collectively this environment may render dopaminergic neurons in the substantia nigra particularly susceptible to Lewy pathology (Plotegher et al., 2014; Sun et al., 2019).

Braak staging is used to describe the spread of Lewy pathology in the brain. It is proposed that the earliest pathological changes occur in stage I in the peripheral nervous system, olfactory system and medulla and in stage II in the pons and grey matter of the spinal cord, which may correspond to prodromal symptoms of hyposmia and constipation as well non-motor symptoms like sleep disturbances and anxiety. By stage III and IV, Lewy pathology is present in the midbrain, basal forebrain, limbic system, thalamus and temporal cortex, and patients begin to present with the cardinal motor symptoms of PD (Braak et al., 2003; Kalia & Lang, 2015). Loss of dopaminergic neurons in the mesolimbic and mesocortical pathways can also occur in PD, which may also contribute to mood and cognitive symptoms, respectively (Kalia & Lang, 2015).

1.1.3. The role of neuroinflammation in Parkinson's disease

Neuroinflammation is also a key feature of PD, predominantly involving microglia cells that are analogous to macrophages in the periphery and are innate mediators of neuroinflammatory responses to pathogens and local damage. Historically, microglia were considered as either resting or activated, but modern advances in single cell sequencing have enabled the identification of multiple transcriptional signatures of microglia that vary depending on age, sex, genetics and disease. On one end of this spectrum of microglial activation states is the classically described quiescent or 'resting' phenotype where microglia adopt a highly arborised morphology characterised by extensive mobile ramifications (Hanisch & Kettenmann, 2007). The main function of these 'resting' microglia is to survey the local microenvironment, detecting signals from surrounding cells that might relate to disruptions to homeostasis (Gao et al., 2023). Factors such as brain derived neurotrophic factor (BDNF) released by microglia and astrocytes in the healthy brain promote neurotransmission and neuroplasticity, highlighting that microglia are not just responsible for clearing pathogens or debris, but also play a crucial supporting role in neuronal functioning (Dougherty et al., 2000; Lynch, 2009; Wilton et al., 2019).

Microglia recognise pathogen-associated molecular patterns (PAMPs) from invading viruses, bacteria or fungi and damage-associated molecular patterns (DAMPs) released by injured or apoptotic cells in the vicinity via pathogen recognition receptors (PRRs), including toll-like receptors (TLRs), C-type lectin receptors and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Upon activation of these PRRs, microglia undergo a phenotypic shift comprised of diverse physical and molecular changes, depending on the stimuli encountered. Reactive microglia retract their branches and adopt an amoeboid morphology to facilitate phagocytosis of foreign materials or debris. Increased expression of PRRs is accompanied by an upregulation in surface proteins involved in antigen presentation and phagocytosis such as major histocompatibility complex (MHC) proteins, complement receptor (CR1, 2 and 4) and cluster of differentiation molecules (CDs) including CD11b, CD68 and CD40 (Cho et al., 2006; Lynch, 2009; McGeer et al., 1988; Ponomarev et al., 2005).

Misfolded proteins in neurodegenerative disease activate TLR signalling. In the PD brain, misfolded α -synuclein activates TLR1/2 and TLR4. This has been mimicked *in vitro* and *in vivo* using lipopolysaccharide (LPS), a component of Gram-negative bacteria known as an endotoxin, which also activates TLR4. TLR4 recognition of LPS or α -synuclein leads to the activation of nuclear Factor κ -light-chain-enhancer of activated B cells (NF κ B), an inducible transcription factor that regulates the expression of inflammatory response genes (Gorecki et al., 2021). When activated, NF κ B translocates to the nucleus and induces the expression of tumour necrosis factor α (TNF α), interleukin 1 β (IL-1 β), IL-6, inducible nitric oxide synthase (iNOS) and ROS (Lull & Block, 2010; Luo et al., 2025a). TLR4 activation can also lead to the downstream induction of type I interferon α (IFN α) and IFN β and chemokines like C-C motif chemokine ligand 5 (CCL5). The release of type I IFNs promotes the

production of chemoattractant molecules like CCL2 and C-X-C motif chemokine ligand 10 (CXCL10), leading to immune cell infiltration and proliferation (Gao et al., 2023). In the healthy brain, microglia reassume a quiescent state once the pathogen or other stimulus is cleared, monitoring the environment for the next trigger. However, persistent microglial activation, proliferation and infiltration to affected brain regions occurs in the diseased brain, which is termed microgliosis.

The presence of chronic neuroinflammation in the PD brain was first established in 1988 when activated microglia were identified in the substantia nigra at post-mortem analysis (McGeer et al., 1988). In the years since, pro-inflammatory $\text{TNF}\alpha$, $\text{IL-1}\beta$, $\text{IFN}\gamma$, IL-6 , IL-2 , iNOS and cyclo-oxygenase 2 (COX2) were found to be elevated in the substantia nigra, striatum and cerebrospinal fluid of PD patients (Chen et al., 2018; Hirsch & Hunot, 2009; Qin et al., 2016; Tansey et al., 2022) and microglia numbers are higher and are more amoeboid in the substantia nigra of PD patients compared to age-matched controls (Smajić et al., 2022). Furthermore, gene variants that are associated with increased risk of PD like *LRRK2*, *SNCA*, *PRKN* and *PINK1* are known to indirectly modulate immune functions (Tansey et al., 2022).

Microglial activation is a common feature of several neurotoxin-based animal models of PD, and in some cases, it precedes neurodegeneration (see section 1.1.5.) (Akiyama & McGeer, 1989; Castaño et al., 1998; Depino et al., 2003). Experimental models have also shown that chronic neuroinflammation in PD and other degenerative conditions drives neuronal cell death and may exacerbate disease progression (**Fig. 1.1**). Cytokine knockdown, microglial depletion or inhibition of microglial activation attenuates dopaminergic cell loss and motor symptoms in these models (Bianchetti et al., 2024; Gordon et al., 2018; Li et al., 2025a; Mount et al., 2007; Wang et al., 2024; Zhang et al., 2025). Furthermore, microglial activation positively correlates with motor symptom severity and negatively correlates with DAT detection in brain imaging (Ouchi et al., 2005).

Long-term use of several classes of anti-inflammatory therapies have been associated with a reduced risk of developing PD. These include anti-TNF treatment for inflammatory bowel disease (Peter et al., 2018), non-steroidal anti-inflammatory drugs like ibuprofen (Chen et al., 2005) and $\beta_2\text{AR}$ agonists like salbutamol for asthma and chronic obstructive pulmonary disease (COPD) (Mittal et al., 2017). Based on the extensive evidence that chronic inflammatory signalling in the substantia nigra drives dopaminergic cell death and that manipulation of immune responses in the PD brain can reduce this pathology, neuroinflammation should be considered as a druggable target for the treatment of PD.

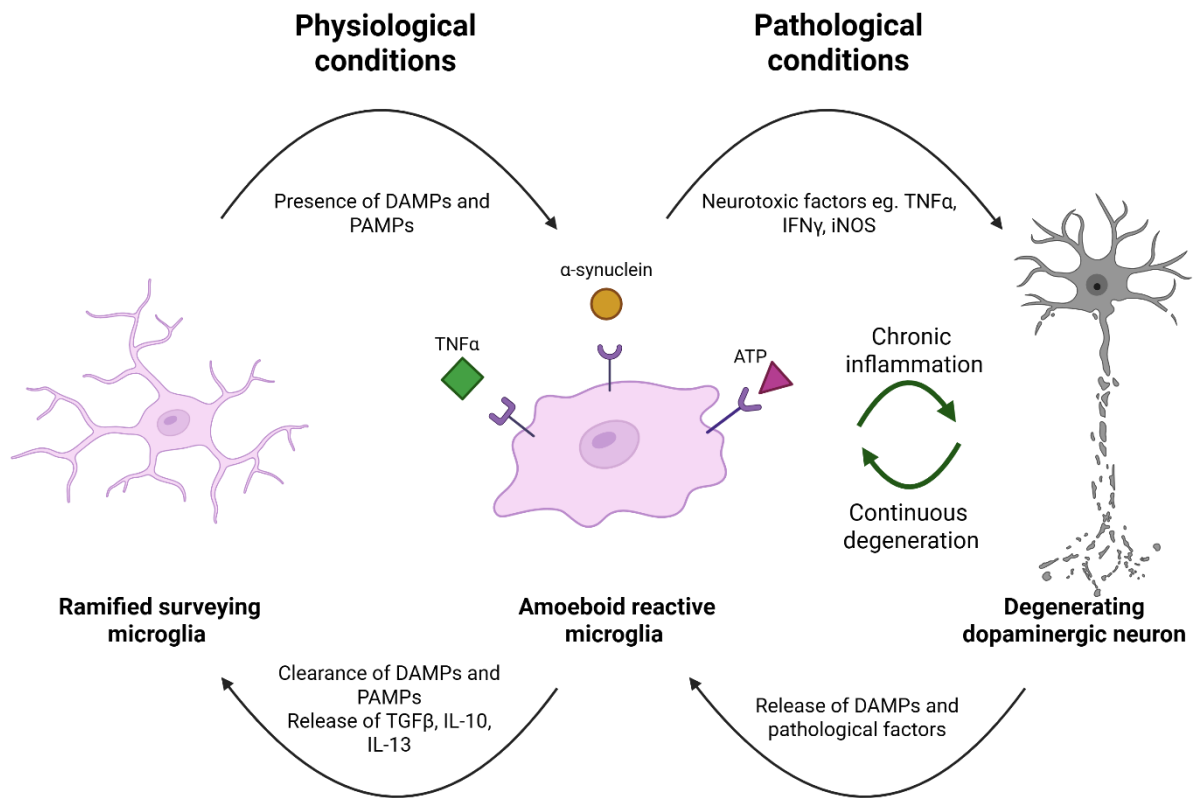


Figure 1.1. Chronic microglial activation in Parkinson's disease drives neurodegeneration.

Under physiological conditions, microglial PRRs are activated by the presence of DAMPs and PAMPs. This causes a change from a ramified to an amoeboid morphology. Activated microglia release a host of pro-inflammatory cytokines including $\text{IL-1}\beta$, $\text{TNF}\alpha$, IL-6 and $\text{IFN}\gamma$ to attack invading species and protect the brain. Once the pathogen has been cleared, microglia release anti-inflammatory factors like transforming growth factor β ($\text{TGF}\beta$), IL-10 and IL-13 to suppress pro-inflammatory signalling and return to homeostasis. In neurodegenerative conditions like PD, however, microglia become chronically activated by the release of DAMPs like ATP from degenerating neurons and misfolded α -synuclein proteins as well as pro-inflammatory cytokines secreted by proximal glial cells. Pro-inflammatory factors $\text{TNF}\alpha$, $\text{IFN}\gamma$ and iNOS are neurotoxic and can accelerate dopaminergic cell death, which in turn further exacerbates microglial reactivity, creating a self-perpetuating cycle of neurotoxicity.

1.1.4. Management of Parkinson's disease

There are no curative or disease-modifying therapies available for PD; current treatments provide symptomatic relief only and are ineffective in the late stages of disease.

Conventional therapies involve targeting the dopaminergic system to replenish nigrostriatal dopamine content. These include dopamine replacements like levodopa (L-DOPA), dopamine agonists like rotigotine and ropinirole, and small molecule inhibitors of enzymes that metabolise dopamine like monoamine oxidase (MAO) A and B and catechol-O-methyl-transferase (COMT) (Gouda et al., 2022; Zahoor et al., 2018). Anticholinergic agents can also be used to restore the balance between cholinergic and dopaminergic signalling in the midbrain and alleviate symptoms (Barrett et al., 2021). However, as the disease progresses and dopaminergic cell loss continues, these strategies become futile.

Invasive therapies also exist like deep brain stimulation (DBS) which involves surgical placement of a device that supplies the basal ganglia with high frequency stimulation (Dallapiazza et al., 2018). While effective in relieving motor symptoms, issues around finetuning of the settings, battery replacement, device migration and neuropsychiatric problems have been reported (Bloem et al., 2021; Gouda et al., 2022). Unilateral lesions are also used to reduce symptoms of tremor in some cases, but these are invasive, irreversible treatment options and are ineffective against bradykinesia and rigidity (Bloem et al., 2021).

In the last decade, no novel medicines have been approved for the treatment of PD. New approvals consist of modified formulations for slow release or alternative routes of administration for existing drugs. Novel treatments are urgently needed that not only alleviate symptoms but can alter the course of disease progression.

1.1.5. Preclinical models of Parkinson's disease

Several preclinical rodent models have been developed using environmental and synthetic neurotoxins that feature degeneration of neurons in the substantia nigra for the testing of anti-Parkinson's therapies. The 6-hydroxydopamine (6-OHDA) model of PD is the most widely used and over the past decades has contributed majorly to our current understanding of dopamine in the healthy and diseased brain (Ungerstedt, 1968). 6-OHDA is a selective catecholaminergic neurotoxin that is typically injected unilaterally into the substantia nigra dopaminergic neurons, microglial reactivity and contralateral motor deficits, and is commonly used to screen drugs targeting dopamine pathways (Hernandez-Baltazar et al., 2017; Konnova & Swanberg, 2018; Marinova-Mutafchieva et al., 2009; Simola et al., 2007). Variations of this model targeting the medial forebrain bundle and the striatum also exist (Dovonou et al., 2023). MPTP and rotenone neurotoxins are also frequently used to induce PD related cell death in mice and rats, respectively (Betarbet et al., 2000; Konnova & Swanberg, 2018; Kopin & Markey, 1988; Smeyne & Jackson-Lewis,

2005). These toxins are injected systemically as they cross the BBB to produce targeted dopaminergic cell death, oxidative stress and neuroinflammation bilaterally (Dovonou et al., 2023). Dosing schedules can vary but subacute to chronic dosing over long periods of time are thought to replicate the human disease trajectory more accurately than intracerebral models (Goldberg et al., 2011). A lack of motor deficits, MPTP resistance in rats and some mouse strains, bilateral pathology and high instances of rotenone-induced mortality are limitations to their laboratory use (Cicchetti et al., 2009; Meredith & Rademacher, 2011)

Several genetic mouse models express mutations in the *SNCA*, *LRRK2*, *PRKN*, *PINK1*, and *GBA1* genes, all of which have been linked with familial forms of PD (Dovonou et al., 2023; Konnova & Swanberg, 2018). However, genetic cases are rare, and these models can produce varying results depending on the promoter used. A number of groups report incomplete degeneration often localised to non-midbrain regions and mild motor deficits in some of these genetically modified mice (Dawson et al., 2010; Dovonou et al., 2023; Peng et al., 2025). Direct injection of pre-formed α -synuclein fibrils to the substantia nigra or striatum has been shown to replicate the spread of misfolded α -synuclein that occurs in PD, but the pathology and parkinsonian-like symptoms can take months to over a year to develop (Luk et al., 2012; Woerman & Luk, 2024). Furthermore, variability between fibril batches is common, with several studies describing partial phenotypes accompanied by modest motor symptoms. Intranigral or striatal delivery of lentiviral or adeno-associated viral vectors that enter local brain cells and interfere with cellular mechanisms to drive α -synuclein overexpression are emerging as powerful tools in understanding PD pathology and for drug screening (Van der Perren et al., 2015). Although there are concerns of reproducibility and vector effects on microgliosis (Huntington & Srinivasan, 2021; Konnova & Swanberg, 2018), the technology involved is rapidly evolving to mitigate these issues.

The PD brain has extensive microgliosis and astrogliosis which contribute to disease pathology, but this feature is often lacking in preclinical disease models. Intranigral injection of the endotoxin LPS was first described by Castaño et al. (1998) as a preclinical model of PD displaying rapid local microglial activation and subsequent reductions in dopamine synthesis as well as loss of dopaminergic cells in the substantia nigra and striatum. The intranigral LPS model also induces motor impairments across various paradigms within weeks of injection (Hoban et al., 2013; Iravani et al., 2005). Furthermore, LPS is reported to trigger α -synuclein overexpression, a feature that is absent in 6-OHDA and MPTP models (Lomeli-Lepe et al., 2024). In the years since it was first characterised, the intranigral LPS model has been used by our laboratory and dozens of others to investigate a broad spectrum of pharmacotherapies for PD ranging from immune modulators targeting the inflammasome, noradrenaline signalling and microglial proliferation to natural products like curcumin and ellagic acid which are known to have anti-inflammatory and anti-oxidant properties (Castano et al., 2002; Khanal et al., 2025; Liu et al., 2023; O'Neill et al., 2020; Sharma et al., 2017; Wang et al., 2017b; Yssel et al., 2018).

The intra-striatal LPS model is also used for preclinical drug screening, however a direct comparison between both models found that intra-striatal injection resulted in transient neuroinflammation, incomplete lesion of the nigrostriatal tract and mild motor deficits (Hoban et al., 2013) and thus is much less frequently utilised. A single systemic injection of a high dose of LPS has also been linked to dopaminergic cell loss in the substantia nigra (Qin et al., 2007). In recent years, the intranasal route has been utilised to deliver LPS to the substantia nigra and striatum of rodents. A chronic dosing regime is required but this route can result in a progressive loss of up to 50% of dopaminergic neurons in the substantia nigra, depletion of striatal dopamine content and the formation of α -synuclein aggregates (He et al., 2013).

1.2. Challenges in CNS drug delivery

1.2.1. The blood-brain barrier

Drug targeting to the brain is a challenge, largely due to the presence of the blood–brain barrier (BBB). This is a highly selective, semipermeable structure comprising many types of brain cells, predominantly vascular endothelial cells, astrocyte end-feet, pericytes and microglia, which act as gatekeepers to the central nervous system (CNS) by controlling the entry of endogenous and exogenous substances (**Fig. 1.2**) (Correale & Villa, 2009). While this structure plays a critical role in regulating CNS functions such as ionic balance, nutrient transport and protection against circulating pathogens, it also hinders the development of therapies targeting brain diseases as many large or hydrophilic drug molecules are prohibited from crossing (Harilal et al., 2020).

Mechanisms for transiently disrupting the BBB have been proposed, including the use of microbubbles and low-frequency ultrasound waves to temporarily allow larger drug molecules entry (Aryal et al., 2014; Kinoshita et al., 2006). However, these processes can render the brain vulnerable to invasion by blood borne toxins or pathogens. Direct drug administration to the brain is highly invasive, can incur serious complications, and is ill-suited for the treatment of long-term diseases that require frequent doses. Due to a growing burden of CNS disorders, there is a demand for novel strategies for the delivery of therapeutics to the brain.

There is evidence of BBB dysfunction in many CNS disorders including PD (Lau et al., 2024). Imaging and post-mortem studies have reported BBB leakage in the substantia nigra and striatum of PD brains (Al-Bachari et al., 2020; Gray & Woulfe, 2015). Several factors including loss of glial cell homeostatic functioning, reduced expression of tight junction proteins like claudin and occludin, downregulation of efflux pumps and physical damage to the cerebrovasculature by α -synuclein pathology or Lewy bodies contribute to a compromised or ‘leaky’ BBB (Lau et al., 2024). Neuroinflammatory processes are associated with increased expression of vascular cell- and intercellular adhesion proteins

(VCAM; ICAM) which promote the infiltration of peripheral immune cells and may exacerbate pathogenesis (Miklossy et al., 2006; Pober & Sessa, 2007).

While a leaky BBB in disease may paradoxically enhance local drug diffusion into the brain, it is not region specific. Alternatively, reduced endothelial cell expression of transporters and receptors required for transport presents as a further challenge to CNS drug targeting in neurodegenerative diseases and may contribute to neuronal vulnerability in affected brain regions as cells are starved of essential nutrients (Ohtsuki et al., 2010).

Several large molecule immunotherapies have failed to show significant efficacy in clinical trials despite promising preclinical evidence of effective clearance of pathological α -synuclein. These include prasinezumab (Roche) and cinpanemab (Biogen). It is likely that the inability of these bulky antibodies to cross the BBB contributed to the lack of clinical benefit observed.

1.2.2. Peripheral drug metabolism

CNS pharmacotherapies are predominantly small molecule drugs, most of which are delivered as oral formulations (Gross, 2015). Broadly speaking, approximately 90% of pharmaceuticals on the global market are oral products (Alqahtani et al., 2021), which are advantageous for patient compliance and reduced healthcare burdens as patients can self-administer. This is important, particularly in the context of diseases like PD, where frequent doses are required over a period of many years. However, these formulations are subject to peripheral metabolism before entering the blood stream and reaching the brain.

Enzymatic conversion and first-pass metabolism in the periphery reduces the amount of active drug that can cross the BBB. For example, in the kidneys, liver and vascular tissue DOPA decarboxylase converts L-DOPA to dopamine which cannot cross the BBB. Hepatic cytochrome P450 enzymes are responsible for the oxidation of drugs like dopamine agonists bromocriptine and ropinirole, increasing drug polarity and hydrophilicity and reducing the likelihood of passive diffusion into the brain (Sevrioukova & Poulos, 2012). Phase II metabolism predominantly involves drug conjugation by glucuronidation, sulfation or acetylation. These processes can increase the polarity of drug molecules and reduce BBB penetration. For example, dopamine agonist apomorphine undergoes hepatic glucuronidation and sulfation, increasing polarity and preventing BBB crossing (El-Bachá et al., 2000). Therefore, apomorphine is typically administered via the parenteral route as a subcutaneous infusion or injection. 3-O-methyldopa (3-OMD), a product of catechol-O-methyltransferase methylation of L-DOPA, is more polar than L-DOPA and cannot passively cross the BBB efficiently. Instead, 3-OMD competes with L-DOPA for binding at the large neutral amino acid transporter 1 (LAT1) expressed by BBB cells, reducing L-DOPA uptake in the CNS. Furthermore, 3-OMD that reaches the brain acts as a partial inhibitor of the DAT, which can reduce the effectiveness of L-DOPA treatment in the midbrain (Lee et al., 2008).

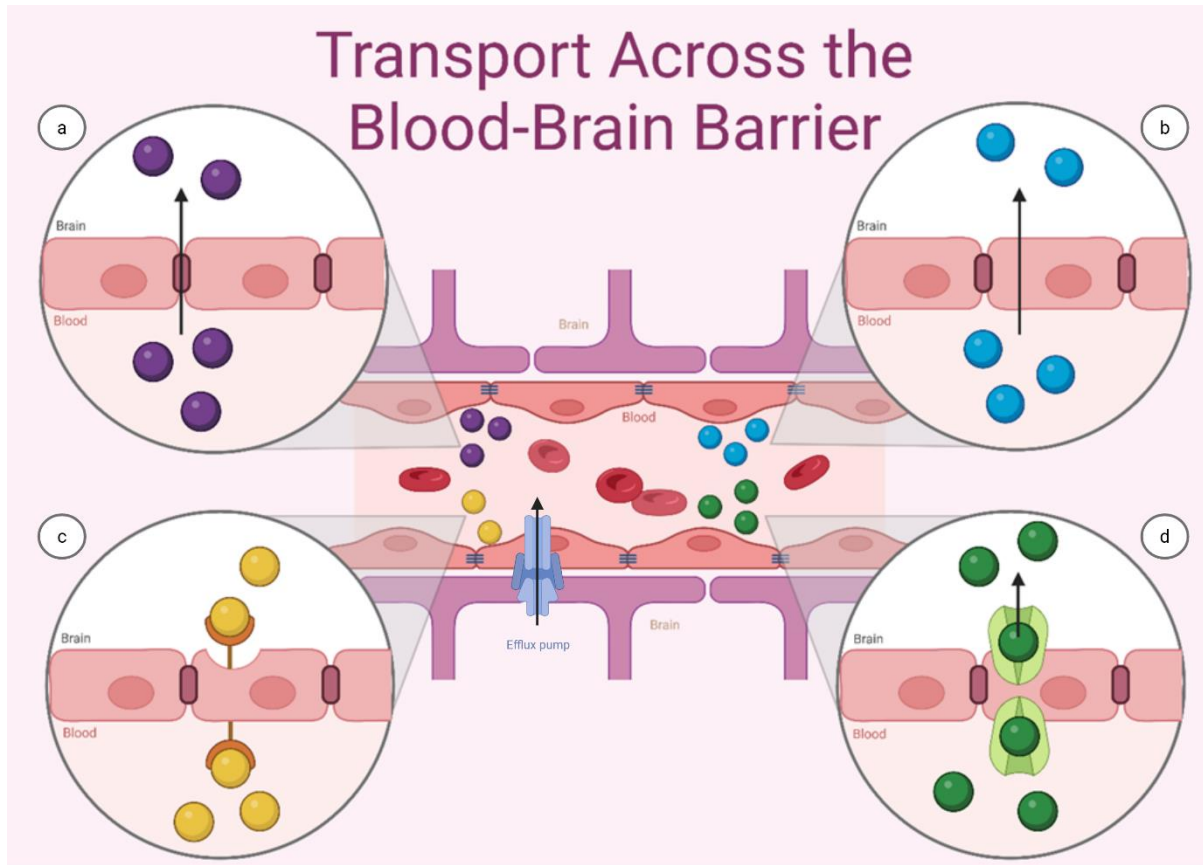


Figure 1.2. Transport across to the CNS is tightly regulated by the BBB.

Transport across the BBB primarily occurs by (a) paracellular transport where small molecules pass between the tight junctions of BBB endothelial cells, (b) passive diffusion of lipophilic molecules across membranes of BBB cells, (c) receptor-mediated transcytosis or (d) carrier-mediated transport. Once in the brain, drugs may be subject to efflux via pumps such as P-glycoprotein located on BBB endothelial cells. Adapted from Maher et al. (2023).

While *in vitro* evidence supports the use of neurotrophic peptides or growth factor mimetics in the treatment of PD, these biologics undergo extensive proteolysis in the periphery by plasma esterases and peptidases and therefore conventional oral or parenteral administration is not possible (Thorne & Frey, 2001). Notably, intracerebral administration of glial derived nerve growth factor (GDNF), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) also proved unsuccessful in clinical trials for PD, which may be attributed to the extensive dopaminergic cell loss that has already occurred upon PD diagnosis (Bondarenko & Saarma, 2021).

Polar, hydrophilic drugs can become substrates for efflux transporters like the ATP-dependent P-glycoprotein (P-gp) expressed by BBB endothelial cells (Lin & Yamazaki, 2003). Therefore, even when drugs manage to enter the CNS, they can be pumped back out into circulation, prohibiting their targeted CNS action. Several PD drugs are reported to be substrates for P-gp including bromocriptine and tolcapone (Bicker et al., 2017; Vautier et al., 2006).

1.2.3. Limitations of Parkinson's disease therapies

PD therapies are notoriously associated with adverse effects, typically caused by the products of peripheral metabolism. L-DOPA, cholinesterase inhibitors and dopamine agonists produce negative side effects including nausea, vomiting and hypotension as well as long-term complications such as dyskinesia (Foltynie et al., 2024). As CNS bioavailability of these drugs is a challenge, high doses must be administered to achieve therapeutic concentrations in the brain. Approximately 1% of orally administered L-DOPA reaches the brain (Aldred & Nutt, 2010), therefore, a large amount of drug remains in the periphery contributing to these off-target effects that can further reduce a patient's quality of life while battling PD.

While strategies are in place to counter these effects, including co-administration of enzyme inhibitors such as carbidopa, there are also side effects associated with these drugs (Leyden & Tadi, 2025). Formulations that protect drugs from peripheral metabolism and improve brain targeting are desperately needed to both improve drug efficacy and patients' quality of life while taking these medicines. Furthermore, the limited efficacy of these treatments and lack of disease-modifying outcomes suggest that novel drug targets need to be identified to address this unmet clinical need.

1.3. Drug repurposing for CNS disease

1.3.1. Advantages of drug repurposing

Drug repurposing is a strategy where an existing drug that has been approved and is in clinical use for one disease is investigated as a new treatment for a different condition (Rani

et al., 2024). The benefits of this process include faster progression to clinical approval, substantially lower development costs, prior pharmacokinetic data and a wealth of evidence of safety across multiple populations in clinical trials and in post-approval pharmacovigilance studies. *De novo* drug discovery from the initial identification of a new compound to clinical approval takes approximately 10 to 15 years, whereas existing drugs can move directly to phase II trials following sufficient preclinical evidence. This in turn reduces the associated costs from upwards of one billion euro to several million euros (Hernández-Parra et al., 2022). The risk and reward ratio for CNS drug discovery rarely favours the latter with only ~10% of newly approved drugs by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) in the past decade indicated for CNS disease (Batta et al., 2020). Therefore, drug repurposing should be seriously considered as a strategy for identifying new therapies across a range of CNS disorders.

1.3.2. Identifying candidates for repurposing

There are several approaches to drug repurposing. Historically, repurposed drugs were chance discoveries. This is true in the case of amantadine, which was prescribed to a PD patient in the 1960s as an antiviral treatment but the patient's physician noted a remarkable improvement in PD symptoms (Schwab et al., 1969). These observations resulted in a clinical trial and amantadine is now approved to alleviate symptoms of dyskinesia in PD. Additionally side-effect profiling of drugs with unexpected neurological or behavioural side effects may give insight into potential CNS activity (Rudrapal et al., 2020).

Retrospective epidemiological studies and review of public health data can be useful in identifying links between drug exposure and disease outcomes. A recently published population-based retrospective cohort study observed a significantly reduced risk of PD in patients treated with dihydropyridine calcium channel blockers for hypertension (Tseng et al., 2021). These findings were supported by preclinical evidence that L-type calcium channels are implicated in dopaminergic stress and degeneration as well as subsequent meta-analyses of nearly three million participants across nine studies confirming the observed correlation (Rudrapal et al., 2020). Despite this, isradipine, a dihydropyridine calcium channel blocker, failed in phase III clinical trials (Maiti & Perlmutter, 2020). Together these studies highlight the complexity of dosing discrepancies between peripheral and CNS targeting of the same drug as well as the timing of interventions as patients with early-stage PD were included in the clinical trial, but the initial observation was that chronic exposure to calcium channel blockers reduced the risk of developing PD.

High-throughput screening (HTS) assays are developed, typically by pharmaceutical companies to test compounds for a particular cellular or molecular output. These assays are scaled up and integrated into robotic systems where large chemical libraries are rapidly screened and potential 'hits' are identified for further testing. HTS has had few successes

in identifying CNS therapies to date with ‘hits’ like minocycline failing in multiple Phase II trials for PD, amyotrophic lateral sclerosis and Huntington’s disease (Gordon et al., 2007; Romero-Miguel et al., 2021). However, HTS has proven useful in the fields of oncology and antiviral therapies.

Modern computational and *in silico* methods have evolved to identify candidates for drug repurposing. These strategies involve the use of transcriptomics, network pharmacology and artificial intelligence (AI)-based predictions to identify potential candidates (Casas et al., 2019; Ferreira & Carneiro, 2025; He et al., 2023). Genome-wide association studies (GWAS) can also be used to identify disease-associated genes and search for drugs known to modulate these targets (So et al., 2019). Current advances in AI and machine learning are transforming the landscape of drug discovery and are likely to revolutionise drug repurposing in the coming years.

1.3.3. Successful drug repurposing for CNS disease

In addition to amantadine, which was repurposed for PD treatment, several other drugs that are now approved for CNS diseases were repurposed from their initial uses. These include anti-hypertensive drug propranolol, which is now used to treat anxiety and migraine, anti-epileptic drugs (AEDs) valproate and topiramate for migraine, AEDs gabapentin and pregabalin as well as antidepressant duloxetine for neuralgia and fibromyalgia, AED clonazepam for panic disorder, AEDs carbamazepine, valproic acid and lamotrigine for bipolar disorder, anaesthetic ketamine for treatment-resistant depression, anti-tussive drug bupropion for major depression, immunosuppressant drug fingolimod and anticancer drug cladribine for multiple sclerosis, AEDs valproate and lamotrigine for bipolar disorder and antihypertensive drugs clonidine and guanfacine for attention deficit hyperactivity disorder (ADHD) (Deng et al., 2024; Neuchat et al., 2023; So et al., 2019; Sprenger et al., 2018)

AEDs are frequently repurposed for the treatment of other brain disorders due to their multiple neurobiological mechanisms of action, which include modulation of ion channels, GABA signalling and glutamate receptor activity. These mechanisms are shared with pathways underlying pain, mood regulation and movement disorders and several AEDs have consequently obtained regulatory approval for these indications (So et al., 2019). Furthermore, these drugs are already known to penetrate the BBB to produce therapeutic effects and have long established safety profiles.

1.3.4. Challenges in drug repurposing

Drug repurposing can encounter patent intellectual property challenges which can increase development costs by up to tenfold. This can deter researchers and investors as the

commercial return is typically lower than that of a new compound. However, reformulating an existing drug or developing new delivery methods can generate novel intellectual property, thereby mitigating these barriers (Hernandez et al., 2017).

When screening drug candidates for repurposing, negative results yielded are rarely published. Therefore, much time is likely wasted screening the same libraries that were elsewhere determined to be invalid. The crux of this problem is a larger issue, where negative data is rarely accepted by journals for publication. If a compound is under commercial development or patent, pharmaceutical companies may also withhold negative data in the event of future licensing of that compound for other indications. Practically, if thousands of compounds are screened it may not be feasible to publish an exhaustive list of negative results.

Several drugs are being investigated for repurposing in PD, including β_2 adrenoreceptor (β_2 AR) agonists that are used to treat asthma and chronic obstructive pulmonary disease (COPD), the anti-cancer agent nilotinib and diabetes medication exenatide (Zahoor et al., 2018). However, there are many candidates for CNS drug repurposing that theoretically warrant further investigation, but peripheral metabolism and BBB impenetrability remain barriers to their success.

1.4. Breathing new life into brain therapies: anti-inflammatory effects of β_2 -adrenoceptor agonists in the CNS

1.4.1. Noradrenaline as an endogenous regulator of neuroinflammation

Noradrenaline signalling in the brain not only regulates cognitive functions, but also neuroinflammatory signalling. This predominantly occurs through the activation of the β_2 AR, a G-protein coupled receptor, which is highly expressed by microglia and astrocytes in the CNS (**Fig. 1.3**) (Mori et al., 2002; O'Neill & Harkin, 2018). Activation of microglial β_2 ARs by noradrenaline suppresses neuroinflammation through inhibition of microglial proliferation and production of IL-1 β , TNF α and iNOS (Dello Russo et al., 2004; Färber et al., 2005; Fujita et al., 1998) and genetic knockdown of the β_2 AR in mice was shown to potentiate LPS-mediated increases in TNF α , IL-1 β and IL-6 expression (Evans et al., 2020). Noradrenaline also binds to the β_2 AR expressed by astrocytes promoting the secretion of growth factors and a neurotrophic environment (Day et al., 2014) and β_2 ARs are expressed by pyramidal and granule cells in the hippocampus and some interneurons play an important role in synaptic plasticity, learning and memory (Maity et al., 2022). Furthermore, the *Ardarb2* mRNA, encoding the β_2 AR was detected in nigrostriatal dopaminergic neurons, but its function in this cell population is not fully understood (Patterson et al., 2022). Inflammatory stimuli like LPS and IFN γ downregulate glial β_2 AR expression, which has implications for neuroinflammation in neurodegenerative disease (Ryan et al., 2020).

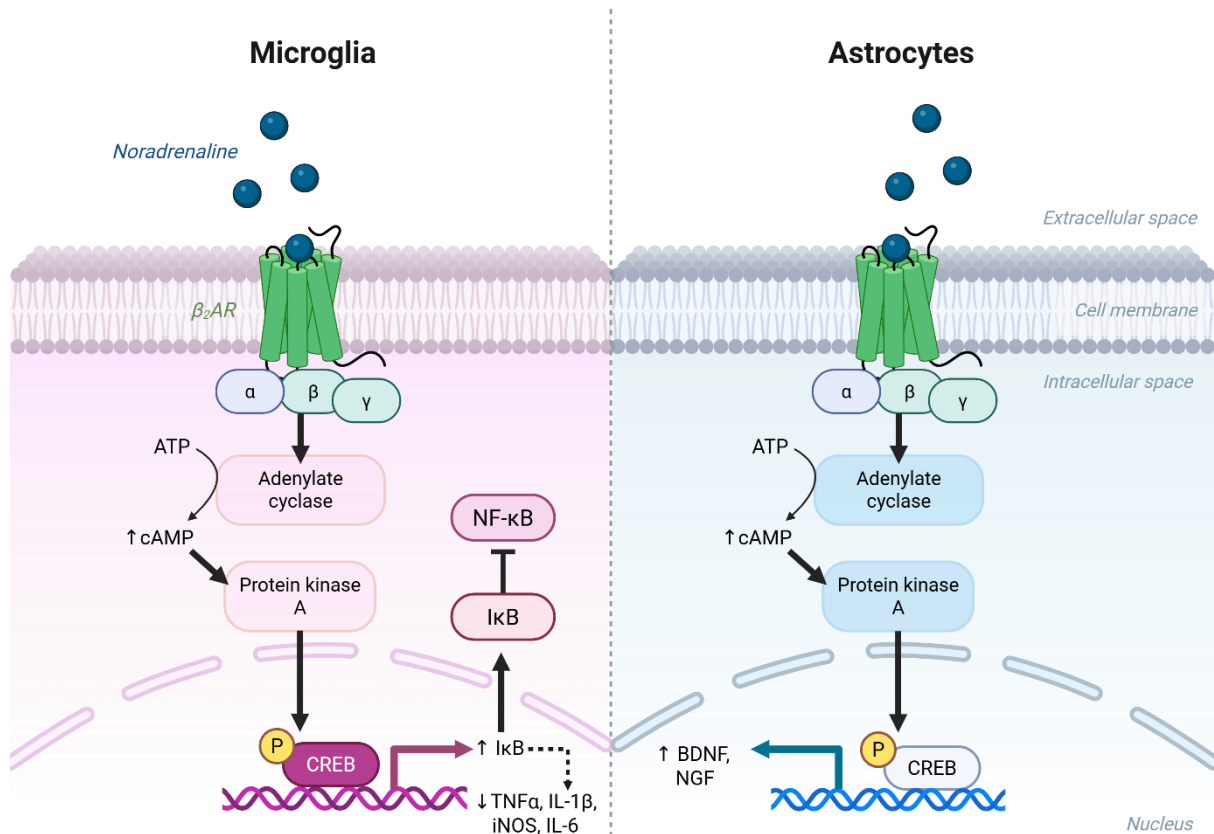


Figure 1.3. Noradrenaline acting at the β_2AR suppresses neuroinflammation and promotes neuronal survival.

Noradrenaline binding to the β_2AR activates adenylyl cyclase conversion of ATP to cyclic adenosine monophosphate (cAMP). The rise in intracellular cAMP activates protein kinase A, which phosphorylates cAMP-responsive element-binding protein (CREB). Phosphorylated CREB translocates to the nucleus and initiates the transcription of inhibitor of nuclear factor κB ($I\kappa B$) in microglia. $I\kappa B$ prevents NF κB from entering the nucleus and therefore inhibits the expression of NF κB -dependent pro-inflammatory genes. In astrocytes, protein kinase A activation also leads to the phosphorylation of CREB, which translocates to the nucleus and initiates the transcription of neurotrophic factors. These include BDNF and NGF, which are translated into proteins and secreted from astrocytes to promote neuronal survival.

The locus coeruleus, a small brainstem nucleus positioned in the upper dorsolateral pontine tegmentum, is the noradrenergic hub of the brain, with extensive projections throughout the brain and spinal cord (Caestecker et al., 2025; Paredes-Rodriguez et al., 2020). Post-mortem examinations of the brain of PD patients revealed that substantial degeneration of locus coeruleus noradrenergic neurons was accompanied by Lewy body pathology in this region, which may precede pathology in the substantia nigra (Chan-Palay & Asan, 1989; Gesi et al., 2000; Marien et al., 2004; O'Neill & Harkin, 2018; Vazey & Aston-Jones, 2012; Zarow et al., 2003).

Indeed, selective lesion of the locus coeruleus by neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) in rodents potentiates neuroinflammation and degeneration in brain regions associated with PD. af Bjerkén et al. (2019) injected rats with DSP4 to deplete locus coeruleus noradrenaline and assessed motor function, microglial reactivity and tyrosine hydroxylase (TH) positive dopaminergic neuronal cell density over a period of 6 months. Animals with a locus coeruleus lesion performed worse than non-lesioned animals on the rotarod test of motor co-ordination and balance, had significantly lower numbers of TH⁺ neurons in the substantia nigra and a higher proportion of reactive amoeboid microglia (af Bjerkén et al., 2019). Furthermore, dopamine β hydroxylase (D β H) is necessary to convert dopamine to noradrenaline and in D β H deficient mice that completely lack noradrenaline, motor dysfunction was more pronounced than in wildtype mice injected with MPTP, despite an 80% loss of nigrostriatal dopamine content (Rommelfanger et al., 2007).

In PD rodent models, DSP4 potentiates microgliosis, dopaminergic cell death in the substantia nigra and motor deficits. DSP4 injection prior to MPTP treatment resulted in greater motor dysfunction in mice that could not be rescued by L-DOPA administration compared to MPTP alone (Archer & Fredriksson, 2006). Similarly, pre-treatment with DSP4 to deplete noradrenaline prior to a systemic LPS challenge led to increased immunoreactivity of microglial markers ionised calcium-binding adaptor protein 1 (IBA1) and CD11b, as well as neuronal oxidative stress and degeneration in the substantia nigra in mice. Furthermore, the authors showed that the combination of DSP4 lesion and LPS-induced neuroinflammation resulted in the manifestation of both motor and non-motor symptoms associated with PD (Song et al., 2019b). In a subsequent study by this group using a mouse line carrying a mutation in the *Snc* gene that encodes α -synuclein, DSP4 injection also amplified motor deficits and degeneration of dopaminergic neurons in the substantia nigra (Song et al., 2020). Furthermore, Song et al. (2019a) demonstrated that a single systemic injection of LPS generates progressive degeneration originating in the locus coeruleus which progresses to the substantia nigra. Pre-treatment with DSP4 further intensified these effects, and depletion of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) activity and subsequent reduction of ROS rescued both neuroinflammation and neuronal cell loss (Jiang et al., 2015; Song et al., 2019a). Consistent

with these findings, it was recently reported that pre-treatment with DSP4 exacerbated dopaminergic cell loss in the unilateral 6-OHDA mouse model of PD (Torrente et al., 2023).

The loss of locus coeruleus noradrenergic signalling in the PD brain may correlate to non-motor symptoms observed in patients, including anxiety and depression. PET imaging studies in PD patients with and without concomitant depression were conducted using [¹¹C]RTI-32, which is a tracer for noradrenaline and dopamine transporter binding. Depressed PD patients displayed reduced [¹¹C]RTI-32 binding in the locus coeruleus and areas of the limbic system, the extent of which was negatively correlated with depression severity (Remy et al., 2005). More recently, similar studies reported that locus coeruleus degeneration was associated with classic motor symptoms of PD including bradykinesia and tremor, as well as non-motor symptoms, including fatigue and depression (Castellanos et al., 2015; Laurencin et al., 2024; Sasaki et al., 2006; Schwarz et al., 2017; Wang et al., 2018).

1.4.2. Pharmacological manipulation of noradrenaline alters inflammatory responses

The administration of noradrenaline reuptake inhibitors (NRIs) increases synaptic and extra-synaptic availability of noradrenaline to promote anti-inflammatory signalling. Intraperitoneal (i.p.) injection of desipramine (15 mg/kg) or atomoxetine (10 mg/kg) reduces the expression of pro-inflammatory *Il1b*, *Tnfa*, *Ccl5*, *Nos2*, *Cd40* and *Cd11b* mRNA in the brains of rats challenged with a systemic LPS injection (O'Sullivan et al., 2009; O'Sullivan et al., 2010). Increased availability of noradrenaline in the rat brain as a result of NRI reboxetine treatment (15 mg/kg; i.p.) also negatively regulates IL-1 β signalling by increasing the expression of *Il1ra* which encodes the IL-1 receptor antagonist (IL-1RA) and *Il1r2* which encodes the IL-1 type II decoy receptor (IL-1RII), (McNamee et al., 2010a). The effects of reboxetine were shown to be associated with an increase in hippocampal and cortical *Il10*, which encodes anti-inflammatory cytokine IL-10 and *Socs3*, which encodes the suppressor of cytokine signaling-3 (SOCS-3) that downregulates the expression of pro-inflammatory mediators via inhibition of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (McNamee et al., 2010b). These treatments were administered along with inhibitory autoreceptor α_2 adrenoreceptor (α_2 AR) antagonist idazoxan (1 mg/kg; i.p.) to further boost noradrenaline release, and were suppressed by β_2 AR antagonist propranolol, indicating a role for the β_2 AR in mediating these anti-inflammatory effects. Furthermore, atomoxetine (3 mg/kg; i.p.) and idazoxan (1 mg/kg; i.p.) treatment protected against the loss of TH⁺ immunoreactivity in the substantia nigra and striatum as well as motor deficits in the intranigral LPS model of PD-related neuroinflammation and degeneration (Yssel et al., 2018).

A canonical study by Mittal and colleagues (2017) reported that long-term use of the β_2 AR agonist salbutamol reduced the risk of developing PD in a large Norwegian cohort and conversely, long-term exposure to β -blockers like propranolol increased the risk of

developing PD. Furthermore, high-throughput gene expression screening identified β_2 AR agonists as regulators of *SNCA* expression, which was confirmed in the MPTP mouse model where β_2 AR agonist clenbuterol (10 mg/kg; i.p.) rescued TH⁺ dopaminergic cell loss in the substantia nigra. The protective effect of clenbuterol was also observed in induced pluripotent stem cell (iPSC)-derived neuronal cells from a patient carrying the *SNCA* triplication associated with familial PD, where the β_2 AR agonist (20 μ M) reduced *SNCA* transcription, attenuated ROS production and increased cell viability (Mittal et al., 2017). Recent clinical studies demonstrated that clenbuterol and salbutamol produced pro-cognitive effects in PD patients which may be linked to an increase in cerebral blood flow (Eijssvogel et al., 2024; Lodeweyckx et al., 2024).

In vitro, noradrenaline increased intracellular cAMP levels, reduced *Tnfa* and *Il6* expression and decreased NO production in LPS-stimulated microglia. This was replicated following β_2 AR activation by terbutaline but not when cells were treated with α_1 , α_2 or β_1 AR agonists (Mori et al., 2002). Both clenbuterol (0.5 mg/kg; i.p.) and formoterol (0.5 mg/kg; i.p.) administration increased mRNA levels of *Il1ra*, *Il1r2*, *Il10* and *Socs3* in the rat brain and these effects were blocked by propranolol (10 mg/kg; i.p.) (McNamee et al., 2010a; McNamee et al., 2010b). Clenbuterol also attenuated the expression of *Il1b*, *Il6*, *Tnfa* and *Cd40* in the rat cortex following systemic LPS challenge (McNamee et al., 2010a).

1.4.3. β_2 AR agonists are anti-inflammatory and neuroprotective in preclinical Parkinson's disease models

The administration of β_2 AR agonists provides disease-modifying effects in pre-clinical PD models. For example, daily salmeterol infusions (0.01 mg/kg) for two weeks conferred neuroprotective effects three months after LPS and MPTP-induced degeneration of dopaminergic neurons in the substantia nigra and rescued performance in the rotarod test of motor function (Qian et al., 2011). A single dose of clenbuterol (0.5 mg/kg, i.p.) was sufficient to protect against increases in *Il1b* and *Tnfa* expression, neutrophil infiltration and cell death in the brains of animals that received an intra-striatal IL-1 β injection (Griffin et al., 2018). Clenbuterol treatment (10 mg/kg; i.p.) was also associated with a reduction in microglial activation, rescued TH cell loss and improved motor dysfunction in mice overexpressing human wildtype α -synuclein, while β_1 AR agonist Xameterol (3 mg/kg; i.p.) enhanced pro-inflammatory signalling and exacerbated dopaminergic cell death in the substantia nigra in this model (Torrente et al., 2023)

Similarly, in the unilateral intranigral LPS inflammatory PD model, daily formoterol treatment (0.1 mg/kg; i.p.) for seven days post-lesion rescued motor deficits in several behavioural paradigms, reduced microgliosis in the substantia nigra and protected against degeneration of the nigro-striatal tract. Formoterol also prevented the progression of dopamine loss and motor symptoms when animals bearing a unilateral LPS lesion were

subsequently challenged with systemic LPS to exacerbate pathology (O'Neill et al., 2020). This has implications for delirium in PD patients, where formoterol therapy may prevent a worsening of symptoms and pathology following secondary infection.

These effects are not just limited to PD, β_2 AR agonists clenbuterol and formoterol are reported to attenuate neuroinflammation and degeneration in animal models of Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Down syndrome (Dang et al., 2014; Le et al., 2025; Teng et al., 2006).

1.4.4. Barriers to clinical use of β_2 AR agonists for treatment of Parkinson's disease

There is surmounting pre-clinical, post-mortem and patient-based evidence that β_2 AR agonists may be useful for modifying the progression of PD and other neurodegenerative diseases through anti-inflammatory and neuroprotective effects. Of these, formoterol and salmeterol are long-acting β_2 AR agonists with a duration of action of at least 12 hours (Hsu & Bajaj, 2025).

Due to structural similarities between α and β AR subtypes, agonists for the β_2 AR can interact with other adrenergic receptors to produce off-target effects. Activation of cardiac β_1 ARs by β_2 AR agonists commonly causes tachycardia when prescribed for the treatment of asthma or COPD (Hsu & Bajaj, 2025). Furthermore, β_2 AR agonist induced arrhythmias have been reported in patients with underlying heart conditions (Sears, 2002). The use of these medicines can also promote glycogenolysis and an increase in blood glucose levels (Lipworth & McDevitt, 1989). While healthy individuals have a lower risk of these off-target actions, these are serious considerations for the use of β_2 AR agonists in PD cohorts. As PD is typically diagnosed in individuals above the age of 60, there is a high possibility that patients may also have heart conditions or diabetes, and prescribing these therapies could be life-threatening.

The negative effects associated with β_2 AR agonists are dose dependent. Therefore, strategies are needed to lower the doses required and improve brain targeting of these drugs to harness their potential in the treatment of PD.

1.5. Nanomedicine for targeted brain therapies

1.5.1. Overview of nanomedicine

The field of nanomedicine offers cutting edge solutions to overcome the challenges of drug delivery to the brain. The term nanomedicine includes a range of nano-sized materials used for medical applications including nanoparticles, micelles, nanodrugs and nanogels (Chan et al., 2010). Nanoparticles are derived from a variety of synthetic, natural and hybrid materials and have been investigated for diagnostic, drug delivery and disease prevention

purposes. Several nanoparticle formulations are approved for clinical use, including inorganic superparamagnetic iron oxide nanoparticles for treating anaemia in chronic kidney disease for the delivery of chemotherapeutic agents and vaccines (Anselmo & Mitragotri, 2021; Cheng et al., 2025; Provenzano et al., 2009; Suzuki & Ishihara, 2021). However, the toxicity and clearance of inorganic nanoparticles remains a concern. Several studies have reported an effect on BBB integrity, oxidative stress, and microglial dysfunction (Neha et al., 2013), therefore making them potentially inadequate as nanocarriers for CNS therapies. Organic nanoparticles have a favourable safety profile and various liposomal, albumin-based, and polymeric materials are FDA approved for biomedical applications (Begines et al., 2020). Preclinical development of several drug carriers has led to the evolution of controlled release systems for drugs associated with systemic toxicity, poor pharmacokinetic profiles and low CNS penetrability.

1.5.2. Polymeric nanoparticles

Polymeric nanoparticles range in size from 1–999 nm and are formulated using synthetic polymers or copolymers of poly(D,L-lactic acid), poly(ϵ -caprolactone), poly(lactic-co-glycolic)acid (PLGA) or natural polymers like chitosan and maltodextrins. They are synthesised by the self-assembly of two or more chains of block copolymers with varying hydrophobicity predominantly using emulsification or nanoprecipitation methods (Chan et al., 2010; Kamaly et al., 2016). PLGA nanoparticles have emerged as popular candidates for CNS drug delivery as they can encapsulate low molecular weight therapies, hydrophilic and hydrophobic drugs, proteins, peptides, vaccine antigens, and gene therapies (**Fig. 1.4**) (De et al., 2022; Kumari et al., 2010; Makadia & Siegel, 2011). Encapsulated therapeutic agents are protected from metabolism, enzymatic degradation, and premature excretion, thus reducing off-target effects and enhancing drug concentration at the target site. As PLGA is an FDA approved polymer, PLGA nanoparticles have been extensively studied as drug delivery systems presenting many advantages; they easily cross the BBB, are biocompatible and stable, allow for controlled release kinetics, have high drug loading capacity, and can be functionalized with surface ligands for targeted drug delivery (Begines et al., 2020; Kamaly et al., 2016; Makadia & Siegel, 2011; Neha et al., 2013; Patel et al., 2012). Furthermore, PLGA nanoparticles are biodegraded by hydrolysis to produce lactic and glycolic acids, which enter the Krebs's cycle and are excreted as carbon dioxide and water (Kamaly et al., 2016; Kumari et al., 2010). Drug release occurs through bulk matrix degradation, however, many environmental factors like pH and the physicochemical nanoparticle characteristics can affect the rate of polymer degradation. Therefore, the release pattern is changeable but typically follows a biphasic profile (Kamaly et al., 2016; Makadia & Siegel, 2011). Increases in PLGA nanoparticle size and concentration, as well as changes in shape, have been reported to cause cytotoxicity *in vitro*, resulting in macrophage

activation and the production of ROS. Nevertheless, the body of evidence suggesting that PLGA is biocompatible far exceeds those that describe toxicity (De et al., 2022).

The first PLGA-based drug formulation, a PLGA-drug depot for cancer treatment, was FDA approved in 1989 and since then several long-acting implants and microsphere PLGA products have reached the global market for treatment of schizophrenia, substance dependence, endocrine dysfunction, various cancers and ophthalmological conditions (Alsaab et al., 2022). Although PLGA-based drug delivery systems are not approved for PD, significant research is being conducted to explore their potential and these systems are beginning to appear in clinical trials (Lababidi & Azzazy, 2025). Recently, a phase III study investigating the efficacy of intramuscular rotigotine PLGA microspheres for the treatment of PD was completed, having demonstrated safety and tolerability in previous trials (NCT04571164). This study demonstrated that weekly intramuscular injection of the dopamine receptor agonist rotigotine-extended release PLGA microspheres significantly improved motor symptoms and quality of life of patients with PD (Sun, 2025). Currently, no PLGA nanoparticles have received regulatory approval, although formulations have entered clinical trials. Notably, PRECIOUS-01, a PLGA nanoparticle loaded with tumour antigen NY-ESO-1 and a natural killer T cell activator, has entered phase I clinical trials to assess the safety, tolerability and efficacy of intravenous treatment for various types of cancer (NCT04751786) (Creemers et al., 2021). This study, a first of its kind, will provide additional information around PLGA nanoparticles in the clinic, with novel insights into drug release from PLGA nanoparticles and the efficacy of such treatments in human populations.

1.5.3. Surface modifications

Surface engineering of PLGA nanoparticles can improve both biocompatibility, brain targeting, stability, and controlled drug release (**Fig. 1.4**). Poly(ethylene glycol) (PEG) is a non-toxic synthetic polymer that is approved for biomedical and pharmaceutical applications. Nanoparticles are commonly coated with PEG in a process called PEGylation. PEGylation of nanoparticles for CNS drug delivery is common and is reported to improve circulation time, biocompatibility, and brain uptake (Nance et al., 2012; Zhi et al., 2021). Owing to its hydrophilicity, neutral charge and solubility, PEGylation increases steric hindrance and circulation time by shielding nanoparticles from opsonisation and aggregation especially when PEG is covalently linked to the polymer rather than adsorbed onto the surface (El-Hammadi & Arias, 2022; Nance et al., 2012). PEG alternatives including poly(ϵ -caprolactone) and chitosan, surfactants like polysorbate 80 and poloxamer 188, monolayer lipids and bilayer lipids can also be chemically grafted or physically adsorbed onto the surface of PLGA nanoparticles to produce similar protective effects (El-Hammadi & Arias, 2022).

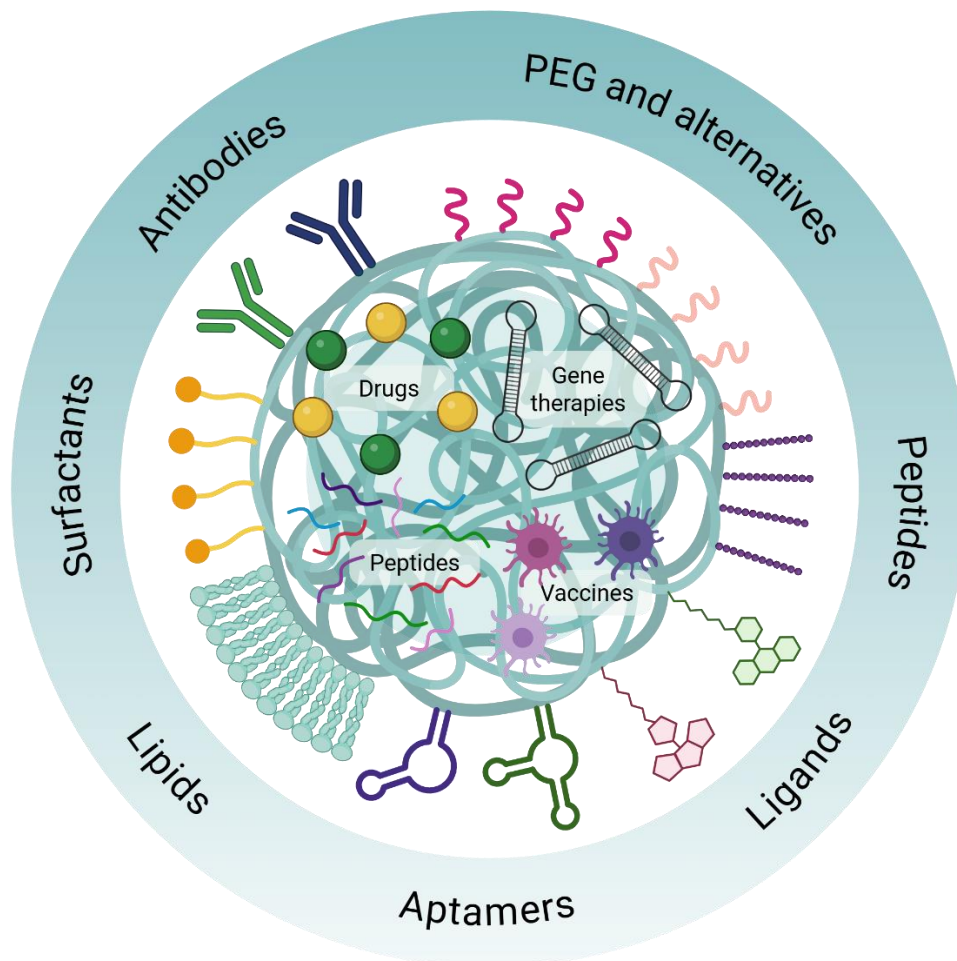


Figure 1.4. PLGA nanoparticle design.

PLGA nanoparticles can be loaded with hydrophobic or hydrophilic drugs, peptides, gene therapies and vaccines for controlled release. The majority of nanoparticles are PEGylated to improve biocompatibility and circulation time. A range of surface moieties may be physically or chemically grafted onto the surface of PLGA nanoparticles. These include targeting ligands, cell penetrating peptides, antibodies and aptamers, to facilitate brain uptake and targeting, as well as surfactants and lipid layers, which can improve BBB crossing and biocompatibility.

Proteins, aptamers, peptides, small molecules, and antibodies can also be physically or chemically attached to the surface of PLGA nanoparticles to improve drug targeting. CNS specific targeting can be achieved using ligands with high affinity for receptors and transporters expressed on the surface of BBB endothelial cells to take advantage of existing carrier-mediated transport and receptor-mediated transcytosis pathways. These ligands include transferrin, lactoferrin, apolipoprotein E and glucose derivatives, which facilitate the brain uptake of nanoparticles through receptor-mediated transcytosis and carrier-mediated transport mechanisms (Bi et al., 2016; Jiang et al., 2014; Loureiro et al., 2016; Wagner et al., 2012).

Cell-penetrating peptides (CPPs) like the transactivator of transcription can also be bound to the surface of nanoparticles through covalent or non-covalent interactions to enhance transport through cell membranes, increasing BBB crossing and cellular uptake of drug-loaded nanoparticles (Annu et al., 2022; Zhang et al., 2021b). Furthermore, CPPs can overcome the P-gp efflux pumps expressed by BBB endothelial cells, which are associated with multi-drug resistance (Halder et al., 2022).

1.5.4. Biocompatibility and brain distribution of PLGA nanoparticles *in vivo*

The safety profile and accumulation of PLGA nanoparticles in peripheral organs and tissues via various routes of administration have been investigated. Fonseca-Gomes et al. (2020) administered high concentrations of PLGA nanoparticles (100 mg/kg; i.p. thrice weekly) and reported no mortality, changes in body weight or signs of toxicity. At the end of this study when organs were harvested, however, PLGA nanoparticles were detected in the spleen and bone marrow of mice, but this was to a lesser extent than solid lipid nanoparticles (SLNs). Furthermore, no signs of pathology were detected in liver, spleen, bone marrow, brain, heart or thymus tissue (Fonseca-Gomes et al., 2020). This is in agreement with Casanova et al. (2022), who reported that i.p. administration of PLGA nanoparticles every 3 days for 43 days had no effect on the body weight of rats (Casanova et al., 2022).

1.5.5. PLGA nanoparticles for drug delivery in Parkinson's disease: preclinical evidence

PLGA nanoparticle-mediated drug delivery has been investigated in animal models of PD and other CNS disorders (summarised **Table 1.1**). This includes clinically used drugs that may benefit from nanocarrier formulations to reduce doses and improve the side effect profile, as well as compounds whose therapeutic potential has not yet been realised due to poor bioavailability or insufficient brain uptake.

Dopamine cannot cross the blood brain barrier and is subject to peripheral enzymes when administered systemically. Monge-Fuentes et al. (2021) reported that i.p. injection of albumin coated PLGA nanoparticles loaded with dopamine effectively crossed the BBB and

improved performance in several behavioural paradigms of mice bearing a 6-OHDA lesion. Moreover, the efficacy of dopamine-loaded PLGA-albumin nanoparticles was greater than systemic administration of L-DOPA at the same concentration (Monge-Fuentes et al., 2021). Tolcapone is a common adjunct to L-DOPA therapy as it is a peripheral and central COMT inhibitor. However, its systemic actions lead to unfavourable side effects such as diarrhoea, vomiting and muscle cramps. It also has a very short half-life so frequent dosing is required. To overcome these constraints, tolcapone was loaded in PLGA nanoparticles and administered (i.p.) to rotenone treated rats. Despite the extensive dosing in this study, non-significant trends towards improved motor function and pathology were observed (Casanova et al., 2022).

Many natural products are reported to have antioxidant, neuroprotective, and anti-inflammatory effects, but due to instability in biological fluids, rapid metabolism, and inability to cross the BBB, they have yet to be harnessed as pharmacotherapies for the treatment of CNS diseases. One such candidate is resveratrol, a natural polyphenolic compound found in red grapes, blueberries and chocolate with reported antioxidant and anti-inflammatory actions. However, the clinical application of these effects has been limited due to low water solubility, chemical instability and short half-life. Loading of lactoferrin coated PLGA nanoparticles with resveratrol and intravenous injection enhanced brain uptake, significantly attenuated dopaminergic cell loss and microgliosis in the substantia nigra and rescued motor deficits in mice treated with MPTP (Katila et al., 2022). Similarly, flavonoids including naringenin, which is typically found in citrus fruits, are reported to have antioxidant and immunomodulatory properties but are poorly soluble in water and subject to peripheral metabolism. When loaded into PLGA nanoparticles and delivered orally, naringenin rescued motor deficits, decreased ROS, increased BDNF and prevented α -synuclein accumulation in the brains of rats injected with the neurotoxin paraquat (Dashputre et al., 2023).

PLGA nanoparticles are also being explored as delivery systems for novel PD treatments. These include the experimental histone deacetylase inhibitor 6 (HDAC6) CAY10603, that prevents the HDAC6-mediated dysregulation of axonal transport that is observed in degenerating neurons and linked to mitochondrial dysfunction. However, CAY10603 has low brain targeting ability. In the methamphetamine-induced PD model, intravenous injection of lactoferrin-PLGA nanoparticles loaded with CAY10603 increased motor activity and co-ordination, striatal dopamine content, dopaminergic cell bodies in the substantia nigra and dopaminergic fibre integrity in the striatum of mice as well as reducing α -synuclein accumulation and microglial reactivity in the substantia nigra and the striatum (Pham et al., 2025).

Table 1.1. PLGA nanoparticles for drug delivery in rodent PD models.

Nanoparticle	Drug	Nanoparticle properties	Model	Route of admin	Dosing regimen	Outcomes	Ref
Albumin-PLGA nanoparticles	Dopamine	Size: 353 nm PdI: 0.5 ZP: -37 mV	6-OHDA mouse model	i.p.	Single post-lesion dose 10 or 20 mg	Restored motor function	Monge-Fuentes et al. (2021)
PLGA nanoparticles	Tolcapone	Size: 182.59 ± 23.94 nm ZP: -26.32 ± 0.48 mV EE: 56.69%	Rotenone rat model	i.p.	3 mg/kg every 3 days for 45 days	Mild motor improvements and reduced gliosis in the substantia nigra	Casanova et al. (2022)
Lactoferrin-PLGA nanoparticles	Resveratrol	Size: 148.2 ± 4.2 nm PdI: 0.12 ± 0.18 ZP: -23.1 ± 3 EE: 75.2 ± 4.1 % LC: 6.1 ± 0.3 %	MPTP mouse model	i.v.	5 mg/kg every second day for 15 days	Attenuated dopaminergic cell loss, microgliosis and loss of motor functions	Katila et al. (2022)
PLGA nanoparticles	Naringenin	Size: 162.1 nm PdI: 0.288 ZP: -9.8 mV EE: 91%	Paraquat rat model	Oral	50 or 100 mg/kg 1 hour before i.p. paraquat daily for 42 days	Prevented motor impairment, reduced ROS and α -synuclein accumulation, increased BDNF	Dashputre et al. (2023)
Lactoferrin-PLGA nanoparticles	CAV10603 HDAC6 inhibitor	Size: 146.7 ± 4.6 nm PdI: 0.09 ZP: -49.2 ± 2.9 mV EE: 20.57 ± 1.21 % LC: 0.69 ± 0.06 %	Meth-amphet-amine mouse model	i.v.	0.2 mg/kg every second day for 14 days (pre-dosing)	Improved motor symptoms, restored striatal dopamine content, protected against cell loss in the nigro-striatal tract, reduced microglial activation in the midbrain, prevented α -synuclein accumulation	Pham et al. (2025)

PLGA: poly(lactic-co-glycolic acid); PdI: polydispersity index (<0.3 indicates nanoparticle uniformity); ZP: zeta potential (surface charge); EE: encapsulation efficiency; LC: loading capacity; 6-OHDA: 6-hydroxydopamine; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; i.p.: intraperitoneal; i.v.: intravenous

Currently, there is no evidence in the literature of PLGA nanoparticles loaded with β_2 AR agonists for the treatment of CNS disease, however, PLGA nanoparticles loaded with formoterol have been developed for renal targeting (Vallorz et al., 2021; Vallorz et al., 2022). Formoterol has also been loaded into SLNs and when administered orally prior to rotenone treatment, formoterol-SLN (10-100 mg/kg) protected against motor impairments and *Snc*a expression in mice (Sola et al., 2023). While nanomedicine provides promising approaches for brain-targeted delivery, systemically administered nanoparticles still face many of the same challenges as free drugs. This is especially true for PLGA nanoparticles, which typically display an initial burst of drug release followed by gradual decay (Kamaly et al., 2016). Several studies have shown that nanoparticle mediated drug delivery achieves higher brain uptake than free drug, but the highest accumulation in the body occurs in the liver, kidneys and spleen (Cruz et al., 2016; Katila et al., 2022; Pham et al., 2025).

1.6. Intranasal drug delivery

1.6.1. Nose-to-brain pathways

Recently, the intranasal route of administration has emerged as a promising approach to enhance brain uptake of drug molecules or biological agents by bypassing the BBB entirely. Drugs are transported from the nasal cavity to the brain parenchyma by direct transport along the olfactory or trigeminal nerves or indirectly via paracellular or transcellular transport across the nasal epithelium and reaches the brain by the blood or cerebrospinal fluid (**Fig. 1.5**). A combination of these pathways likely contributes to drug transport from the nasal cavity to the brain, depending on the properties of the drug formulation.

1.6.2. Intranasal therapies used in the clinic for CNS disorders

In the past 30 years, intranasal formulations have been approved for use in the treatment of headaches, seizures, depression and opioid overdose (**Fig. 1.6**). This route of administration not only enhances drug delivery to the brain, but also reduces systemic exposure to the drug, preventing drug metabolism and adverse effects. Several intranasal therapies have been investigated for PD, but these are mostly still in the preclinical phase. Early phase clinical trials of intranasal insulin, glutathione and apomorphine have demonstrated safety, tolerability and early indications of improved motor functions in PD patients (Johnson et al., 2025; Mischley et al., 2017; Novak et al., 2019; Thijssen et al., 2022). The safety and tolerability of erythropoietin has also been demonstrated, and it has now also progressed to phase II/III trials to investigate its therapeutic effects (García-Llano et al., 2021). Recently published results for an intranasal formulation of L-DOPA reported motor improvement with only mild and transient side effects (Nomoto et al., 2025). Furthermore, a phase I study found that the intranasal delivery of human neural stem cells

was generally safe, well-tolerated with preliminary indications of improved motor function within 6 months (Jiang et al., 2024).

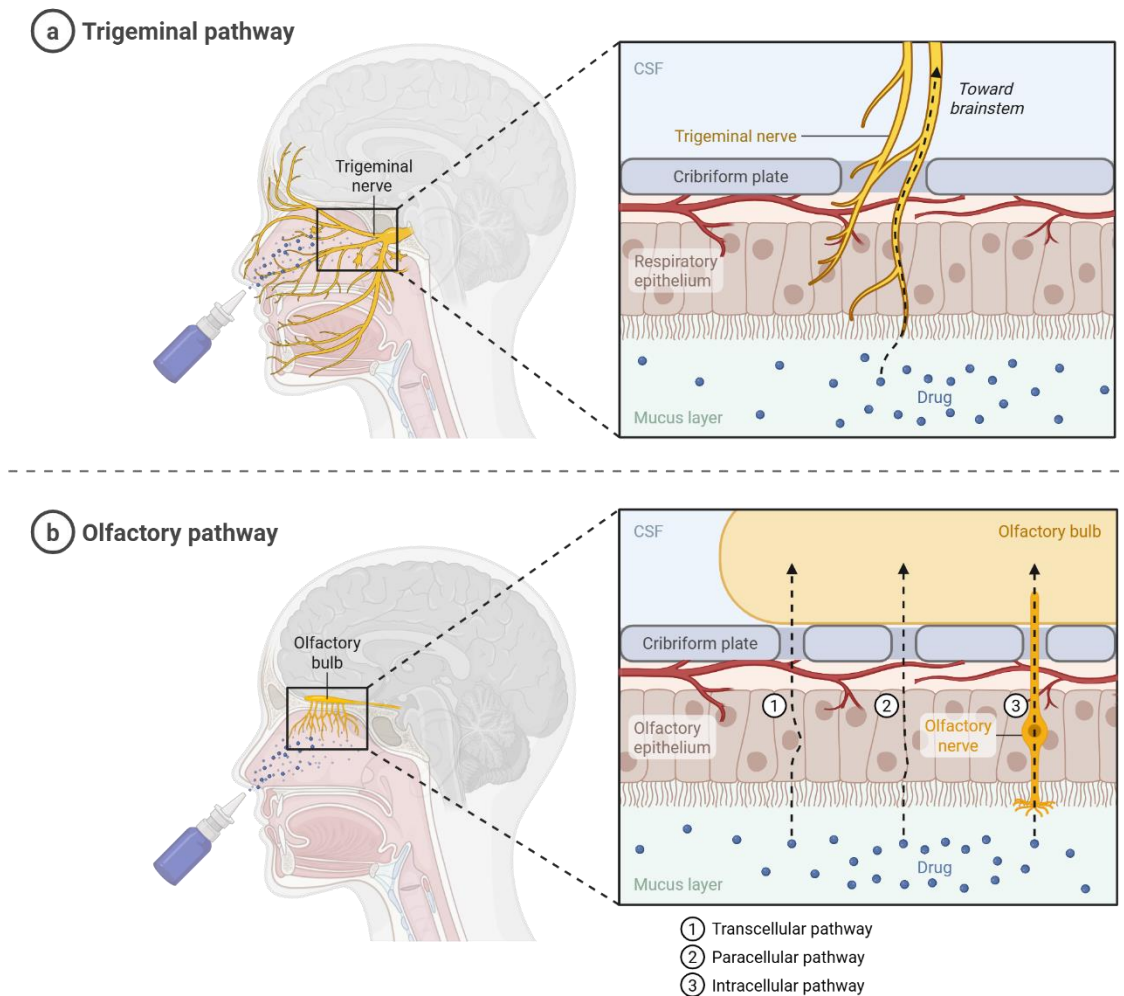


Figure 1.5. Nose-to-brain drug delivery.

(a) In the respiratory region of the nasal cavity, drug travels via the trigeminal nerve to the brain stem or along its ophthalmic and maxillary branches to reach the forebrain. (b) In the olfactory region, transcellular, paracellular or intracellular transport mechanisms facilitate drug movement across the olfactory epithelium and the cribriform plate to the olfactory bulb and then adjacent brain regions including the amygdala, piriform cortex and hypothalamus. Drug molecules may also enter the general circulation via capillaries in the nose and cross the BBB to reach the brain.



Figure 1.6. Timeline of approvals of intranasal formulations for CNS disorders.

1.6.3. Limitations of intranasal drug administration

Despite its immense potential in drug delivery for CNS disease, intranasal administration is associated with some limitations that may hamper its clinical use.

The nasal cavity is a small surface area for absorption, restricting the volume that can be administered (Wen & Ren, 2024). Therefore, for effective dosing, highly concentrated formulas must be used, which are associated with manufacturing challenges and risk of overdose. The intranasal route is unsuitable for high molecular weight and lipophobic drugs as they are less likely to be transported across the lipophilic mucus layer and across the nasal epithelium (Kaur et al., 2016). If formulations are swallowed or inhaled, the drug enters the gastrointestinal tract and systemic circulation where it undergoes peripheral metabolism and must subsequently cross the BBB. These processes reduce CNS bioavailability and increase the risk of off-target effects (Chung et al., 2023; Keller et al., 2022). Furthermore, this route of administration must be carefully considered for drugs that may alter local pH or affect blood flow, as changes in these factors may interfere with the immune host defence mechanisms in the nose (Keller et al., 2022). Mucociliary clearance can also impede drug delivery to the brain. This occurs every 15-20 minutes leaving a very short window for drug absorption to take place. However, the rate of mucociliary clearance is slower in PD patients (Saha et al., 2023).

1.6.4. Biomaterials for intranasal drug delivery

Nanoparticles can be employed to overcome challenges with intranasal drug delivery. Quantification of drug levels in brain tissue following intranasal administration of nanoparticles loaded with anti-Parkinson's drugs L-DOPA and rotigotine revealed higher concentrations than those achieved via alternative routes, including standard oral, injectable and intranasal solution formulations (Ahmad et al., 2022a; Bi et al., 2016; Kou et al., 2025; Yan et al., 2018). Furthermore, Yan et al. (2018) reported that intranasal delivery of lactoferrin-coated PLGA nanoparticles more effectively targeted rotigotine to the striatum compared to other brain regions and peripheral tissues.

In situ gelling systems that respond to pH, temperature or ionic concentration are advantageous as they increase residence time in the nasal cavity to facilitate drug transport to the brain. Nasal gels are typically prepared using mucoadhesive polymers such as xanthan gum, cellulose, chitosan and poly vinyl alcohol (Kaur et al., 2016). The use of such hydrogels is associated with enhanced safety, improved drug efficacy, less frequent dosing and fewer adverse effects (Zielińska et al., 2022).

Chitosan is a commonly used excipient for intranasal drug formulations and can be incorporated into the nanoparticle design or can be used to make hydrogels for nasal drug delivery. Chitosan is a natural biodegradable polymer that can form electrostatic interactions with negatively charged mucin and cell membranes in the nasal cavity to reduce clearance. Furthermore, chitosan reversibly promotes the opening of tight junctions, enhancing drug transport to the brain (Rassu et al., 2016).

Higher brain concentrations of dopamine agonist ropinirole were detected in the brains of rats following the intranasal administration of *in situ* gel delivery systems compared to injectable formulations (Khan et al., 2010). Furthermore, Wang et al. (2020) demonstrated that drug concentrations in the olfactory bulb, cerebrum, cerebellum and striatum were higher when rotigotine was incorporated into a PLGA-poloxamer-based-gel system and delivered intranasally, compared with intranasal delivery of rotigotine-PLGA nanoparticles without the gel and injection of a rotigotine solution.

1.6.5. Intranasal biomaterial-mediated drug delivery in preclinical Parkinson's models

Thus far, investigations in preclinical PD models using intranasal delivery of drug-loaded biomaterials have not combined PLGA nanoparticles and hydrogel systems, but evidence of drug efficacy exists for each system in isolation (summarised in **Table 1.2**).

Intranasal administration of PLGA nanoparticles loaded with dopamine, L-DOPA, rotigotine, bromocriptine and metformin rescued motor deficits and dopaminergic cell loss in several PD rodent models (Arisoy et al., 2020; Cong et al., 2025; Gambaryan et al., 2014; Tang et al., 2019; Yan et al., 2018). Furthermore, the efficacy of drug released from PLGA

nanoparticles following intranasal delivery was greater than standard oral L-DOPA treatment (Arisoy et al., 2020; Cong et al., 2025; Gambaryan et al., 2014; Yan et al., 2018).

Sridhar and colleagues reported that intranasal administration of MAO-B inhibitor selegiline and dopamine agonist pramipexole via a thermosensitive poloxamer 407-based gel improved locomotor activity and increased dopamine levels in the brains of rats injected with rotenone. In both studies intranasal gel-mediated drug delivery was superior to oral and nasal solution drug formulations (Sridhar et al., 2020; Sridhar et al., 2018). Several natural products are reported to have antioxidant and neuroprotective properties, but low brain bioavailability hinders their clinical use. Incorporation of the creatine prodrug dodecyl creatine ester, and the plant derived compounds albiflorin and rhynchophylline into nasal gel formulations, followed by intranasal administration, attenuated motor deficits and striatal dopamine depletion in rodent models of PD (Chen et al., 2022; Disdier et al., 2025; Lin et al., 2023).

A recent study investigating bambuterol, a prodrug of β_2 AR agonist terbutaline, incorporated drug loaded liposomes into a thermosensitive gel for intranasal administration. Intranasal administration increased brain: plasma bambuterol concentrations compared to injected free drug in mice. Furthermore, in mice that received a stereotactic injection of misfolded amyloid β ($A\beta_{1-42}$) oligomers into the CA1 area of the hippocampus, intranasal administration of a bambuterol-liposome-gel (0.3 mg/kg daily in 9 μ l) improved long-term spatial memory and promoted neuronal survival (Groo et al., 2025). This study underscores the neuroprotective potential of β_2 AR agonists and the advantages of intranasal formulations for treating neurodegenerative diseases. Intranasal delivery of β_2 AR agonists, however, has not yet been explored in PD models.

Table 1.2. Biomaterials for intranasal drug delivery in rodent PD models.

Drug delivery system	Drug	Nanoparticle properties	Model	Dosing regimen	Volume	Outcomes	Ref
PLGA nanoparticle	L-DOPA	Size: 250 ± 50 nm EE: 10 ± 2 %	6-OHDA rat model	1 mg/kg daily for 13 weeks	Not disclosed	Improved motor function	Gambaryan et al. (2014)
Lactoferrin PLGA nanoparticles	Rotigotine	Size: 118.4±12.4 nm ZP: -21.94 ± 2.83 mV EE: 88.07 ± 6.01 % LC: 8.01 ± 0.05 %	6-OHDA rat model	2 mg/kg twice daily for 3 weeks	200 µl	Protected against dopaminergic cell loss in the nigro-striatal tract	Yan et al. (2018)
Lactoferrin and borneol co-modified PLGA nanoparticles	Dopamine	Size: 175.3 ± 9.6 nm PdI: <0.02 ZP: -15.7 ± 0.86 mV EE: 25.43 ± 5.32 %	6-OHDA rat model	20 days (dose not disclosed)	Not disclosed	Increased striatal dopamine content	Tang et al. (2019)
WGA-PLGA nanoparticles	L-DOPA	Size: 720.0 ± 87.39 nm PdI: 0.672 ± 0.073 ZP: -2.86 ± 0.440 mV EE: 50.47 %	MPTP mouse model	16 mg/kg daily for 7 days	20 µl	Improved locomotor activity	Arisoy et al. (2020)
Low-molecular weight protamine and lactoferrin co-modified PLGA nanoparticles	Bromocriptine	Size: 248.53 ± 16.25 nm PdI: 0.274 ZP: -2.63 ± 0.74 mV EE: 73.30 ± 0.74 % LC: 10.11 ± 0.1 %	Haloperidol mouse model	0.22 mg/kg daily for 2 weeks (30 mins after haloperidol injection)	Not disclosed	Improved haloperidol-induced hypokinesia and rigidity, antioxidant effects	Cong et al. (2025)
Biomimetic PLGA nanoparticles (coated with BV2 microglial cell membrane)	Metformin	Size: 252.2 nm PdI: 0.281 ZP: -20 mV EE: 10.76% LC: 3.98%	MPTP mouse model	100 mg/kg thrice weekly for 3 weeks	Not disclosed	Reversed motor impairments, attenuated dopaminergic cell loss, prevented α-synuclein	Li et al. (2025b)

Table 1.2. continued.

						accumulation, reduced microgliosis	
Poloxamer 407- chitosan nasal gel	Selegiline	N/A	Rotenone rat model	1 mg/kg daily for 10 days	25 µl	Improved motor function and restored dopamine levels, increased MAO-B activity	Sridhar et al. (2018)
Poloxamer 407-guar gum nasal gel	Pramipexole	N/A	Rotenone rat model	0.3 mg/kg daily for 10 days	20 µl	Increased locomotor activity and increased dopamine levels	Sridhar et al. (2020)
Glycyrrhizic acid nanogel	Albiflorin	N/A	Paraquat mouse model	0.075, 0.15 or 0.3 mg/kg once daily for 3 days	5 µl	Reversed motor deficits, protected against dopaminergic cell loss and reduced α -synuclein accumulation	Chen et al. (2022)
Poloxamer 407/188- PEG- β cyclodextrin nasal gel	Rhynchophylline	N/A	MPTP mouse model	10 mg/kg daily for 10 days	Not disclosed	Restored locomotor functions, rescued dopaminergic cell loss	Lin et al. (2023)
Polysorbate-caprylic nasal emulsion	Dodecyl creatine ester (creatine prodrug)	N/A	6-OHDA rat model	13.3 mg/kg daily for 5 weeks	25 µl	Improved motor performance, rescued nigro-striatal dopamine content, enhanced BDNF	Disdier et al. (2025)

PLGA: poly(lactic-co-glycolic acid); PEG: poly(ethylene glycol); Pdl: polydispersity index (<0.3 indicates nanoparticle uniformity); ZP: zeta potential (surface charge); EE: encapsulation efficiency; LC: loading capacity; 6-OHDA: 6-hydroxydopamine; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; WGA: wheat germ agglutinine; L-DOPA: levodopa.

1.7. Thesis aims

Given the heterogeneity of PD and the lack of effective treatments, novel therapeutic formulations are desperately needed. Emerging strategies of drug repurposing, nanoparticle delivery systems and intranasal drug administration are potential approaches for overcoming the challenges of drug delivery to the brain. The overarching aim of this thesis was to develop an anti-inflammatory drug-loaded nanoparticle system for intranasal delivery to the LPS rat model of PD-associated neuroinflammation and degeneration. This comprised of both *in vitro* and *in vivo* validation.

Chapter 3 aimed to establish primary cortical glial cultures that are responsive to inflammatory stimuli that could be used in future experiments to test cellular compatibility with nanoparticles. To achieve this, cells were stimulated with inflammatory stimuli IFN γ and LPS, and changes in glial gene expression and the effects of glial conditioned media on neuronal complexity were assessed.

Chapter 4 aimed to synthesise and characterise polymeric nanoparticles (PEG-PLGA). The compatibility of these nanoparticles was then tested in primary rat neuronal and glial cells to investigate whether unloaded PEG-PLGA may initiate an inflammatory response before progressing to drug loading and release studies.

Chapter 5 aimed to load PEG-PLGA nanoparticles with the candidate anti-inflammatory drug β_2 AR agonist formoterol (Form-PEG-PLGA) and study drug release mechanisms. Furthermore, Form-PEG-PLGA was incorporated into a mucoadhesive and thermosensitive chitosan-based hydrogel and drug release from this system was investigated for future *in vivo* intranasal applications. It was then necessary to test the anti-inflammatory effects of formoterol released from PEG-PLGA in an *in vitro* model of neuroinflammation.

Chapter 6 aimed to determine whether cargo released from the chitosan hydrogel could reach the brain following intranasal administration. This was investigated using manganese as a contrast agent for MRI in rats. This study then progressed to assess whether Form-PEG-PLGA-gel could reduce pro-inflammatory responses in the rat brain following systemic LPS exposure when delivered via the intranasal route.

Chapter 7 aimed to investigate whether intranasal delivery of Form-PEG-PLGA-gel could attenuate the microglial reactivity, dopaminergic cell loss and motor deficits induced by the stereotactic injection of LPS directly into the substantia nigra in rats.

Chapter 2: Materials & Methods

2.1. Materials

2.1.1. Animals

Male and female Wistar rats

Trinity College Comparative
Medicine Unit

Standard rat chow

Trinity College Comparative
Medicine Unit

2.1.2. General laboratory equipment

Analytical balance

Fisher scientific

Autoclave

Mason Technology

Benchtop large centrifuge

Sorvall

Benchtop mini centrifuge

Thermo Fisher Scientific

Biosafety cabinet

Chemflow, Chemical Systems
Control

Gyro-rocker

Stuart

Hot-stir plate

VWR

pH meter

Mettler Toledo

Vortex

Scientific Industries

2.1.3. General laboratory chemicals and reagents

Absolute Ethanol

Trinity College Hazardous
Materials

Biocidal

Trinity College Hazardous
Materials

Deionised water

TCIN

Dimethyl sulfoxide

Sigma-Aldrich

Ethanol, molecular grade

Trinity College Hazardous
Materials

Formoterol hemifumarate

Selleckchem

Hydrochloric acid

Sigma-Aldrich

Lipopolysaccharide
(Escherichia coli O111:B4 serotype)

Sigma-Aldrich

Methanol, HPLC grade	Trinity College Hazardous Materials
Phosphate buffered saline 10X (sterile)	Sigma-Aldrich
Potassium dihydrogen phosphate (KH_2PO_4)	Scientific laboratory supplies
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium dihydrogen phosphate (NaH_2PO_4)	Sigma-Aldrich
Sodium hydroxide (NaOH)	Sigma-Aldrich
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Ultrapure water, Milli-Q® system	Merck
Virkon	Trinity College Hazardous Materials
Whatman #2 filter paper	Sigma-Aldrich

2.1.4. General laboratory consumables

Conical tubes (15 ml, 50 ml)	Falcon
Micropipette tips, filtered (10, 20, 100, 200, 1000 μl)	Sarstedt
Micropipette tips, unfiltered (10, 20, 100, 200, 1000 μl)	Sarstedt
Micropipettes (p10, p20, p100, p200, p1000)	Eppendorf
Microtubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf
Pasteur pipettes (10 ml, 3.5 ml)	Sarstedt
Syringes (1 ml, 5 ml)	BD, Luer-Lok

2.1.5. Nanoparticle preparation, characterisation and drug release

Acetonitrile, HPLC grade	Trinity College Hazardous Materials
Amicon®Ultra Centrifugal 3 kDa filter tubes	Millipore, Sigma-Aldrich
Analytical balance	Scientific laboratory supplies
Benchtop centrifuge	Eppendorf
Spectra/Por® dialysis membrane, 50 kDa	Spectrum, Fischer Scientific
Disposable folded capillary cuvette	Malvern Panalytical
Calcium chloride (CaCl_2)	Sigma-Aldrich

Carboxylic acid poly(ethylene glycol)-block-poly(lactide-co-glycolide)	Sigma-Aldrich
Glass vials (5 mL, 10 mL)	Thermo Fisher Scientific
Glucose	Sigma-Aldrich
Hot-stir plate	Stuart
HPLC auto-injector SIL-10AD	Shimadzu
HPLC guard column C18	Phenomenex
HPLC guard column cartridges	Phenomenex
HPLC Hypersil™ BDS C18 column	Thermo Fisher Scientific
HPLC pump LC-10AT	Shimadzu
HPLC solvent degasser DGU-14A	Shimadzu
HPLC system controller SCL-10A	Shimadzu
HPLC UV detector SPD-10A	Shimadzu
HPLC vials (1.5 ml)	Thermo Fisher Scientific
JEM 2100 microscope	JEOL Ltd.
Magnesium chloride (MgCl ₂)	Sigma-Aldrich
Multi Reax 115V agitator	Heidolph
Needles 21G, 120 mm	B.Braun
Orbital shaker-incubator ES-20	Grant Bio
Orthophosphoric acid	Sigma-Aldrich
Potassium chloride (KCl)	Sigma-Aldrich
Potassium dihydrogen orthophosphate (KH ₂ PO ₄)	
Regenerated cellulose 0.45 µm syringe filter	Sartorius Ltd.
Sodium bicarbonate	Sigma-Aldrich
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium dihydrogen phosphate (NaH ₂ PO ₄)	Sigma-Aldrich
Syringe 1mL	Henke-ject
Syringe pump	KDS, Sigma-Aldrich
Tetrahydrofuran	Sigma-Aldrich
Ultrasonic bath	Fisher Scientific
Zetasizer Nano series Nano-ZS ZEN3600	Malvern Panalytical

2.1.6. Hydrogel preparation

Acetic acid	Sigma-Aldrich
Chitosan (MW 310,000-275,000)	Biosynth
β -glycerophosphate disodium salt hydrate	Sigma-Aldrich

2.1.7. Cell culture

B-27 Supplement	Sigma-Aldrich
Cell culture flasks (T75 cm ²)	Sarstedt
Cell culture plates (24-well, 96-well)	Sarstedt
Cell strainers (40 μ m)	Fisher
CoolCell™ cell freezing container	Corning®
Cryogenic vial (2 ml)	Sigma-Aldrich
Dulbecco's modified Eagle medium: F12	Sigma-Aldrich
EDTA vacuette tubes (9 ml)	Thermo Fisher Scientific
Foetal Bovine Serum- heat inactivated	Sigma-Aldrich
Fungizone	Sigma-Aldrich
Glass round coverslips (13 mm)	Thermo Fisher Scientific
Glutamax	Sigma-Aldrich
Haemocytometer	Thermo Fisher Scientific
Histopaque®-1077	Sigma-Aldrich
Laboratory carbon dioxide (CO ₂)	BOC gas
Lactate dehydrogenase assay	Thermo Fisher Scientific
Laminar flow hood, Optimale 18	ADS Laminaire
Neurobasal™ medium, Gibco™	Thermo Fisher Scientific
Penicillin-streptomycin	Sigma-Aldrich
Petri dishes (90 mm)	Fisher
Poly-D-lysine hydrobromide	Sigma-Aldrich
Poly-L-lysine hydrobromide	Sigma-Aldrich
Rat GM-CSF	Peprtech
Recombinant rat IFN γ	Peprtech
RPMI, Gibco™	Thermo Fisher Scientific
SteriCycle CO ₂ incubator	Thermo Fisher Scientific
Sterile filters (0.22 μ m)	Sigma-Aldrich
Sterile scalpels	Sarstedt

Trypan Blue (0.4%)	Sigma-Aldrich
Trypsin-EDTA solution (0.25%)	Sigma-Aldrich
96-well microplate reader	Biotek

2.1.8. Cell lysis, RNA isolation, CDNA manufacture and PCR

High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific
Nanodrop spectrophotometer	Thermo Fisher Scientific
NucleoSpin RNA, Mini kit	Macherey-Nagel
MicroAMP™ fast optical 96-well PCR plate	Thermo Fisher Scientific
Microplate seal	Agilent
QuantStudio5 Real-time PCR machine	Thermo Fisher Scientific
PCR plates	Thermo Fisher Scientific
PCR primers	Thermo Fisher Scientific
rDNase	Macherey-Nagel
RNase-free micropipette tips (10, 200, 1000 µl)	Sarstedt
RNase-free tubes (1 ml, 0.5 ml, 0.2 ml)	Axygen® Corning
RNase-free water	Thermo Fisher Scientific
RNaseZap	Thermo Fisher Scientific
Taqman™ fast advanced mastermix	Thermo Fisher Scientific
Thermal cycler	Bio Rad
β-mercaptoethanol	Sigma-Aldrich

2.1.9. Immunocytochemistry

Alexa Fluor 488 goat anti-rabbit (A11008)	Thermo Fisher Scientific
Alexa Fluor 546 goat anti-mouse (A11003)	Thermo Fisher Scientific
Bovine serum albumin	Sigma-Aldrich
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Aldrich
Epifluorescence microscope	Axio Imager.Z1, Carl Zeiss
Epifluorescence microscope camera	Axiocam 305 mono, Carl Zeiss
Glass microscope slides (76mm x 26 mm)	Epredia
Mouse anti-MAP2 (M1406)	Sigma-Aldrich
Normal goat serum	Sigma-Aldrich
Paraformaldehyde solution (37%)	Sigma-Aldrich
Rabbit anti-IBA1 (019-19741)	Wako, Fujifilm

ProLong™ Diamond antifade mountant, Invitrogen™	Thermo Fisher Scientific
---	--------------------------

2.1.10. Behavioural testing

D-amphetamine	Sigma-Aldrich
Polycarbonate cylinder	N/A
Rotarod	Ugo-Basile
Video camera	Canon, Logitech

2.1.11. Stereotactic surgery

Alcohol swabs	Roche's chemist, Bray
Buprecare (Buprenorphine)	Trinity College Comparative Medicine Unit
Chlorohexidine solution, 4%	Roche's chemist, Bray
SHIYANG III high speed dental drill	SJK Dental Corporation Ltd.
Drill bit replacements (224RF 018)	Meisinger
Hamilton® Neuros needles (33/20/3)	Hamilton, Switzerland
Hamilton® Neuros (7002 KH SYR) 5 µl syringe	Hamilton, Switzerland
Isoflurane vaporiser, Vet Tech 3 VT1499	Vet Tech
Isoflurin® (Isoflurane)	Trinity College Comparative Medicine Unit
LACRI-LUBE eye ointment	Trinity College Comparative Medicine Unit
Lidocaine topical cream, 5%	Trinity College Comparative Medicine Unit
Xylocaine topical spray (10 mg lidocaine/spray)	Trinity College Comparative Medicine Unit
Medical oxygen (O ₂)	BOC gas
Meloxicam	Trinity College Comparative Medicine Unit
Syringe needles (25 G x 13 mm)	BD, Luer-Lok
Povidone iodine, 10%	Trinity College Comparative Medicine Unit
Sterile saline (0.89% NaCl)	Trinity Pharmacy, Dublin 2
Sterile scalpel blades, 21 blade	Thermo Fisher Scientific
Surgibond tissue adhesive	Trinity College Comparative Medicine Unit

2.1.12. Intranasal drug administration

Venflon™ catheter	BD Luer-Lok
Isoamyl acetate	Thermo Fisher Scientific

2.1.13. Transcardial perfusion and MRI

Euthasol (sodium pentobarbital)	Trinity College Comparative Medicine Unit
Dissection tools	Fine Science Tools
Isopentane (2-methylbutane)	Trinity College Hazardous Materials
Manganese	Sigma-Aldrich
MRI 7T	Bruker
Paraformaldehyde	Sigma-Aldrich
Perfusion pump	Gilson
Sucrose	Sigma-Aldrich
Syringe needles (23 G x 13 mm)	BD, Luer-Lok

2.1.14. Post-mortem tissue processing

Chromium (III) potassium sulfate ($\text{KCr}(\text{SO}_4)_2$)	Thermo Fisher Scientific
Cryostat CM1850	Leica
Gelatin	Sigma-Aldrich
Glass slide coverslips (60 mm x 22 mm)	Sigma-Aldrich
Microtome blades (MX35 Ultra™, 35°, 80 mm)	Epredia
7T MRI	Bruker
Optimal cutting temperature compound	Sakura Finetek
Polytron PT 1300 D handheld homogeniser	Kinematica AG
Super-frost glass microscope slides (75 mm x 25 mm x 1mm)	Epredia

2.1.15. Immunohistochemistry

DPX mounting media	Thermo Fisher Scientific
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

Glass slide coverslips (76 mm x 26 mm)	Sigma-Aldrich
Hydrogen peroxide, 30%	Sigma-Aldrich
Light microscope, BX51	Olympus
Light microscope digital camera, DRP72	Olympus
Microscope slides (76 mm x 26 mm)	Epredia
Microtome blades (MX35 Ultra™, 35°, 80 mm)	Epredia
Mouse anti-TH (MAB318)	Millipore
Normal goat serum	Sigma-Aldrich
Normal horse serum	Sigma-Aldrich
PAP pen	Sigma-Aldrich
Rabbit anti-IBA1 (019-19741)	WAKO, FUJIFILM
Xylene	Trinity College Hazardous Materials
3,3'-diaminobenzidine	Sigma-Aldrich

2.1.16. Software

ANYmaze version 7.52	Stoelting
BioRender	Biorender.com
CellSens™	Olympus
Quant Studio Design and Analysis version 2.7.0.	Applied Biosystems, Thermo Fisher Scientific
ImageJ version 1.54	National Institute of Health
Lab Solutions, version 5.71 SP1	Shimadzu
MATLAB (2025a)	MathWorks
Nanodrop 2000	Thermo Fisher Scientific
Prism 10	Graphpad
G*Power	Heinrich Heine Universität Düsseldorf
Zeiss ZEN 3.8	Carl Zeiss
Zetasizer software	Malvern

2.2. Pharmaceutical methods

2.2.1. Nanoparticle synthesis

Nanoparticles were prepared using a nanoprecipitation technique, as previously described by de Oliveira et al. (2013). Briefly, 20 mg of carboxylic acid-terminated poly(ethylene glycol)-block-poly(lactide-co-glycolide) copolymer (Sigma-Aldrich) was dissolved in 2 ml of tetrahydrofuran and added dropwise to 5 ml ultrapure water using a syringe pump under constant stirring to obtain PEG-PLGA nanoparticles. Residual tetrahydrofuran was evaporated in a fume hood overnight under constant agitation and the particles were filtered through a 0.45 μm regenerated cellulose syringe filter to remove aggregates. PEG-PLGA nanoparticles were isolated using Amicon®Ultra 3 kDa centrifugal filter tubes (Millipore) that were spun at 1,000 x g for 30 mins. Isolated nanoparticles were stored at 4°C until further use.

To load particles with drug, formoterol hemifumarate (Selleckchem) was reconstituted in dimethyl sulfoxide (DMSO, 100%) (50 mM/21 mg/ml) before being added to the organic phase containing polymer (65% w/w of polymer). Drug-loaded nanoparticles are herein referred to as Form-PEG-PLGA.

2.2.2. Nanoparticle characterisation

The size and uniformity of PEG-PLGA nanoparticles were determined by measuring the hydrodynamic diameter (diameter of particles in solution) and polydispersity index (Pdl), respectively, using dynamic light scattering (DLS). The zeta (ζ) potential (surface charge) was measured by electrophoretic light scattering (ELS) using a Zetasizer Nano series Nano-ZS ZEN3600 (Malvern Instruments) equipped with a red laser ($\lambda = 633 \text{ nm}$). Measurements were performed at 25°C with an equilibration time of 60 seconds.

DLS determines the hydrodynamic diameter based on the Brownian motion of particles in suspension, where smaller particles diffuse faster, producing more rapid fluctuations in scattered light intensity. ELS measures the electrokinetic potential of nanoparticles, termed the ζ potential, which is the potential at the slipping plane of a particle as it moves under an electric field (Bhattacharjee, 2016). For characterisation, PEG-PLGA were diluted in ultrapure water ($\sim 0.1 \text{ mg/ml}$), samples were sonicated to ensure a polydisperse suspension and loaded into a folded capillary cuvette. Five measurements were taken for each sample and the average hydrodynamic diameter (nm) and ζ -potential (mV) of PEG-PLGA were calculated for each batch.

Transmission electron microscopy (TEM) of PEG-PLGA was performed by Dr. Raman Bekarevich (School of Chemistry, Trinity College Dublin) and Ms. Almudena Moreno-Borralló (School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin) using a JEOL 2100 microscope at 200 kV.

2.2.3. Encapsulation efficiency and High-Performance Liquid Chromatography (HPLC) coupled to UV detection

Drug encapsulation was quantified by measuring the amount of unencapsulated drug in the filtrate after nanoparticle isolation using high-performance liquid chromatography (HPLC) coupled to UV detection based on the method described by Nadarassan et al. (2007). Briefly, chromatographic separations were carried out at room temperature using a Hypersil™ BDS C18 (250 mm x 4.6 mm x 5 µm) analytical column (Thermo Fisher Scientific), which was protected by a guard column fitted with C18 cartridges (4 x 3 mm) (Phenomenex). The mobile phase consisted of 5 mM potassium dihydrogen orthophosphate (KH₂PO₄) and acetonitrile (80:20, v/v) adjusted to pH 2.5 using orthophosphoric acid and was delivered at a flow rate of 1 ml/min. The injection volume for each sample was 25 µl. Stock solution of formoterol hemifumarate (50 mM/21 mg/ml) prepared in DMSO (100%) was diluted to 1 mg/ml in mobile phase and a serial dilution was performed for a standard curve (**Fig. 2.1**). Filtrate samples were diluted 1:4 in mobile phase and compared to standards to determine the amount of unencapsulated formoterol. The encapsulation efficiency (EE) of Form-PEG-PLGA was calculated using **equation 1**. The loading capacity (LC) of the synthesised nanoparticles was calculated using **equation 2**.

Equation 1:

$$\text{Encapsulation Efficiency (\%)} = \left(\frac{\text{Total drug added} - \text{free drug}}{\text{Total drug added}} \right) \times 100$$

Equation 2:

$$\text{Loading capacity (\%)} = \left(\frac{\text{Mass of encapsulated drug}}{\text{Mass of nanoparticles}} \right) \times 100$$

2.2.4. Hydrogel preparation

Hydrogel with thermosensitive and mucoadhesive properties was prepared based on the method described by Perkušić et al. (2023). High molecular weight chitosan was dissolved in 0.1M acetic acid to achieve a final 2% (w/v) gel concentration under constant stirring and gentle heat. Formoterol or Form-PEG-PLGA were added slowly to the chitosan solution to achieve 2.2 mg of drug per ml of gel. β-glycerophosphate disodium salt (βGP) was dissolved in ultrapure water to achieve a final 18.8% (w/v) gel concentration and added to the chitosan-drug/nanoparticle solution at a rate of 15 ml/hour under constant stirring. Gels were prepared fresh for *in vivo* administration or were solidified overnight at 37°C for drug release studies.

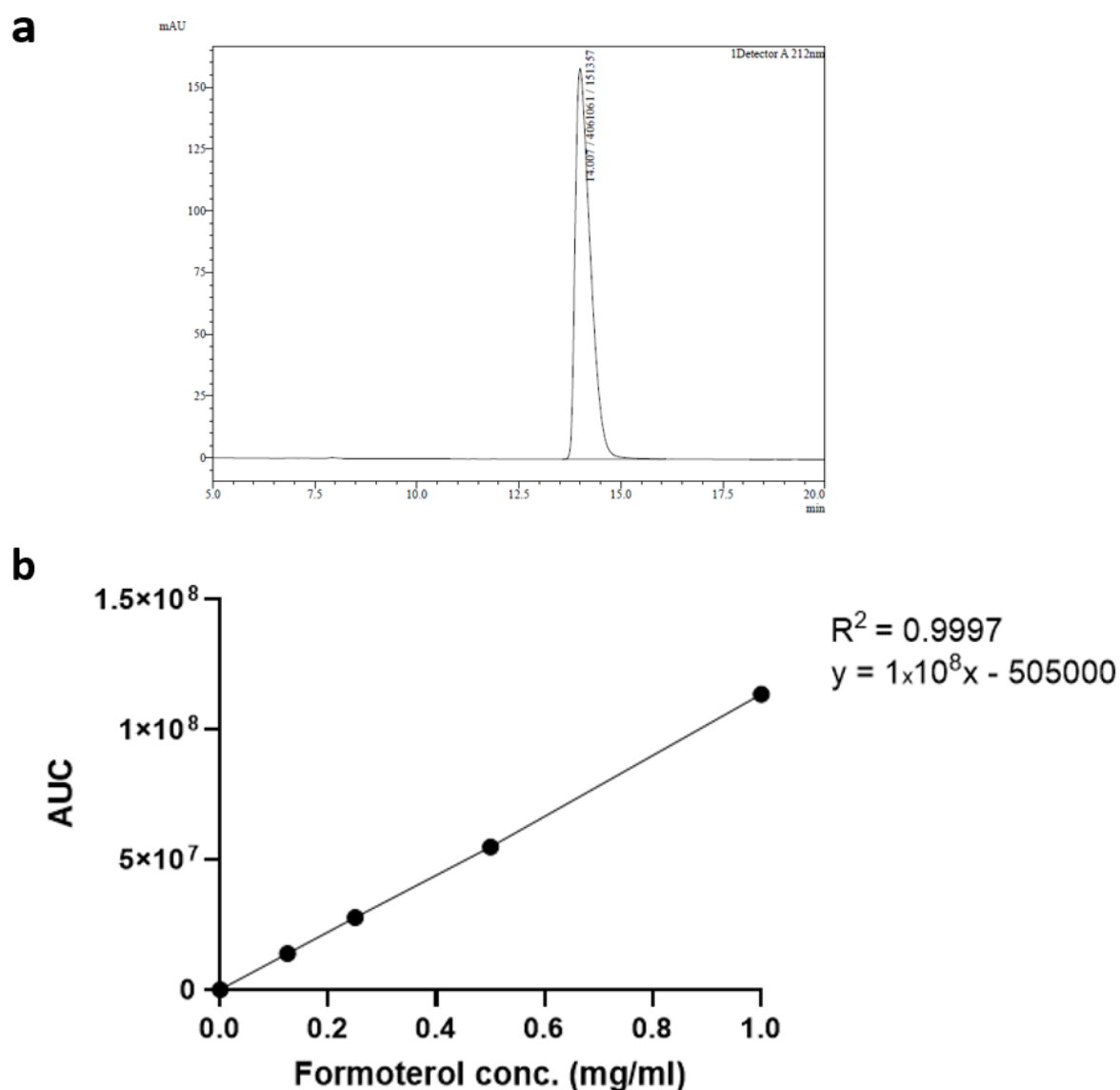


Figure 2.1. Formoterol hemifumarate detection by HPLC-UV.

(a) Sample chromatogram for formoterol with a retention time of ~14 minutes when detected by HPLC-UV. (b) Standard curve for calculating the amount of unencapsulated formoterol in the filtrate following nanoparticle isolation. AUC: area under the curve of the chromatogram.

2.2.5. Drug release

The experimental set-up for testing drug release from Form-PEG-PLGA and Form-PEG-PLGA-hydrogel is depicted in **Fig. 2.2**.

Form-PEG-PLGA or free drug was suspended in 1 ml of ultrapure water (amount of formoterol was equal to 100 µg/ml) and loaded into a 50 kDa dialysis membrane sealed at both ends. The membrane was completely submerged in pre-warmed 10 ml of artificial cerebrospinal fluid (aCSF; **Table 2.1**) to mimic CNS conditions and placed in an orbital shaker set to 37°C and 200 rpm. The release media (aCSF) was completely removed and replaced every 10 minutes for one hour, then every 30 minutes until the four-hour timepoint, every hour until the six-hour timepoint, every two hours until the 12-hour timepoint and lastly at 24 hours.

Hydrogels containing Form-PEG-PLGA or free formoterol were solidified overnight at 37°C (amount of formoterol was equal to 100 µg/ml). 10 ml of pre-warmed 1x phosphate buffered saline (PBS, pH 6.5) was added into the glass vial containing hydrogels to mimic conditions in the nasal cavity. Hydrogels were placed in an orbital shaker set to 37°C and 200 rpm. The release media (PBS) was completely removed and replaced every 10 minutes for one hour, then every 30 minutes until the four-hour timepoint, every hour until the six-hour timepoint, every two hours until the 12-hour timepoint and daily until the 72-hour timepoint.

Drug release was quantified using the HPLC-UV method described in section 2.2.3. A stock solution of formoterol hemifumarate was prepared in aCSF or PBS (100 µg/ml) and a serial dilution was performed to prepare standards for a standard curve. The % drug release for each time point was calculated using **equation 3**.

Table 2.1. aCSF ingredients.

Ingredients	Concentration
Sodium chloride (NaCl)	119 mM
Sodium bicarbonate (NaHCO ₃)	26.2 mM
Calcium chloride (CaCl ₂)	2.5 mM
Potassium chloride (KCl)	2.5 mM
Magnesium chloride (MgCl ₂)	1.3 mM
Sodium dihydrogen phosphate (NaH ₂ PO ₄)	1 mM
Glucose	11 mM
HPLC grade water (1 L)	-

Equation 3:

$$\text{Drug release (\%)} = \left(\frac{\text{Drug released}}{\text{Starting drug concentration}} \right) \times 100$$

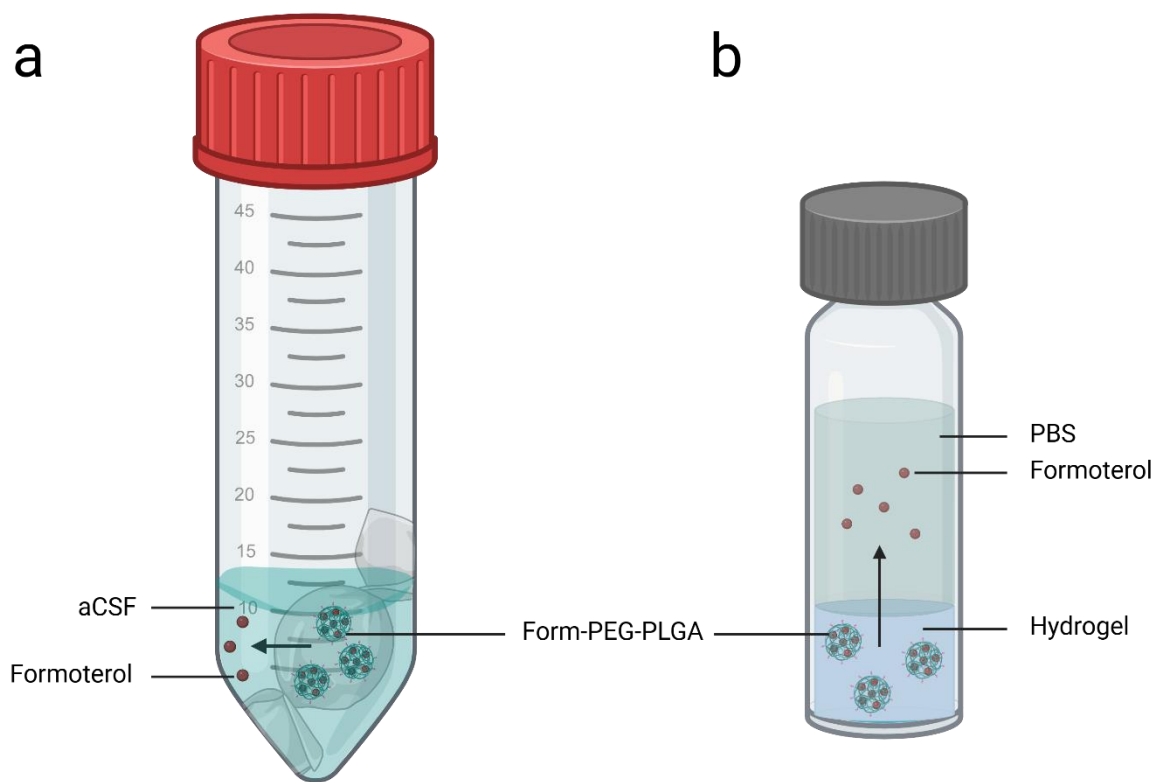


Figure 2.2. Drug release experiments.

Experimental set-up for measuring drug release from (a) Form-PEG-PLGA nanoparticles and (b) Form-PEG-PLGA-hydrogel.

2.2.6. Mathematical models of drug release

The Korsmeyer-Peppas and Weibull models of drug release are among the most frequently reported to describe drug release from polymeric nanoparticles (Garcia et al., 2023; Lopez-Mitjavila et al., 2025).

The Korsmeyer-Peppas model was applied to the first 6 time points of formoterol release from nanoparticles and the nanoparticle-hydrogel system using **equation 4**, where Mt is the amount of drug released at time t , M_{∞} is the total amount of drug released after infinite time, K is the Korsmeyer-Peppas rate constant and n is the diffusional exponent, which determines the release mechanism.

Equation 4: $\frac{Mt}{M_{\infty}} = Kt^n$

Drug release from Form-PEG-PLGA was analysed using the Weibull model (**equation 5**), which describes the cumulative fraction released as a function of time using the α scale and β shape parameters.

Equation 5: $\% release_{\infty}^0 = 1 - \exp \left[- \left(\frac{t}{\alpha} \right)^{\beta} \right]$

To interpret drug release from the Form-PEG-PLGA-hydrogel dual release system, a double Weibull model (**equation 6**) was applied as described by Ruiz-Picazo et al. (2024), which describes dissolution in two sequential stages of release from a matrix. Each stage of release is described by α scale and β shape parameters.

Equation 6:

$$\% release_{\infty}^0 = Fmax_1 + \left((Fmax_2 \cdot Fmax_1) \cdot \left(1 - \exp \left[- \left(\frac{t - gt}{\alpha_2} \right)^{\beta_2} \right] \right) \right)$$

2.3. Cell culture methods

2.3.1. Aseptic technique

All cell culture work was conducted using aseptic techniques in a sterile laminar flow hood (Laminaire). Cells were maintained in a sterile humidified incubation chamber (Thermo Scientific) at 37°C and 5% carbon dioxide. Sterile reagents and autoclaved consumables were used for all cell culture work.

2.3.2. Preparation of cell culture media

All ingredients were filter sterilised prior to batch preparation of cell culture media (**table 2.2**). Media was pre-warmed in a sterile water bath at 37°C before use in culturing, media changes or the application of experimental treatments.

Table 2.2. Cell culture media ingredients.

Culture media	Ingredients (v/v)
Plating Neurobasal media (pNBM)	6% foetal bovine serum (FBS) 1% Glutamax 1% penicillin-streptomycin 0.1% fungizone
Feeding Neurobasal media (fNBM)	1% Glutamax 1% B27 neuronal supplement 1% penicillin-streptomycin 0.1% fungizone
Plating Dulbecco's Modified Eagle media (pDMEM)	10% foetal bovine serum (FBS) 1% penicillin-streptomycin 0.1% fungizone
Feeding Dulbecco's Modified Eagle media (fDMEM)	10% foetal bovine serum (FBS) 1% penicillin-streptomycin 0.1% fungizone 0.1% granulocyte-macrophage colony-stimulating factor (GM-CSF)
Treatment Dulbecco's Modified Eagle media (tDMEM)	1% penicillin-streptomycin 0.1% fungizone

2.3.3. Preparation of primary cortical rat neuronal cultures

Cortical neurons were isolated from the brains of 1–2-day-old Wistar rat neonates. Briefly, rats were decapitated and the brain was removed from the skull. Olfactory bulbs, the cerebellum and any surface blood vessels were removed before finely chopping the cortical tissue with a sterile scalpel. Cortical tissue was then dissociated in trypsin-ethylenediaminetetraacetic acid (EDTA) for 2 minutes at 37°C. pNBM was used to deactivate trypsin activity, the tissue was triturated and centrifuged at 2000 rpm for 3

minutes at 21°C. The supernatant was discarded, the pellet reconstituted in 5 ml of pNBM and passed through a 40µm nylon cell strainer. The cell suspension was centrifuged again at 2000 rpm for 3 minutes at 21°C. The supernatant was discarded and cells were resuspended in 1 ml of pNBM. Cells were counted using the trypan blue exclusion method using a haemocytometer. Cells were plated in 100 µl of pNBM at a density of 3×10^5 cells/ml on coverslips pre-coated with Poly-D-lysine to promote cell adhesion. After 2-3 hours, a further 200 µl of pNBM was added to each well. 15-24 hours after plating, the pNBM was completely removed from each well and replaced with fNBM. Media was changed every 5-7 days using fNBM until cells were mature at day *in vitro* (DIV) 14 when they were considered mature for use in experiments.

2.3.4. Preparation of primary cortical rat glial cultures enriched for microglia

Cortical glia were isolated from the brains of 2-3-day-old Wistar rat neonates. Brain tissue was isolated as described above and incubated in pDMEM for 20 minutes at 37°C. The tissue was then triturated and passed through a 40µm nylon cell strainer. The cell suspension was centrifuged at 2000 rpm for 3 minutes at 21°C. The supernatant was removed and cells were resuspended in 1 ml of pDMEM, which was either added directly into T-75 flasks pre-coated with poly-L-lysine containing 3 ml of pDMEM or plated 100 µl per well of a 24-well plate pre-coated with poly-L-lysine for mixed glial cultures. 2-3 hours after plating, a further 5ml of pDMEM was added to each flask or 200ul was added to each well of the 24-well plate and 24 hours later the pDMEM was completely removed and replaced with fDMEM. Media in T-75 flasks and 24-well plates was changed every 3-4 days. When cells in T-75 flasks reached DIV 8-12 cultures were plated and enriched for microglia. Briefly, T-75 flasks were rocked for 3-4 hours at 200 rpm in a sterile orbital shaker maintained at 37°C. The microglia, now lifted into the media, were collected in a fresh falcon tube. The bottom of the flask was briefly scraped in a further 1 ml of fDMEM to collect some but not all astrocytes, which remain adhered to the base of the flask, and added to the microglial cell suspension. Cells were centrifuged at 2000 rpm for 5 minutes at 37°C. The supernatant was discarded and glial cultures enriched for microglia were plated in 100 µl of fDMEM in 24-well plates precoated with Poly-L-lysine at a density of 3×10^5 cells/ml cells per well. A further 200 µl of fDMEM was added to each well 2-3 hours after plating and media was changed every 3-4 days until cells were mature at DIV 14 and ready for use in experiments.

2.3.5. Cell culture treatments

All cell culture treatments were applied as a 50% media change to avoid cells becoming reactive to a media change. Glial media was changed to pDMEM 2-3 days before applying treatment as GM-CSF can increase baseline microglial reactivity. All glial treatments were

applied in tDMEM (see **table 2.2**) for the collection of conditioned media as FBS can cause cytotoxicity and the production of ROS in neuronal cultures. Isolated nanoparticles were sterilised under UV light for 30 minutes prior to cell treatments. Stock solutions of lipopolysaccharide (LPS) or Interferon γ (IFN γ) were prepared under aseptic conditions in PBS or 0.1% bovine serum albumin (BSA), respectively before being diluted to working concentrations in the appropriate cell culture media. Nanoparticles were sterilised under UV light for 30 mins and immediately applied to cells in culture media. Conditioned media (CM) was collected from enriched microglial cultures where stated, and stored at -20°C until use. CM was centrifuged at 2000 rpm for 3 minutes at room temperature and the supernatant was transferred to mature neurons as a 50% media change.

2.3.6. Peripheral blood mononuclear cell (PBMC) cell treatments

Blood collection from healthy donors, PBMC isolation and cell preparation were carried out by Mr. Christopher Sheridan (School of Medicine, Trinity College Dublin). Ethical approval was obtained from the Faculty of Health Sciences Research Ethics Committee at Trinity College Dublin (reference no. 230905). Briefly, up to 60 ml of blood was obtained by forearm venepuncture in two male and one female healthy volunteers aged 26-29 and collected in 9 ml ethylenediaminetetraacetic acid (EDTA) vacuette tubes. Blood was mixed with an equal volume of PBS and 2% FBS. This mixture was added to 15mL of Histopaque density gradient cell separation medium and spun at 600 x g for 25 minutes. Following this, 10- 15 ml of the PBMC “buffy coat” layer was removed, washed with PBS and 2% FBS and spun at 300 x g for 8 minutes twice. The cell pellet was resuspended in 1 ml of complete RPMI and cells were counted using the trypan blue exclusion method and a haemocytometer. Cells were resuspended in cryopreservation media (90% FBS and 10% DMSO) in a dropwise manner and aliquoted into 2 ml cryovials at a density of 800,000 to 1,200,000 PBMC/ml. Cryovials were stored for 24h in a CoolCell container in a -80°C freezer before being transferred to a long-term storage box. Cells were thawed at 37°C and pre-warmed complete RPMI medium was added into the cryovial to achieve a 1:1 dilution. The diluted cell suspension was transferred to 8 ml of pre-warmed complete RPMI medium. Cells were centrifuged at 250 x g for 7 minutes and the pellet was resuspended in the desired volume of warm complete RPMI medium. Cell number and viability were determined using a hemacytometer with the trypan blue method. The cell suspension was diluted to a final working concentration of 5×10^6 PBMCs/ml in room temperature complete RPMI medium and 160 μ l of cell suspension was added into wells in a 24-well plate. Cells were allowed to settle for 1 hour before treatments were applied in 40 μ l of pre-warmed RPMI culture media. Treatments were applied for 24 hours, as previously described by (Crowley et al., 2015), who demonstrated robust inflammatory signalling at this timepoint. Cells were then lysed and RNA isolated for

downstream PCR analysis as described in section 2.3.8. Culture media was also collected for LDH assay as described in section 2.3.7.

2.3.7. Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released into culture media upon damage to the cell membrane. Therefore, the presence of LDH in culture media is an indication of cytotoxicity. In this assay, extracellular LDH is quantified by an enzymatic reaction whereby 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 to a red formazan product that can be measured at 490nm. The quantity of formazan is directly proportional to the amount of LDH present in cell culture media.

LDH assays were performed according to manufacturer's instructions (Invitrogen). 30 μl of glial or neuronal cell culture media was loaded into wells of an optically clear 96 well plate in duplicate and incubated with 30 μl of reaction mixture for 30 minutes protected from light and on a gyrorocker. After 30 minutes, 30 μl of stop solution was then added to each well and the absorbance was read at 490 nm and 680 nm on a microplate reader (Synergy HT, BioTek Instruments). Background absorbance values at 680 nm were subtracted from 490 nm to quantify LDH release from experimental groups. Extracellular LDH is presented as absorbance expressed as a percentage of controls, where 100% represents baseline LDH. Negative controls (fresh culture media not exposed to cells) and positive controls containing LDH supplied within the assay kit were also included in the assay to confirm test results.

2.3.8. Cell lysis, RNA isolation, cDNA synthesis and qRT-PCR

The Macherey-Nagel™ NucleoSpin™ ribonucleic acid (RNA) isolation kit (Macherey Nagel) was used in accordance with manufacturer's instructions to lyse cells and isolate ribonucleic acid (RNA). Cell lysis buffer containing β -mercaptoethanol and kit RA1 buffer (1:100) was applied to cells for 5 minutes. Cell lysates were scraped into RNase-free tubes and stored at -80°C overnight or until used.

Samples were thawed on ice and mixed with a micropipette to homogenise lysate following freeze-thaw. Cell lysate was passed through a violet ring filter and centrifuged at 11,000 x g for 1 minute to clear the lysate. Samples were diluted 1:1 with 70% culture-grade RNase-free ethanol (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 11,000 x g for 30 seconds to bind RNA to the filter. Sample were washed with membrane desalting buffer and centrifuged at 11,000 x g 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 deoxyribonucleic acid (DNA). rDNase was deactivated with RAW2 buffer and centrifuged at 11,000 x g for 30 seconds. RA3 buffer was passed through the filter and centrifuged first for 30 seconds at 11,000 x g, then passed through again and centrifuged for 2 minutes at 11,000 x g. The blue ring filter was placed into an RNase-free Eppendorf tube, and RNA eluted in RNase-free water by centrifugation at 11,000 x g for 1 minute. RNA was then stored at -80°C until use.

The amount of RNA in each sample was quantified using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific). The amount of RNA in each sample was equalised to the lowest sample using RNase-free water. Complimentary DNA (cDNA) strands were manufactured using a high-capacity cDNA reverse transcription kit using equalised RNA templates in a 1:1 ratio with reverse transcription master mix according to manufacturer's instructions (Applied Biosystems, ThermoFisher Scientific). cDNA was synthesised in a thermocycler (MJ Research) at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and 4°C to ∞. Samples were diluted 1:1 in RNase-free water and stored at -20°C until use.

Quantitative real time polymerase chain reaction (qRT-PCR) was performed by adding 5 µl of Taqman mastermix with 0.5 µl of primer for the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 0.5µL of primer for the target gene (see **table 2.3**) to 4 µl of cDNA in a reaction plate. Loaded plates were sealed a briefly centrifuged to drive liquid to the bottom of the well. qRT-PCR was performed using the QuantStudio™ 5 system with an initial denaturation at 50°C for 2 minutes and 95°C for 20 seconds, followed by a cycling stage of denaturation at 95°C for 1 second and annealing and extension at 60 °C for 2 minutes. The amount of fluorescence at the end of each cycling stage is detected and relates to gene amplification. Data were analysed with the QuantStudio™ Design and Analysis software using the comparative CT method ($\Delta\Delta Ct$).

Table 2.3. Rat and human PCR primers.

Species	Primers
Rat	<i>Gapdh</i> : Rn01775763_g1 (VIC-MGB)
	<i>Il1b</i> : Rn00580432_m1 (FAM-MGB)
	<i>Tnfα</i> : Rn00562055_m1 (FAM-MGB)
	<i>Nos2</i> : Rn00561646_m1 (FAM-MGB)
	<i>Cd40</i> : Rn01423584_g1 (FAM-MGB)
	<i>Cd68</i> : Rn01495634_g1 (FAM-MGB)
Human	<i>GAPDH</i> : Hs02786624_g1 (VIC-MGB)
	<i>IL1B</i> : Hs01555410_m1 (FAM-MGB)
	<i>TNFα</i> : Hs00174128_m1 (FAM-MGB)
	<i>IL6</i> : Hs00174131_m1 (FAM-MGB)

2.3.9. Immunocytochemistry

Ionised calcium-binding adaptor protein 1 (IBA1) is widely used as a marker for microglia. Mature mixed glia were fixed with 4% paraformaldehyde (PFA) for 20 minutes followed by three PBS washes. Cells were blocked for 30 minutes with 5% goat serum in 0.05% Triton-X in PBS (PBS-T). Rabbit anti-IBA1 primary antibody [011-27991] was prepared in blocking buffer (1:1000) and applied overnight at 4°C. Cells were washed and goat anti-rabbit Alexa Fluor 488 [A11008] (1:1000) secondary antibody was prepared in PBS and applied for 2 hours at room temperature in a light-protected environment. Coverslips were washed and cells were counterstained with for DNA, 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (1:10,000 in deionised water) for 20 minutes at room temperature in a light protected environment. Coverslips were mounted on glass microscope slides using ProLong™ Diamond antifade mounting media, and stored in a light protected environment at 4°C.

Microtubule associated protein 2 (MAP2) is a cytoskeletal protein expressed abundantly throughout mature neurons and was used as a marker to visualise and assess neuronal complexity (**fig.2.3a**). Mature neurons were stained as above except for cells were blocked for 2 hours using 5% normal goat serum (NGS) and 5% BSA in 0.1% PBS-T and mouse anti-MAP2 primary antibody [M1406] was prepared in blocking buffer (1:1000) and applied overnight at 4°C. A goat anti-mouse Alexa Fluor 488 secondary antibody [A11029] in 0.1% PBS-T (1:500) was applied as above.

2.3.10. Imaging of MAP2+ neurons and Sholl analysis

MAP2 stained neurons were imaged at 20X magnification using an AxioImager Z1 epifluorescent microscope with a Zeiss AxioCam 305 mono microscope camera and Zeiss ZEN 3.8 software, where approximately 10-15 cells in randomly selected locations on each coverslip were imaged for analysis (three coverslips per treatment group). A constant exposure was maintained throughout each coverslip.

Sholl analysis is a method of quantifying neuronal complexity (Gutierrez & Davies, 2007; Sholl, 1953). The soma, primary neurites and axon terminals were identified manually for each neuron and MATLAB (R2025b) was used to perform an adapted fast Sholl method for analysis by overlaying 25 concentric rings 10 µm apart to each neuron imaged, beginning at the identified soma (**fig 2.3b**). 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 'ith' segment, 'b_i' is the number of branches in the 'ith' segment and 't_i' is the number of neurite terminals in the 'ith' segment. This equation was programmed into MATLAB to allow for a semi-automated process to quantify the number of primary neurites extending from the soma, the length of these neurites and the extent of neuritic branching. The

number of intersections with the concentric rings was also quantified to produce a resulting Sholl profile.

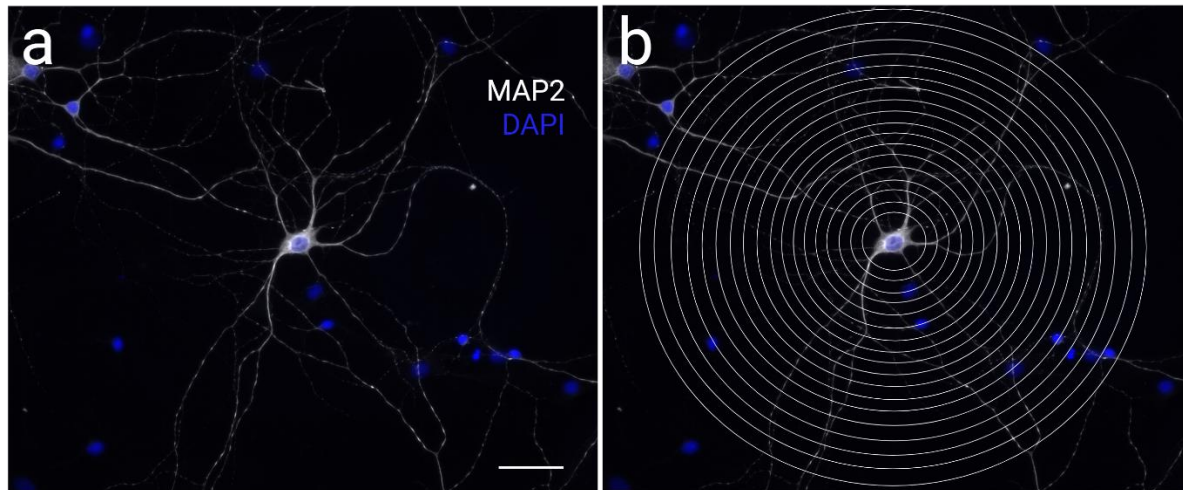


Figure 2.3. Sholl analysis of MAP2⁺ neurons.

(a) Representative image of MAP2 stained mature primary cortical rat neuron and (b) visualisation of overlaying Sholl analysis concentric rings at 10 μm intervals. The Sholl profile of neuronal complexity is constructed by quantifying the number of times the neuritic branches intersect with the rings at 10-260 μm from the cell soma. Magnification 20X, scale bar = 50 μm . MAP2: microtubule associated protein 2 (neuronal marker); DAPI: 4',6-diamidino-2-phenylindole (nucleic marker).

2.3.11. Imaging of IBA1⁺ microglia and cell counts

Fluorescently stained microglia were visualised using an Axiolmager Z1 epifluorescent microscope with a Zeiss AxioCam 305 mono microscope camera and Zeiss ZEN 3.8 software. 6-8 locations on each coverslip were randomly selected for imaging and analysis.

The Image J cell counter plugin was used to count the number of IBA1⁺ cells and the number of IBA1⁻ cells (DAPI staining only) to calculate the average percentage of microglial cells in each culture.

2.4. *In vivo* methods

2.4.1. Animals

Animal experiments were carried out 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 (case reference no. 7031980),

and performed under project licence AE19136/P186 granted by the Health Products Regulatory Authority in line with the PREPARE (planning research and experimental procedures on animals: recommendations for excellence) guidelines and the 3R's (Replacement, Reduction and Refinement). 4-6-month-old male and female Wistar rats obtained from the Comparative Medicine Unit in Trinity College Dublin were pair-housed with access to food and water *ad libitum* and environmental enrichment. Animals were housed in a climate-controlled room at 21°C ($\pm 2^\circ\text{C}$) with 50% humidity ($\pm 10\%$) and maintained on a 12:12-hr light–dark cycle. Animals were handled to familiarise them with the experimenter prior to behavioural tests or drug administration.

2.4.2. Power analysis and randomisation

An a priori power analysis was performed using G*Power (version 3.1.9.7) to determine the required sample size for chapter 7 and appendix I. Based on immunohistochemical data from the intranigral LPS rat model of PD (males only) (O'Neill et al., 2020) (Group 1: mean = 106, standard deviation = 29; Group 2: mean = 68, standard deviation = 35), the analysis indicated that 10 subjects per group were required to achieve an actual power of 0.82 ($\alpha = 0.05$, critical $t = 1.73$, degrees of freedom = 18).

Simple randomisation was performed using excel and baseline behaviour scores were assessed to ensure balanced baseline performance across groups.

2.4.3. Drug administration

For intranasal drug administration animals were lightly anaesthetised in an induction chamber with 3.5% inhalational isoflurane and oxygen at a flow rate of 2L/min. Once a sufficient plane of anaesthesia was achieved (approximately 3.5 mins), animals were removed from the induction chamber and placed in the supine position (on their back). A pre-loaded syringe fitted with a catheter cut to approximately 9 mm was placed in the nasal cavity and the gel containing drug or drug-loaded nanoparticles was slowly administered. The animal's head was kept in position for a further 20 seconds to allow the gel to set. Rats were placed in heated recovery bed with isoamyl acetate (a compound that smells like apples) applied to the bedding to encourage the animals to sniff as they awoke. When fully recovered from anaesthesia, animals were returned to their home cage. The procedure specific humane endpoints included excessive bleeding through the nose, aspiration pneumonia and suffocation, but these were not met in this study.

For i.p. LPS injection, rats were secured in dorsal recumbency (on their back) with their head tilted slightly downwards to shift organs away from the i.p. cavity. The needle was inserted at a 45° angle into the lower right abdominal quadrant to avoid injecting the caecum or bladder. Body temperature was monitored using a rectal probe. The procedure

specific humane endpoints included excessive bleeding or infection at the injection site and persistent sickness behaviour, but these were not met in this study.

2.4.4. MRI

The brains of animals administered intranasal manganese (Mn^{2+}) in solution or loaded in hydrogel (see section 2.2.4) as a contrast agent were scanned post-mortem. All tissues exhibit an intrinsic baseline longitudinal relaxation time (T_1) as water molecules realign when the magnetic field is applied. Contrast agents, like Mn^{2+} , have T_1 relaxivity properties that shorten the time it takes for water protons in a specific tissue to realign. Therefore, areas where more contrast agent has accumulated appear brighter in a T_1 -weighted MRI scan. MR images of the olfactory bulb and frontal lobe were recorded as previously described by Gobbo et al. (2012) with the assistance of Assistant Prof. Oliviero Gobbo (School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin) and Dr. Christian Kerskens (Trinity College Institute of Neuroscience, Trinity College Dublin). Briefly, a Bruker 7T BioSpec 70/30 USR system (Bruker, Ettlingen Germany) equipped with a 1H receive-only 8 x 1 rat surface array coil, 72mm transmit coil, BGA9S gradients (675mT/m) and Paravision 7 software was used to record T_1 -weighted 3D images. Images were acquired using gradient echo sequences (Resolution = $137 \times 137 \times 500 \mu m^3$, Field of View = $35 \times 35 \times 8 mm^3$, Matrix = $256 \times 256 \times 16$, repetition and echo times (TR/TE) = 21.3/7.5 ms, $\alpha = 15^\circ$, number of averages = 4, acquisition time = 6.33 min). A total of 16 slices were acquired, yielding a slice thickness of 0.5 mm.

Mn^{2+} -related signal enhancement was evaluated on three separate MRI slices located at the level of the olfactory layer and ventricle (Paxinos: interaural 14.7, Bregma 5.7), ventral orbital cortex and olfactory ventricle (Paxinos: interaural 13.2, Bregma 4.2) and primary and secondary motor cortex, lateral ventricle, olfactory tubercle and lateral olfactory tract (Paxinos: interaural 10.6, Bregma 1.6). Resulting images were converted to heat maps in image J to visualise signal intensity.

2.4.5. Tissue harvesting and preparation for RNA isolation

Animals were euthanised by CO_2 asphyxiation, decapitated and brains were dissected to isolate cortical tissue and the striatum. Spleen tissue was also dissected. All tissue was weighed, transferred to RNeasy Lysis Buffer and stored at $-80^\circ C$ until further use. The Macherey-Nagel™ NucleoSpin™ RNA isolation kit (Macherey Nagel) was used to lyse 30 mg of tissue using a polytron PT 1300D handheld tissue homogeniser (Kinematica AG) and for RNA isolation as outlined in section 2.3.8. cDNA synthesis and PCR were also carried out as described in section 2.3.8.

2.4.6. Stereotactic surgery

Surgical procedures were performed using aseptic technique. Animals were anaesthetised with an initial induction of 5% inhalational isoflurane, which was gradually reduced to and maintained at 1.8-2% with an oxygen flow rate of 2L/min throughout the surgery. After induction, the rats head was shaved and the surgical area cleaned with alcohol swabs and 2% chlorhexidine solution. Topical anaesthesia was applied to the ears, and the animal's head was secured on the stereotactic frame using non-rupture ear bars. Body temperature was maintained during the procedure using a heating pad and was monitored throughout using a rectal probe. Lacrilube was applied to the animal's eyes to prevent dryness during the procedure. The surgical area was cleaned again with chlorhexidine and betadine anti-septic. Lidocaine spray was applied topically as a local anaesthesia and a midline incision was made with a sterile 10 blade scalpel. Connective tissue was removed to reveal bregma and lambda. The co-ordinates of bregma and lambda were noted to ensure the head was flat and to use as a reference for locating the substantia nigra. A burr hole was drilled on the left hemisphere at the coordinates anterior-posterior (AP): -5.3 mm from bregma, mediolateral (ML): - 2.0 mm from bregma, dorsal-ventral (DV): -8.5 mm from skull (males) or -7.5 mm from skull (females). The pre-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 intranigral injection was delivered at a rate of 1 µl/min. After injection, the needle was left in place for an additional 5 minutes and slowly removed over 2 minutes to allow diffusion into the targeted brain region and avoid efflux as the needle was withdrawn. The incision was cleaned and glued closed with surgical adhesive. Warmed saline (0.89% sodium chloride, subcutaneous (s.c.) was administered during surgery to prevent dehydration and maintain overall fluid balance. The animal was removed from the stereotactic frame and allowed to recover from anaesthesia in a heated recovery bed. Buprenorphine (0.03 mg/kg, s.c.) and meloxicam (1.5 mg/kg, s.c) were administered immediately after surgery. Meloxicam administration was continued once daily for 3 days post-surgery. Animals were pair-housed in a clean cage post-surgery, provided with soft food and were monitored closely. The procedure specific humane endpoints included anaesthesia-related hypothermia, keratoconjunctivitis sicca, severe post-operative pain, rapid weight loss, wound infection not ameliorated by antibiotics, persistent reopening of surgical wound and progression of motor impairments that prevents the animal from feeding. One animal reached a humane endpoint due to the motor impairment that prevented the animal from feeding.

Following tissue analysis, 2 animals were excluded from all behavioural results and post-mortem analysis due to the absence of an LPS lesion in the substantia nigra.

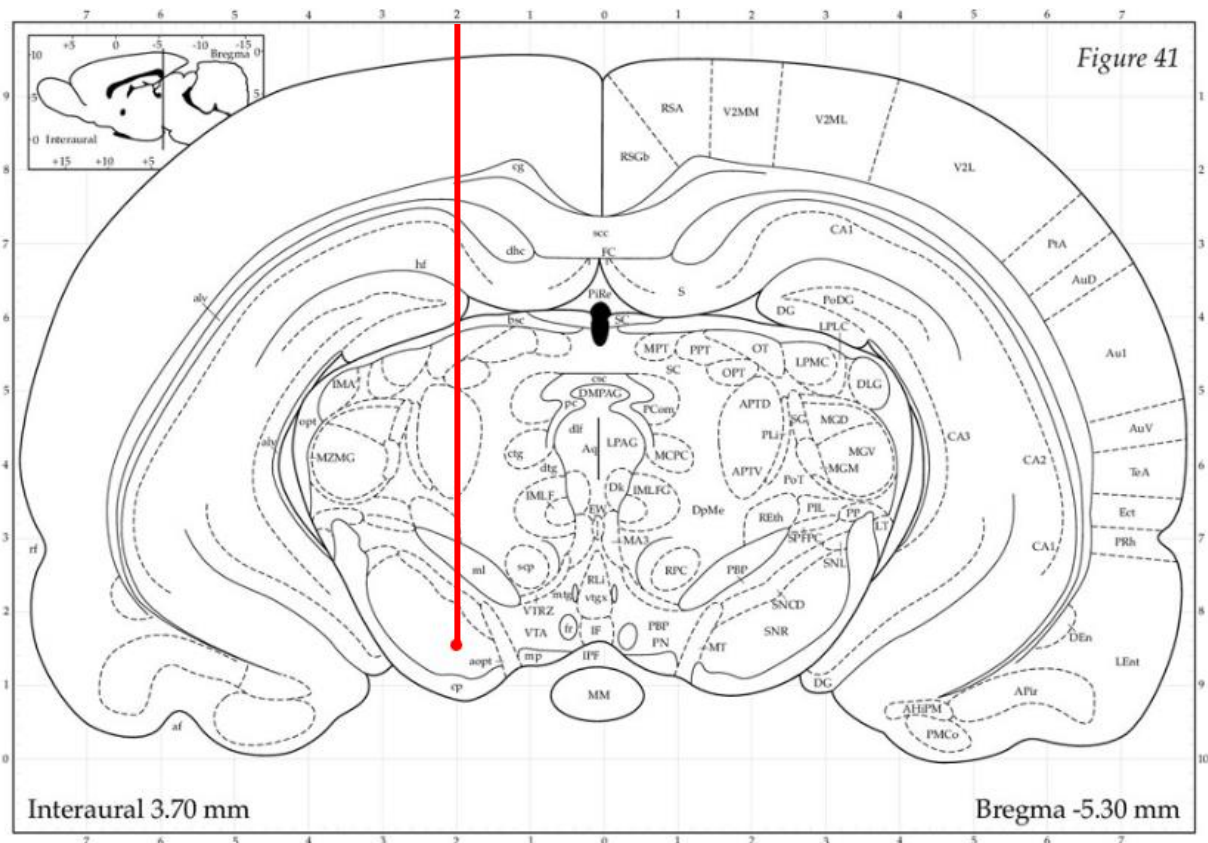


Figure 2.4. Stereotactic injection of LPS to the substantia nigra.

Schematic showing the stereotactic co-ordinates for the substantia nigra in male rats (image based on male rat brain anatomy), where anterior-posterior (AP): -5.3 mm from bregma, mediolateral (ML): -2.0 mm from bregma, dorsal-ventral (DV): -8.5 mm from skull. Female co-ordinates differ in the DV axis where the needle is lowered to -7.5 mm from the skull. Image adapted from Paxinos and Watson Rat Brain Atlas (2006).

2.4.7. Cylinder test

The cylinder test was used to assess forelimb asymmetry following unilateral lesion of the nigro-striatal tract and was performed as previously described by (Schallert et al., 2000). Briefly, rats were placed in a glass cylinder (19 cm diameter x 50 cm height for females and 29 cm diameter x 44 cm height for males) and their exploratory behaviour of the apparatus was recorded for 5 minutes using a camcorder. The cylinder test was performed once at baseline and once two weeks post-surgery. The number of wall and floor placements were counted. A wall placement was recorded when the animal's forepaw contacted the wall of the cylinder after rearing. A floor placement was counted when a rat's forepaw made contact with the base of the cylinder when landing from a rearing. Simultaneous wall and floor placements were also recorded. Forelimb use was assessed as total (wall + floor), total ipsilateral (same side as lesion) and total contralateral (opposite side to lesion) forepaw placements, both pre- and post-lesion. Contralateral limb use was further assessed by

examining contralateral contacts as a percentage of ipsilateral contacts and the ratio of contralateral to ipsilateral forelimb use. A forelimb asymmetry score was calculated for each animal (**equation 7**), where a score of 1 indicates equal forelimb use, greater than 1 indicates bias towards the right forelimb (contralateral side) and less than 1 indicates bias towards the left forelimb (ipsilateral side). To encourage exploration upon second exposure to the apparatus post-surgery, the cylinder test was performed in different rat behavioural rooms at baseline and post-lesion.

Equation 7:

$$\text{Forelimb asymmetry index score} = \left(\frac{\text{contralateral} - \text{ipsilateral}}{\text{Total combined}} \right) \times 100$$

2.4.8. Stepping test

The stepping test was used to evaluate forelimb akinesia post-unilateral lesion of the nigro-striatal tract (Olsson et al., 1995). The rat is held by the experimenter with all paws except for one forepaw restrained. The animal is held at a 45° angle from a table and walked in a forwards and backwards direction (10 seconds in each direction) over 90 cm. This is repeated 3 times with each fore paw in one trial. At baseline, 3 training trials were performed followed by two test trials conducted by a single researcher. Post-lesion, one training session was conducted followed by two test trials. Average scores across test days were calculated for ipsilateral and contralateral forward, backward and combined steps. Contralateral limb use was further assessed by examining contralateral steps as a percentage of ipsilateral steps, the ratio of contralateral to ipsilateral steps and forelimb asymmetry scores.

2.4.9. Rotarod test

The rotarod test is used to assess gross motor coordination and balance in rodents (Dunham & Miya, 1957). The apparatus consists of a rotating rod of diameter 6 cm elevated 30 cm from the base. On the first day of training, rats were introduced to the apparatus at a constant speed of 10 rpm and encouraged to remain walking on the rotating rod for 300 seconds. On the subsequent two days of training, a steady ramp increase in speed from 10 to 40 rpm over a 300 second period was introduced. During training, rats were placed back on the rotarod after falls. Baseline rotarod scores were recorded over two separate test days and the average scores were calculated. Post-lesion, one training session was conducted followed by two test trials over two consecutive days. Both training and test sessions consisted of three trials with a 30-minute rest period between trials. The average latency to fall was calculated for each animal pre- and post-lesion.

2.4.10. Amphetamine-induced rotations test

The amphetamine-induced rotation test is a paradigm to assess the extent of damage to the nigrostriatal tract post-lesion (Ungerstedt & Arbuthnott, 1970). Animals were administered D-amphetamine (5 mg/kg, s.c.) and placed in a glass cylinder (19 cm diameter x 50 cm height for females and 29 cm diameter x 44 cm height for males). Amphetamine administration causes dopamine release in the nigrostriatal tract. Unilateral lesion of this nerve bundle results in higher dopamine release by neurons in the intact hemisphere in response to amphetamine, causing a higher number of rotations ipsilateral to the lesioned hemisphere. The animal's rotational behaviour was recorded for one hour using a Logitech webcam connected to ANYmaze behavioural tracking software (Stoelting, version 7.52). Clockwise, anticlockwise and the total number of rotations were recorded. The ratio of ipsilateral to contralateral rotations, net ipsilateral rotations and ipsilateral rotations per minute were calculated from this data. A washout period of 4 days was allowed prior to euthanasia for the acute effects of amphetamine on the dopaminergic system to wear off.

2.4.11. Transcardial perfusion

Rats were briefly anaesthetised (5% isoflurane, 2L/min oxygen) until the animal lost its righting reflex and a terminal dose of sodium pentobarbital was administered (100 mg/kg, i.p.). When all reflexes were lost, a thoracotomy was performed to expose the chest cavity of the rat. A 23-gauge needle was placed in the left ventricle of the still beating heart and rats were perfused with 1x PBS (**Table 2.4**) at a rate of 15ml/min for 5-10 minutes using a perfusion pump (Gilson). The right auricle was cut as an exit for the perfusate. When the perfusate and liver were clear, PBS was replaced with 4% PFA in 0.2M PBS (**Table 2.5**) at a rate of 15ml/min for 5-10 minutes until tissues were adequately fixed. Animals were decapitated and the brain was removed. Brains were post-fixed for a further 24 hours in 4% PFA before being transferred to a 30% sucrose solution for 48-72 hours for cryopreservation prior to snap-freezing using ice cold isopentane.

Table 2.4. 10X PBS recipe for tissue work.

Ingredients	Concentration
Sodium chloride (NaCl)	3M
Sodium dihydrogen phosphate (NaH_2PO_4)	104 mM
Potassium dihydrogen phosphate (KH_2PO_4)	34 mM
dH ₂ O (2L)	-
Concentrated hydrochloric acid (HCl) or 10N sodium hydroxide solution (NaOH) to adjust to pH 7.4	-

Table 2.5. 0.2M PBS recipe for tissue work.

Ingredients	Concentration
Sodium phosphate dibasic (Na_2HPO_4)	160 mM
Sodium dihydrogen phosphate (NaH_2PO_4)	38 mM
dH_2O (2L)	-
Concentrated hydrochloric acid (HCl) or 10N sodium hydroxide solution (NaOH) to adjust to pH 7.4	-

2.4.12. Tissue processing for immunohistochemistry

All brains were embedded in optimal cutting temperature media (OCT) before cryosectioning. Coronal sections of 20 μm thickness were collected of the striatum and substantia nigra in series. Sections were mounted directly onto gelatine-coated slides and stored at -80°C until further use.

2.4.13. Immunohistochemistry

Slide mounted tissue sections were thawed at room temperature and rehydrated in three 5-minute 1x PBS washes. Endogenous peroxidase activity was quenched in 2.5% hydrogen peroxide (H_2O_2) in 100% methanol (MeOH) for 20 minutes at room temperature. Tissue was permeabilised by three 5 minute 0.3% PBS-T washes. Slides were dunked in 1x PBS to remove excess PBS-T, and a hydrophobic barrier was applied around sections using a PAP pen to ensure that reagents were evenly dispersed on tissue sections. Blocking of non-specific binding sites was performed with 10% normal goat or horse serum in 1x PBS, for IBA1 and TH staining respectively, for two hours at room temperature in the humidified chamber. Sections were coated in rabbit anti-IBA1 primary antibody (WAKO; 019-19741; 1:1000) or mouse anti-TH primary antibody (Millipore; MAB138; 1:2000) prepared in 1x PBS overnight at room temperature in the humidified chamber. The following day, slides were washed with 1x PBS three times for 10 minutes per wash and incubated with biotinylated goat anti-rabbit secondary antibody for IBA1 or biotinylated horse anti-mouse for TH in 1x PBS (1:200) for two hours in the humidified chamber followed by three 10-minute 1x PBS washes. Sections were then incubated with avidin-biotinylated horse radish peroxidase (HRP) complex (ABC) in 1x PBS for 2 hours at room temperature in the humidified chamber, which was then washed off with two 10-minute 1x PBS washes. The 3,3'-diaminobenzidine (DAB) reaction was performed by submerging slides in DAB (0.5 mg/ml) solution prepared in 0.1M PBS oxidised by 0.4% H_2O_2 solution for 2-4 minutes. The DAB reaction was stopped by placing slides in deionised water for 5 minutes. Dehydration took place by passing slides through increasing ethanol gradients of 70%, 90%, 95% and 100% for 5 minutes each. Sections were cleared by 5 minutes in xylene. Coverslips were

applied to slides using DPX mounting media and were allowed to dry in the fume hood overnight.

2.4.14. Imaging and analysis of IBA1⁺ microglia and TH⁺ dopaminergic neurons

Sections were imaged using an Olympus BX51 light microscope with an Olympus DRP72 camera and CellSens software. Three images were acquired at 20X magnification per hemisphere of IBA1⁺ and TH⁺ cells to capture the entire substantia nigra for analysis. ImageJ software (version 1.54) was used to convert images to 8-bit, create a mask and count the number of IBA1⁺ or TH⁺ cells per image. A tagged skeleton of IBA1⁺ cells was also created from the mask to analyse the area of individual microglial cells (**Fig. 2.5**). The average number of IBA1⁺ or TH⁺ cells and the average area of individual IBA1⁺ cells within the substantia nigra of each hemisphere was counted per section which included a minimum of 3 sections per animal. Cell counts and cell area in the lesioned substantia nigra are expressed as a % of the intact hemisphere.

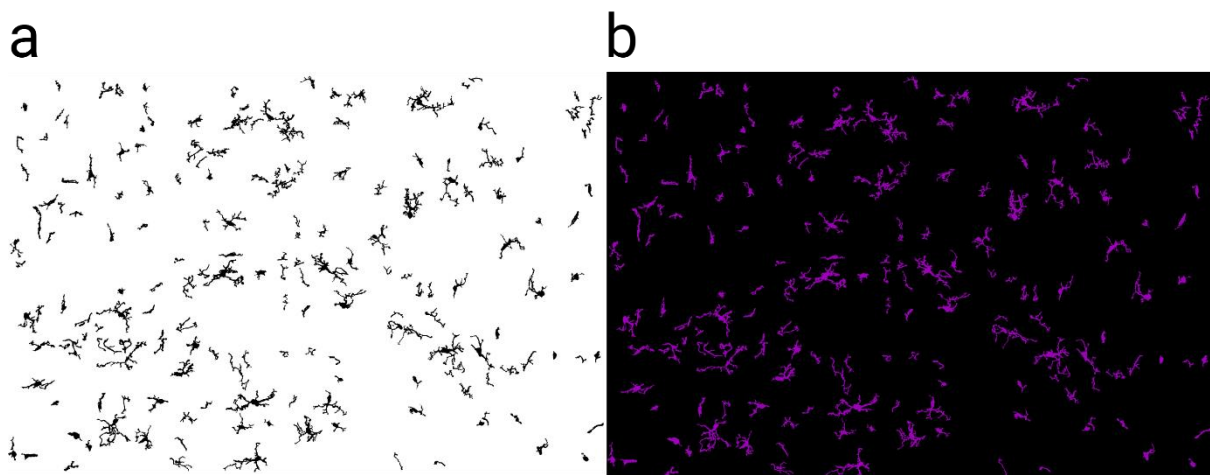


Figure 2.5. Analysis of IBA1⁺ microglia using ImageJ.

(a) Mask created of grayscale 8-bit DAB images for automated cell counting and (b) tagged skeleton for analysis of individual cell area.

2.5. Data analysis

In *in vitro* experiments (chapter 3-5), 3-4 replicates were included for analysis. For cell culture work, one biological replicate consists of three technical replicates from an individual rat brain. For *in vitro* imaging, approximately 10 cells were analysed per technical replicate. In *in vivo* investigations (chapter 6 and 7), each animal represents one biological replicate.

Data were analysed using GraphPad Prism 10. Outliers were detected using the outlier function of GraphPad Prism. Data were tested for normality using the Shapiro-Wilk test prior to parametric analyses. Where appropriate, student's t-test, one-way analyses of variance (ANOVA), ordinary two-way ANOVA or repeated measures two-way ANOVA were performed. Kruskal Wallis test by ranks was performed for analysis of non-parametric data. Where statistically significant effects were shown following a one-way ANOVA or Kruskal Wallis test, Dunnett's or Dunn's multiple comparisons test were performed, respectively, to identify group differences compared to controls. Where statistically significant effects were shown following a two-way ANOVA, Tukey's or Šídák's multiple comparisons tests were performed to identify differences between groups. Results were deemed significant when $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), or $P < 0.0001$ (****) and are represented as mean $\pm$ standard error of the mean (SEM).

Chapter 3: Establishing rat primary glial cultures that respond to inflammatory stimuli

3.1. Introduction

Broadly speaking, the cells that make up the CNS are divided into neurons, which transmit electrical and chemical signals throughout the brain and beyond, and their supporting glial cells, namely microglia and astrocytes (Purves, 2001). *In vitro* cultures of these cells have been fundamental in advancing our understanding of the brain on a cellular and molecular level and are valuable tools for testing drug therapies and biomaterials prior to their further evaluation in preclinical disease models.

Establishing neuronal cultures is a challenging process as neurons are non-dividing cells. To overcome this, immortalised cell lines like human SH-SY5Y, mouse N2a cells and rat PC12 cells are commonly used in neuroscience as they are relatively cheap, fast growing and can withstand several passages and freeze-thaw cycles (Gordon et al., 2013). Several glial cell lines have also been immortalised, such as mouse BV2 microglia and human U87MG astrocytes (Saura, 2007; Timmerman et al., 2018). However, there are several limitations to the use of immortalised cell lines, especially when they are tumour derived as their immortalisation can misrepresent normal cell functions. SH-SY5Y cells are an immature phenotype, and heterogenous population which do not accurately reflect neurons *in vivo* (Gordon et al., 2013). Furthermore, glial cell lines display an altered transcriptome compared to primary cells leading to aberrant inflammatory responses (Henn et al., 2009).

Primary cultures are established by isolating cells directly from fresh brain tissue and are advantageous as they more accurately reflect neuronal and glial behaviour *in vivo*. Typically, 2-3-day old neonatal rodent brains are optimal for culturing neurons and glia from most CNS regions as this timeframe is after the peak of neurogenesis and just before oligodendrogenesis (Saura, 2007). In typical mixed glial cultures, astrocytes are the predominant cell type and these are often referred to as astroglial-enriched cultures. As the protocol for isolating neurons and glia from the brain somewhat overlap, initially there may be some glia present in neuronal cultures and vice versa. However, the culture medium used, and maintenance techniques can decrease the percentage of the non-target cells. Other cell types can also be present, particularly in glial cultures, in small, often negligible proportions, including oligodendrocytes, various precursor cells, ependymal cells, fibroblasts and endothelial cells (Saura, 2007).

There are several limitations to the use of primary cultures as they are difficult to establish and maintain and often produce insufficient cell yields. Culture purity is a challenge and ethical approval is required (Gordon et al., 2013; Saura, 2007). These cultures are also more time consuming than cell lines as there is a lag time between the initial plating of cells and

performing experiments on mature cultures. Therefore, the decision to use immortalised or primary cells is highly dependent on experimental design and measurable outcomes.

Glial cultures are particularly useful in examining the brain's innate immune response to various stimuli. Microglia and astrocytes express an array of PRRs, which when activated stimulate the expression and release of pro-inflammatory cytokines, chemokines, nitric oxide (NO) and ROS (Purves, 2001). IL-1 β and TNF α are pro-inflammatory cytokines that are secreted in response to TLR activation and are encoded by the *Il1b* and *Tnfa* genes, respectively, in rodents. IL-1 β and TNF α orchestrate and amplify inflammatory responses by downstream induction of cytokines IL-6 and IL-1 α , chemokines CCL2, CCL5, and CXCL10 as well as NO and ROS (Kim et al., 2010; Mir et al., 2008; Pugazhenthir et al., 2013; Todd et al., 2019; Van Kralingen et al., 2013). Furthermore, inhibition of IL-1 β and TNF α in animal models of PD, rescues dopaminergic neuronal loss (Arai et al., 2004; McCoy et al., 2006). NO is generated by iNOS, encoded by the *Nos2* gene. During chronic neuroinflammation, dysregulated NO acts as a neurotoxin and propagates neurodegeneration in PD (Molina et al., 1998). Co-stimulatory factors such as CD40, CD80 and CD86 are upregulated by microglia in response to IFN γ and TNF α and are involved in antigen presentation and activation of T cell response, which leads to exacerbated inflammatory responses in the diseased brain (Benveniste et al., 2004).

The transfer of glial conditioned media (CM) to neurons creates a model of cross-talk where the impact of pro-inflammatory mediators released by microglia and astrocytes on neuronal survival and growth can be tested.

3.2. Aims

The aim of this study was to establish reproducible enriched microglial cultures that are responsive to pro-inflammatory stimuli for use in the subsequent testing of biomaterials. T

3.3. Experimental design

Primary glia were isolated from the neonatal rat cortex to generate mixed glial cultures and enriched microglial cultures (see methods section 2.3). Mature enriched microglial cultures were stimulated with IFN γ (10-200 ng/ml) or LPS (10-200 ng/ml) for 24 or 6 hours, respectively. Cells were lysed, RNA was isolated and cDNA was synthesised for PCR gene expression analysis. Untreated mixed glial cultures and enriched microglial cultures were fixed and immunocytochemistry for IBA1 was performed. Cells were imaged using fluorescent microscopy and IBA1⁺ microglia were counted. Glial cell culture media was collected for LDH assay and for transfer to primary neuronal cultures isolated from neonatal rat cortex for 24 hours. Primary neurons were fixed and immunocytochemistry for the neuron specific MAP2 was performed. Neurons were imaged using a fluorescent microscope and morphology was analysed to create a Sholl profile of neuronal complexity. Neuronal culture media was also collected for LDH assay.

3.4. Results

3.4.1. Enriched microglial cultures

Glial cultures were established that were enriched for microglia (**Fig.3.1b**) and compared to standard mixed glial preparations (**Fig. 3.1a**). Unpaired t-test showed a mean 55% increase in the percentage of IBA1⁺ microglia in enriched cultures compared to a standard mixed glial protocol ($P<0.0001$).

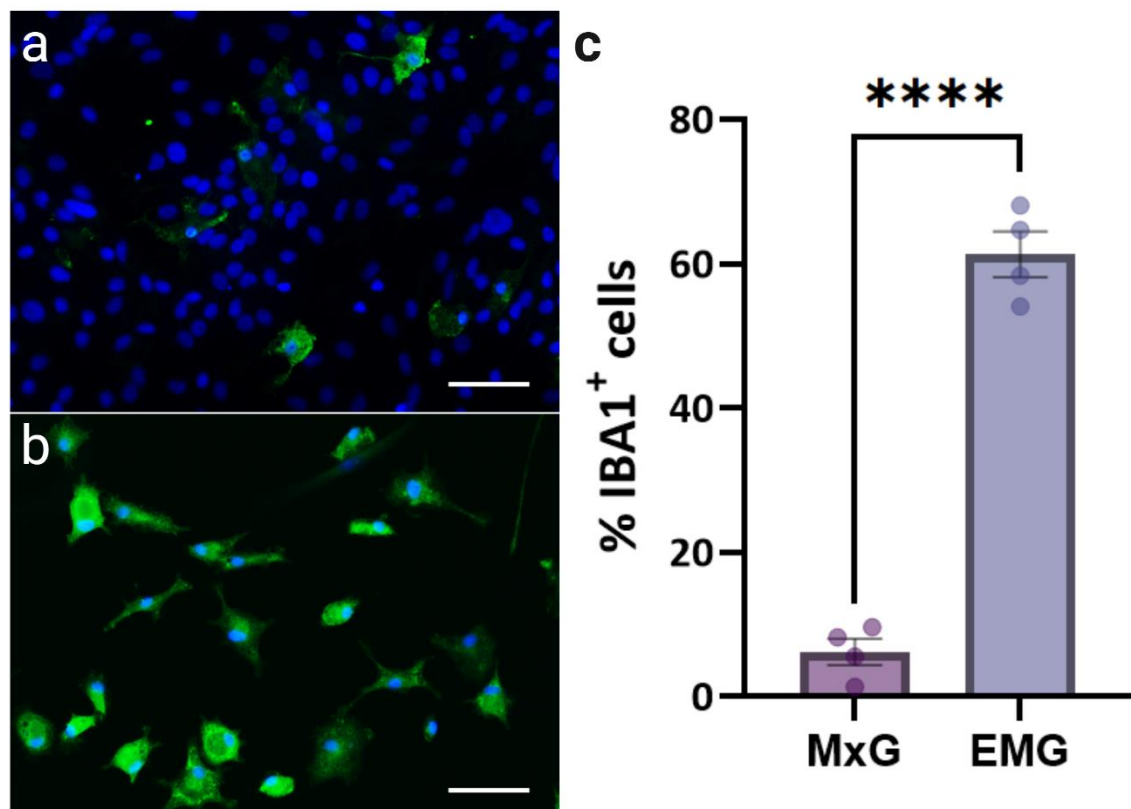


Figure 3.1. IBA1⁺ cells in mixed and enriched microglial cultures.

Representative images of IBA1⁺ microglia in primary (a) mixed glial and (b) enriched microglial cultures isolated from the neonatal rat cortex. (c) Cell counts were performed in Image J. Data were analysed by unpaired t-test and are expressed as the mean $\pm$ SEM, where $N=4$. Significant differences between groups are denoted by * where **** $P<0.0001$. Magnification 20X, scale bar = 50 μ m (a, b). IBA1: ionised calcium-binding adaptor molecule 1 (microglial marker); DAPI: 4',6-diamidino-2-phenylindole (nucleic marker); MxG: mixed glial cultures; EMG: enriched microglial cultures.

3.4.2. IFN γ stimulation of enriched microglial cultures increased mRNA levels of pro-inflammatory markers after 24 hours

One-way ANOVA of extracellular LDH showed an effect of IFN γ [$F_{(4, 15)} = 4.451$, $P < 0.05$] (**Fig.3.2a**). Dunnett's multiple comparisons revealed that LDH release in enriched microglia exposed to 200 ng/ml of IFN γ had lower extracellular LDH compared to vehicle control ($P < 0.05$).

One-way ANOVA of fold change in *I1b* mRNA showed an effect of IFN γ [$F_{(4, 15)} = 6.303$, $P < 0.01$] (**Fig.3.2b**). Dunnett's multiple comparisons revealed that *I1b* transcripts were higher in enriched microglia exposed to 200 ng/ml of IFN γ compared to vehicle control ($P < 0.01$).

One-way ANOVA of fold change in *Tnf α* mRNA showed no effect of IFN γ (**Fig.3.2c**).

One-way ANOVA of fold change in *Nos2* mRNA showed an effect of IFN γ [$F_{(4, 15)} = 76.91$, $P < 0.0001$] (**Fig.3.2d**). Dunnett's multiple comparisons revealed that *Nos2* transcripts were higher in enriched microglia exposed to 10, 50, 100 and 200 ng/ml of IFN γ compared to vehicle control (all $P < 0.0001$).

One-way ANOVA of fold change in *Cd40* mRNA showed an effect of IFN γ [$F_{(4, 15)} = 9.162$, $P < 0.001$] (**Fig.3.2e**). Dunnett's multiple comparisons revealed that *Cd40* transcripts were higher in enriched microglia exposed to 10 and 50 ng/ml IFN γ ($P < 0.01$) and 100 and 200 ng/ml of IFN γ ($P < 0.001$) compared to vehicle control.

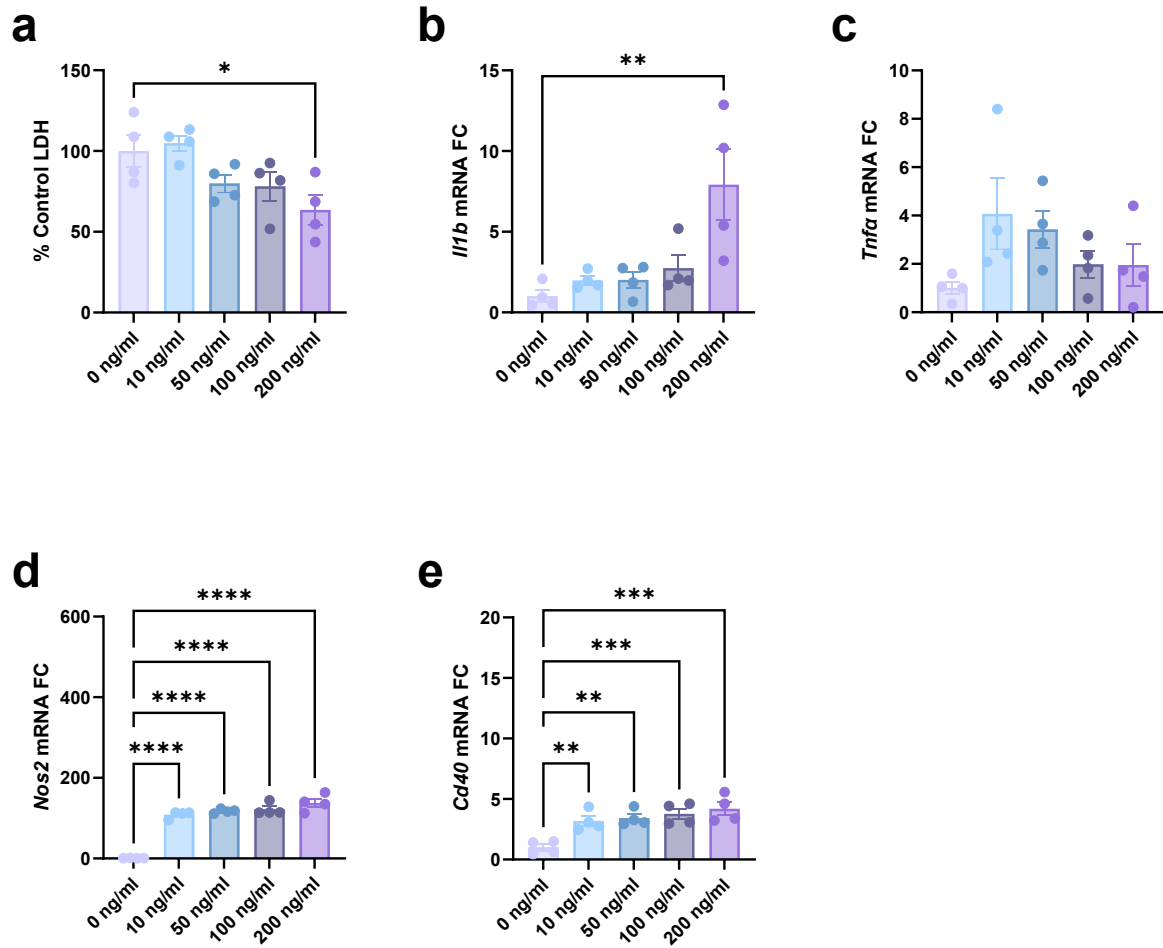


Figure 3.2. IFN γ promotes a pro-inflammatory response in enriched microglial cultures.

Enriched microglial cultures were stimulated with IFN γ (10-200 ng/ml) for 24 hours. (a) Extracellular LDH as a % of control and fold change (FC) in (b) *Il1b*, (c) *Tnfa*, (d) *Nos2* and (e) *Cd40* mRNA. Data were analysed by one-way ANOVA followed by Dunnett's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=4. Significant differences between groups are denoted by * where ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

3.4.3. LPS stimulation of enriched microglial cultures increased mRNA levels of pro-inflammatory markers after 6 hours

One-way ANOVA of extracellular LDH showed an effect of LPS [$F_{(4, 10)} = 12.71$, $P < 0.001$] (**Fig.3.3a**). Dunnett's multiple comparisons revealed that extracellular LDH in enriched microglia exposed to 100 and 200 ng/ml of LPS had higher extracellular LDH compared to vehicle control ($P < 0.01$ and $P < 0.001$, respectively).

One-way ANOVA of fold change in *Il1b* mRNA showed an effect of LPS [$F_{(4, 10)} = 28.20$, $P < 0.0001$] (**Fig.3.3b**). Dunnett's multiple comparisons revealed that *Il1b* transcripts were higher in enriched microglia exposed to 10, 50, 100 and 200 ng/ml of LPS compared to vehicle control (all $P < 0.0001$).

One-way ANOVA of fold change in *Tnfα* mRNA showed an effect of LPS [$F_{(4, 10)} = 4.369$, $P < 0.05$] (**Fig.3.3c**). Dunnett's multiple comparisons revealed that *Tnfα* transcripts were higher in enriched microglia exposed to 50, 100 and 200 ng/ml of LPS compared to vehicle control (all $P < 0.05$).

One-way ANOVA of fold change in *Nos2* mRNA showed an effect of LPS [$F_{(4, 10)} = 43.65$, $P < 0.0001$] (**Fig.3.3d**). Dunnett's multiple comparisons revealed that *Nos2* transcripts were higher in enriched microglia exposed to 10, 50, 100 and 200 ng/ml of LPS compared to vehicle control (all $P < 0.0001$).

One-way ANOVA of fold change in *Cd40* mRNA showed an effect of LPS [$F_{(4, 10)} = 48.88$, $P < 0.0001$] (**Fig.3.3e**). Dunnett's multiple comparisons revealed that *Cd40* transcripts were higher in enriched microglia exposed to 10, 50, 100 and 200 ng/ml of LPS compared to vehicle control (all $P < 0.0001$).

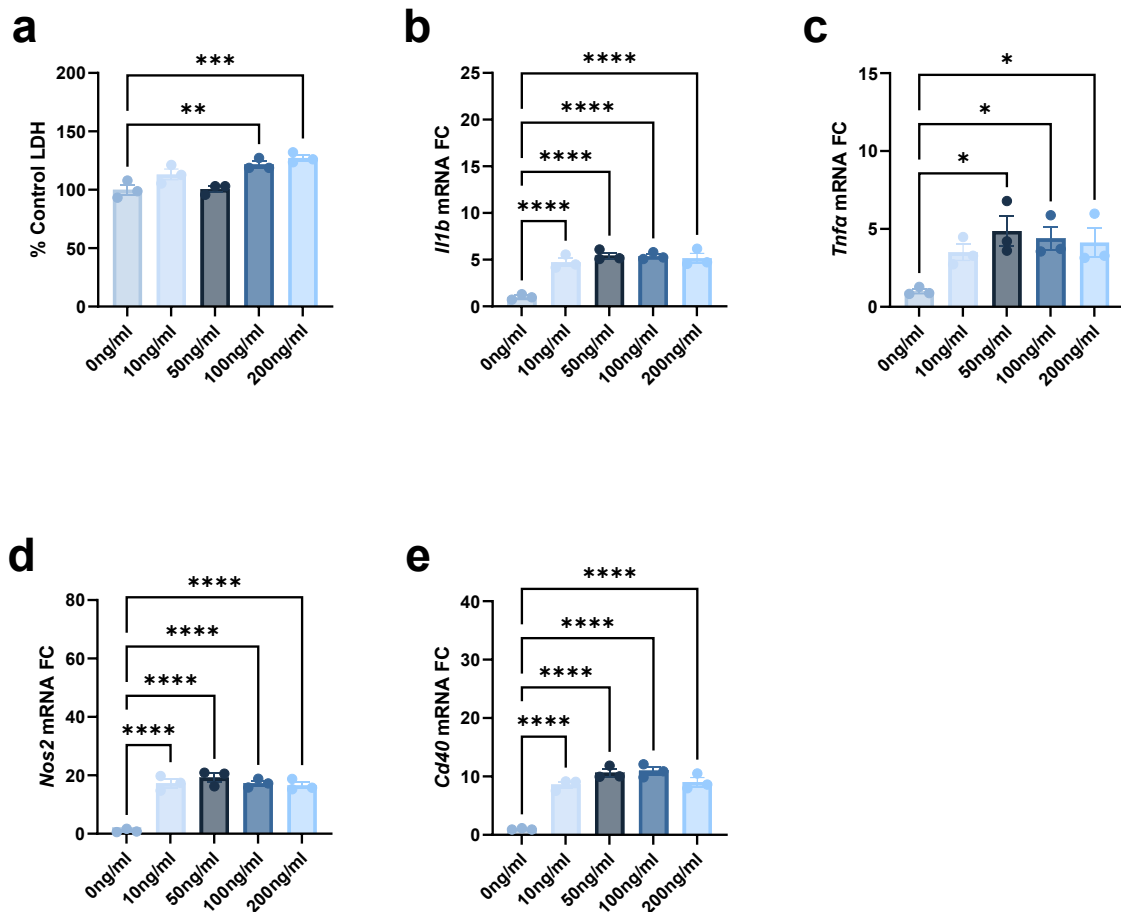


Figure 3.3. LPS promotes a pro-inflammatory response in enriched microglial cultures.

Enriched microglial cultures were stimulated with LPS (10-200 ng/ml) for 6 hours. (a) Extracellular LDH as a % of control and fold change (FC) in (b) *Il1b*, (c) *Tnfa*, (d) *Nos2* and (e) *Cd40* mRNA. Data were analysed by one-way ANOVA followed by Dunnett's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=3. Significant differences between groups are denoted by * where ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

3.4.4. Transfer of conditioned media from IFN γ and LPS-treated enriched microglia for 24 hours reduces neuronal complexity

One-way ANOVA of extracellular LDH showed no effect of CM (**Fig.3.4a**).

One-way ANOVA of the number of primary neurites showed an effect of CM [$F_{(2, 6)} = 11.81$, $P < 0.01$] (**Fig.3.4b**). Dunnett's multiple comparisons revealed that the number of primary neurites in neurons treated with IFN γ CM and LPS CM were lower than vehicle control ($P < 0.05$ and $P < 0.01$, respectively).

One-way ANOVA of the number of branches showed an effect of CM [$F_{(2, 6)} = 12.63$, $P < 0.01$] (**Fig.3.4c**). Dunnett's multiple comparisons revealed that the number of branches in neurons treated with IFN γ CM and LPS CM were lower than vehicle control ($P < 0.01$ and $P < 0.05$, respectively).

One-way ANOVA of neurite length showed an effect of CM [$F_{(2, 6)} = 12.35$, $P < 0.01$] (**Fig.3.4d**). Dunnett's multiple comparisons revealed that the neurite length of neurons treated with IFN γ CM and LPS CM were lower than vehicle control ($P < 0.05$ and $P < 0.01$, respectively).

Two-way repeated measures of the Sholl profile showed an effect of distance [$F_{(25, 150)} = 96.34$, $P < 0.0001$], CM [$F_{(6, 150)} = 32.38$, $P < 0.0001$] and a distance x CM interaction [$F_{(50, 150)} = 5.048$, $P < 0.0001$] (**Fig.3.4e**). Tukey's multiple comparisons revealed a reduction in the complexity of neurons treated with IFN γ CM compared to vehicle CM treated neurons at 10-30 μm ($P < 0.05$), 40-90 μm ($P < 0.0001$), 100-110 μm ($P < 0.01$) and 120-150 μm ($P < 0.05$) from the cell soma. Tukey's multiple comparisons also revealed a reduction in the complexity of neurons treated with LPS CM compared to vehicle CM treated neurons at 10-30 μm ($P < 0.05$), 40-100 μm ($P < 0.0001$), 110-120 μm ($P < 0.01$) and 130-150 μm ($P < 0.05$) from the cell soma. Statistical significance is displayed in fig. 3.4e as the minimum threshold reached ($P < 0.05$).

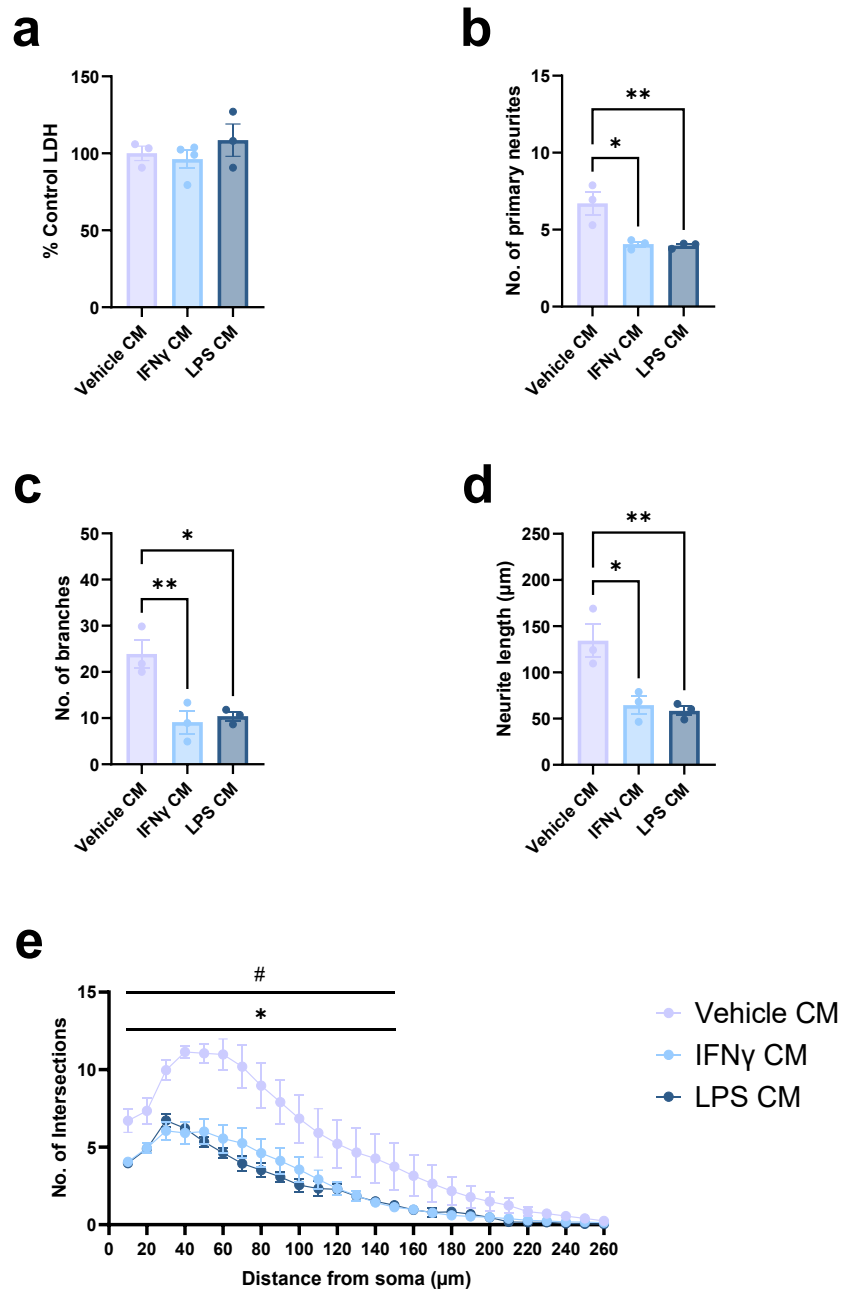


Figure 3.4. CM from IFN γ or LPS treated enriched microglial cultures reduces neuronal complexity.

CM from glia exposed to IFN γ (50 ng/ml) or LPS (100 ng/ml) for 24 hours was transferred to primary neuronal cultures for a further 24 hours. (a) Extracellular LDH as a % of control, (b) the number of primary neurites, (c) the number of branches, (d) neurite length and (e) the Sholl profile of neuronal complexity. Data were analysed by one-way ANOVA (a-d) or two-way repeated measures ANOVA followed by Tukey's multiple comparisons (e) and are expressed as the mean $\pm$ SEM, where N=3. Significant differences between groups are denoted by * where * $P < 0.05$ and ** $P < 0.01$ (a-d). Significant differences in (d) are denoted by * or # for IFN γ CM and LPS CM, respectively, compared to vehicle CM where $P < 0.05$.

3.5. Discussion

At the outset of this project, we aimed to establish a robust *in vitro* platform for the subsequent testing of biomaterials for CNS drug delivery. Initial mixed glial protocols yielded cultures that had very low microglia populations and were unresponsive to classic pro-inflammatory stimuli like IFN γ and LPS over a range of concentrations and time points (Tyndall, PhD thesis). There are several factors that can contribute to low microglial yields in a mixed culture; culture technique, species and age of neonates, lack of supplemental factors like GM-CSF, media used and the frequency of media changes (Saura, 2007).

To overcome this problem, we set out to establish mixed glial cultures that were enriched for microglia (enriched microglial cultures) by first using a shaking protocol to isolate the major glial sub-populations (Tamashiro et al., 2012) and then manipulating the cultures to achieve the desired proportion of each cell type (see section 2.3.4). The enriched microglial cultures had a significantly higher percentage of microglia than the previous mixed glia protocol. Microglia and astrocytes account for up to 20% and 40% of the total glial cell population in the brain, respectively. Therefore, the increased number of microglia in enriched cultures presented here does not represent *in vivo* conditions. Pure microglia cultures are reported to undergo a transcriptional switch upon isolation and introduction to a new *in vitro* environment (Gosselin et al., 2017; Marsh et al., 2022). This can lead to baseline reactivity, where cells are unresponsive to subsequent stimulation or responses can be exaggerated. Other glial cell types, namely astrocytes, were re-introduced to enriched microglia at lower densities to combat this effect.

To investigate the origins and proportions of the remaining 40% of cells in these cultures techniques such as flow cytometry or immunocytochemistry for astrocyte marker glial fibrillary associated protein (GFAP), mature oligodendrocyte marker 4 (O4) or oligodendrocyte precursor cell marker nerve/glial antigen2 (NG2) may be adopted. Typically, CNS cells do not fare well when cultured in isolation and therefore a 'pure' culture does not reflect homeostatic phenotypes. Therefore, the presence of other cell types in these cultures may lead to more physiologically relevant results than absolute or pure microglia cultures

There is a stimuli-dependent temporal response of microglia that should be considered in experimental design. *Tnfa* and *Nos2* expression begin to increase after 12 hours of IFN γ exposure and remain elevated until 48 hours post-treatment in primary microglia (Jiang et al., 2022). *Cd40*, on the other hand, only peaks 24 hours post-IFN γ exposure and remains upregulated for a further 48 hours (Nguyen et al., 1998). Therefore, to determine whether enriched microglia were responsive to IFN γ , cells were stimulated for 24 hours prior to mRNA analysis. *Il1b* was included in this panel for comparative purposes, however IFN γ is not a robust inducer of *Il1b* signalling, which is reflected in this study as the maximum test concentration was required to promote its expression.

LPS induced mRNA expression of pro-inflammatory markers tends to peak shortly after the initial stimulation in microglia but also varies between markers. Das et al. (2016) reported that LPS (10-100 ng/ml) mediated expression of pro-inflammatory *Tnfa*, *Il1a*, *Il1b*, *Cxcl10*, *Ccl4* and *Ccl5* peaked 4 hours post-treatment in primary microglia. While *Nos2* and *Cd40* expression in primary microglia begins to rise after 4 hours of LPS exposure, mRNA levels typically peak 8 and 24 hours later, respectively (Dello Russo et al., 2004; Qin et al., 2005). To encompass these dynamic LPS responses, we exposed primary microglia to LPS for 6 hours before mRNA analysis.

Enriched microglial cultures produced robust *Il1b*, *Tnfa*, *Nos2* and *Cd40* responses following direct stimulation with a range of concentrations of IFN γ and LPS. Microglial pro-inflammatory responses to LPS are well characterised *in vitro* and are synonymous with *in vivo* phenotypes (Hoogland et al., 2015; Lund et al., 2006). However, bacterial activation of microglia *in vivo* is rare due to the presence of the BBB. The application of pro-inflammatory cytokines like IFN γ or TNF α to primary microglial cultures re-enacts physiological mechanisms of activation. In the present study we show that enriched microglial cultures are responsive to the classic inflammatory stimulus LPS as well as an intrinsic trigger IFN γ .

Furthermore, the presence of extracellular LDH in culture media was analysed to determine the viability of cultures. Interestingly, LPS but not IFN γ induced cytotoxicity at high concentrations. Concentrations of IFN γ and LPS used to stimulate microglia *in vitro* varies, but use of 50 ng/ml of IFN γ and 100 ng/ml of LPS are common practice in primary microglia cultures (He et al., 2024; He et al., 2021; Jiang et al., 2022; Woolf et al., 2025). As 50 ng/ml of IFN γ and 100 ng/ml of LPS are frequently reported in the literature and both elicited pro-inflammatory signalling in enriched microglial cultures in the present study, CM was collected from enriched microglia treated with 50 ng/ml of IFN γ or 100 ng/ml LPS and transferred to neurons to establish an *in vitro* model of inflammation-driven changes in neuronal complexity.

CM from glia treated with IFN γ or LPS was not toxic to neurons in this study. The Harkin lab have previously reported that IFN γ CM does not affect neuronal viability (O'Reilly et al., 2021). However, when microglia are exposed to high concentrations of LPS the resulting CM induces apoptosis in neuronal cells (Dai et al., 2015; Nakao et al., 2022).

The Harkin lab has also previously shown that the direct application of IFN γ to pure primary neurons does not affect their complexity or viability but IFN γ treated glial CM reduces neuronal outgrowth in these cultures (O'Farrell et al., 2017; O'Reilly et al., 2021). Furthermore, in organotypic hippocampal slice cultures, an LPS mediated loss of synapses was attenuated by the depletion of microglia (Sheppard et al., 2019), highlighting the role of microglia-specific secreted pro-inflammatory markers on neuronal function.

Collectively these results demonstrate that enriched microglial cultures have sufficient proportions of microglia to produce robust and reliable responses to pro-inflammatory

stimuli and the resulting CM negatively impacts neuronal morphology. Therefore, these cells are a suitable platform for testing the biocompatibility of biomaterials.

Chapter 4: Biomaterial preparation and biocompatibility *in vitro*

4.1. Introduction

Polymeric nanoparticles are emerging as potential candidates for controlled drug delivery to multiple systems, including the brain and for the treatment of a range of neurological disorders. Several synthetic polyesters and natural polymers can be used to synthesise nanoparticles including PLGA, poly(D,L-lactic acid), poly(ϵ -caprolactone), chitosan, alginate and maltodextrins. Of these, PLGA is a particularly attractive material for use in biomaterials as it is an FDA and EMA approved copolymer that is used in a variety of medicinal products available on the global market, including long-acting drug depots, implants and suture materials (Kamaly et al., 2016). In a recent randomised clinical trial, local delivery of a thermosensitive gel containing chitosan coated-PLGA nanoparticles loaded with antibiotic ciprofloxacin was found to have superior efficacy over standard treatments in endodontic infections (NCT05475444) (Arafa et al., 2023). Moreover, phase I clinical trials investigating the safety, tolerability and efficacy of intravenous PLGA nanoparticles loaded with immunotherapy PRECIOUS-01 for the treatment of various types of cancer are ongoing in the Netherlands and results are expected in 2026 (NCT04751786) (Creemers et al., 2021). When completed, this study is likely to give important insights into the safety, pharmacokinetics and disease-modifying effects of PLGA-mediated drug delivery in humans following systemic administration.

PLGA nanoparticles are particularly advantageous as drug nanocarriers as they are biocompatible, tuneable, encapsulate a wide range of drug classes including hydrophobic, hydrophilic and varying molecular weight drugs, display controlled release kinetics and are reported to cross the BBB *in vitro* (Begines et al., 2020; Kamaly et al., 2016; Neha et al., 2013; Patel et al., 2012; Zhang et al., 2021a). PLGA nanoparticles are also biodegradable; PLGA is degraded by hydrolysis of its ester linkages resulting in lactic and glycolic acid water soluble monomers, which can enter cells via monocarboxylate transporters or by diffusion, respectively. Intracellular lactic and glycolic acids are used up in the Krebs cycle and are excreted from the body as water and CO₂ (Alghareeb et al., 2024; Alsaab et al., 2022).

PLGA nanoparticles are constructed using bottom-up techniques, namely nanoprecipitation, solvent evaporation, salting-out or dialysis (Kamaly et al., 2016; Rao & Geckeler, 2011). These synthesis methods can yield particles with distinct physicochemical characteristics. Both size and surface charge of nanoparticles affect cellular uptake and distribution in biological systems. Small nanoparticles with a diameter of 5 nm or less are rapidly cleared from the circulation by the kidneys, while nanoparticles that are 200 nm or larger can become lodged in the spleen (Jo et al., 2015; Sahin et al., 2017; Soo Choi et al.,

2007). Size also affects BBB penetration and smaller polymeric nanoparticles display increased transport across *in vitro* BBB models (Kulkarni & Feng, 2013).

Due to favourable electrostatic interactions with negatively charged cell membranes, cationic nanoparticles are more easily internalised by cells than neutral or anionic nanoparticles. However, the feasibility of cellular uptake also results in the rapid clearance of positively charged nanoparticles from the circulation by macrophages as well as increased liver accumulation, which results in prompt plasma clearance and reduced bioavailability (Jo et al., 2015). Positively charged nanoparticles may interact with blood components, potentially causing haemolysis, platelet aggregation and toxicity (Di et al., 2021; Mehrizi et al., 2021). While negatively charged PLGA nanoparticles can interact with platelets, this has not been reported to cause or interfere with platelet aggregation (Bakhaidar et al., 2019; Bakhaidar et al., 2020). Cationic particles have been shown to cause cytotoxicity and disrupt the integrity of the BBB, whereas such effects are not reported for neutral and anionic nanoparticles (Han et al., 2007; Lockman et al., 2004). PLGA nanoparticles are typically negatively charged but this can vary depending on the synthesis method used and may be altered by surface modification or coating.

Surface engineering can improve particle biocompatibility, brain targeting, stability, and controlled drug release. Polymers like PEG, PCL, chitosan, and PEG-based surfactants like polysorbate 80 and poloxamer 188 can be chemically grafted or adsorbed onto the surface of PLGA nanoparticles. The hydrophilicity of these moieties increases steric hindrance and circulation time (Priya et al., 2023). PEGylation of nanoparticles for CNS drug delivery is common and is reported to improve the circulation time, biocompatibility, and brain uptake, even in pathological conditions (Nance et al., 2012; Sánchez-López et al., 2018; Zhi et al., 2021). Typically, polymers used for coating PLGA nanoparticles are either positively charged or neutral and can therefore be used to alter surface charge.

The biocompatibility of PLGA nanoparticles has been evaluated *in vitro*, where the viability of human SH-SY5Y neuroblastoma cells, monocytes, 16 HBE epithelial cells used to model the BBB and a 'BBB on a chip' model, rodent PC12 catecholaminergic neurons, brain endothelial cells, primary microglia and primary astrocytes, or murine hippocampal neurons, N2a neuroblastoma cells, and N9 microglia were all unaffected by PLGA nanoparticles (Cano et al., 2020; Chatzitaki et al., 2020; Garcia et al., 2023; Jeon et al., 2019; Katila et al., 2022; Kaur et al., 2022; Sánchez-López et al., 2018; Vanden-Hehir et al., 2019; Wang et al., 2022). Even prolonged PLGA nanoparticle exposure was deemed safe in primary neuronal-mixed glial co-cultures where no effects on viability or neuronal morphology were reported with particle concentrations up to 2.5 mg/ml (Grabrucker et al., 2011). Furthermore, 20 mg/ml of PLGA nanoparticles was non-toxic to 16HBE cells (Meng et al., 2018), highlighting the broad safety margin of these materials in CNS cell cultures.

Owing to the simple synthesis methods, size, wide range of drugs that can be loaded and the ability of these particles to cross the BBB for controlled release, PLGA nanoparticles are

a promising candidate for enhanced drug delivery to the CNS. Despite extensive preclinical validation, the translation of polymeric nanoparticles to CNS applications remains limited by stability, reproducibility, and predictable immune interactions.

4.2. Aims

The aim of this chapter was to prepare PEG-PLGA nanoparticles that are compatible with neurons and enriched microglia *in vitro*. We hypothesised that PEGylated PLGA nanoparticles of sub-100 nm in size would exhibit negligible immunogenic activation in primary enriched microglia and would not compromise neuronal morphology.

4.3. Experimental design

PEG-PLGA nanoparticles were prepared using a nanoprecipitation method and a carboxylic acid PEG-block-PLGA co-polymer and assessed for size and surface charge (see methods section 2.2.1. and 2.2.2). Primary enriched microglia were isolated from the neonatal rat cortex and cultures were enriched for microglia (see methods section 2.3). When mature, cultures were exposed to PEG-PLGA (0-1000 $\mu\text{g/ml}$) or IFN γ (50 ng/ml) as a positive control for 24 hours. Cells were lysed, RNA was isolated and cDNA was synthesised for PCR gene expression analysis. PEG-PLGA (0-1000 $\mu\text{g/ml}$) were directly applied to mature primary neuronal cultures isolated from the neonatal rat cortex for 24 hours and cells were fixed and immunocytochemistry for the neuronal marker MAP2 was performed. CM from enriched microglia was transferred to neuronal cultures for a further 24 hours, before cells were fixed and stained for MAP2. All neurons were imaged using a fluorescent microscope and morphology was analysed to create a Sholl profile of neuronal complexity. Enriched microglial and neuronal cell culture media was collected in each experiment for LDH cytotoxicity assay.

4.4. Results

4.4.1. Characterisation of PEG-PLGA

The average hydrodynamic size of PEG-PLGA was 96 ± 5 nm (**Fig. 4.1a**) with a Pdl of 0.097 ± 0.01 . ζ -potential measurements showed that PEG-PLGA had a surface charge of -24 ± 4 mV (**Fig. 4.1b**). The spherical shape of PEG-PLGA and size uniformity were confirmed by TEM (**Fig. 4.1c, d**).

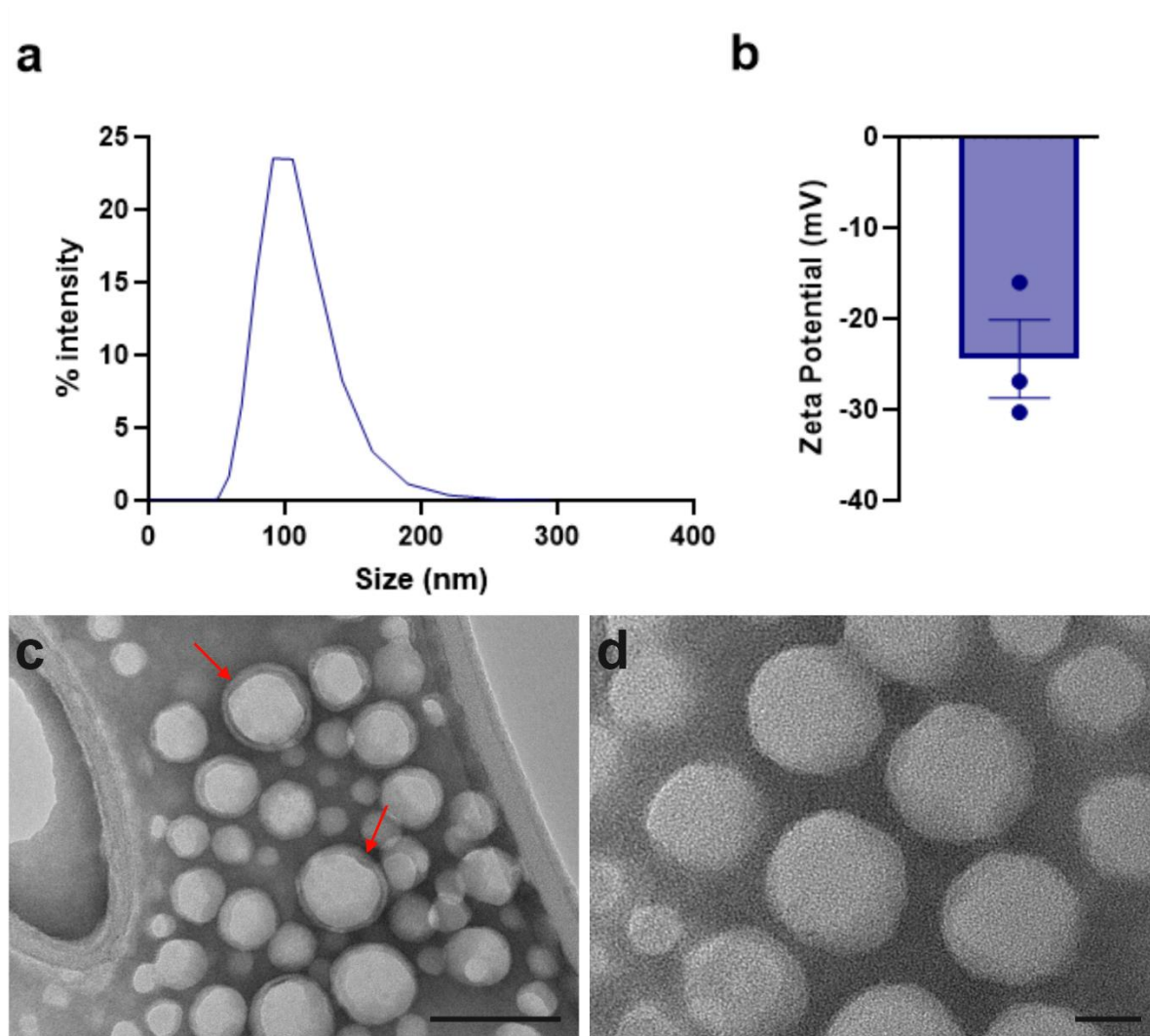


Figure 4.1. PEG-PLGA characterisation.

(a) Hydrodynamic size distribution, (b) ζ -potential measurement and (c) and (d) transmission electron microscopy (TEM) images of PEG-PLGA nanoparticles, where arrows highlight a faint outer corona consistent with PEG. Data represents the average size frequency (a) and the mean $\pm$ SEM (b), where N=3 separate batches of nanoparticles. Direct magnification 80000X, scale bar = 100 nm (c) and direct magnification 200000X, scale bar = 20 nm (d). TEM was performed by Dr. Raman Bekarevich (School of Chemistry, Trinity College Dublin) and Ms. Almudena Moreno-Borrillo (School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin).

4.4.2. PEG-PLGA are biocompatible with primary rat enriched microglial cultures

One-way ANOVA of extracellular LDH showed an effect of PEG-PLGA [$F_{(5, 12)} = 8.03$, $P < 0.01$] (**Fig.4.2a**). Dunnett's multiple comparisons revealed that LDH release in enriched microglia exposed to 1000 $\mu\text{g/ml}$ of PEG-PLGA had higher extracellular LDH compared to untreated control ($P < 0.01$).

One-way ANOVA of fold change in *I11b* mRNA showed an effect of PEG-PLGA [$F_{(6, 21)} = 13.65$, $P < 0.0001$] (**Fig.4.2b**). Dunnett's multiple comparisons revealed an increase in *I11b* mRNA in enriched microglia exposed to 400 and 1000 $\mu\text{g/ml}$ of PEG-PLGA compared to untreated control ($P < 0.05$ and $P < 0.0001$, respectively).

One-way ANOVA of fold change in *Tnf α* mRNA showed an effect of PEG-PLGA [$F_{(6, 21)} = 3.259$, $P < 0.05$] (**Fig.4.2c**). Dunnett's multiple comparisons revealed an increase *Tnf α* mRNA in enriched microglia exposed to IFN γ (50 ng/ml) ($P < 0.01$) but not PEG-PLGA when compared to control.

Kruskal-Wallis ANOVA of fold change in *Nos2* mRNA showed an effect of PEG-PLGA [$H_{(6)} = 24.17$, $P < 0.001$] (**Fig.4.2d**). Dunn's multiple comparisons revealed an increase in *Nos2* mRNA in enriched microglia exposed to IFN γ (50 ng/ml) ($P < 0.001$) and 1000 $\mu\text{g/ml}$ of PEG-PLGA ($P < 0.01$) compared to control.

Kruskal-Wallis ANOVA of fold change in *Cd40* mRNA showed no effect of IFN γ or PEG-PLGA (**Fig.4.2e**).

Kruskal-Wallis ANOVA of fold change in *Cd68* mRNA showed no effect of IFN γ or PEG-PLGA (**Fig.4.2f**).

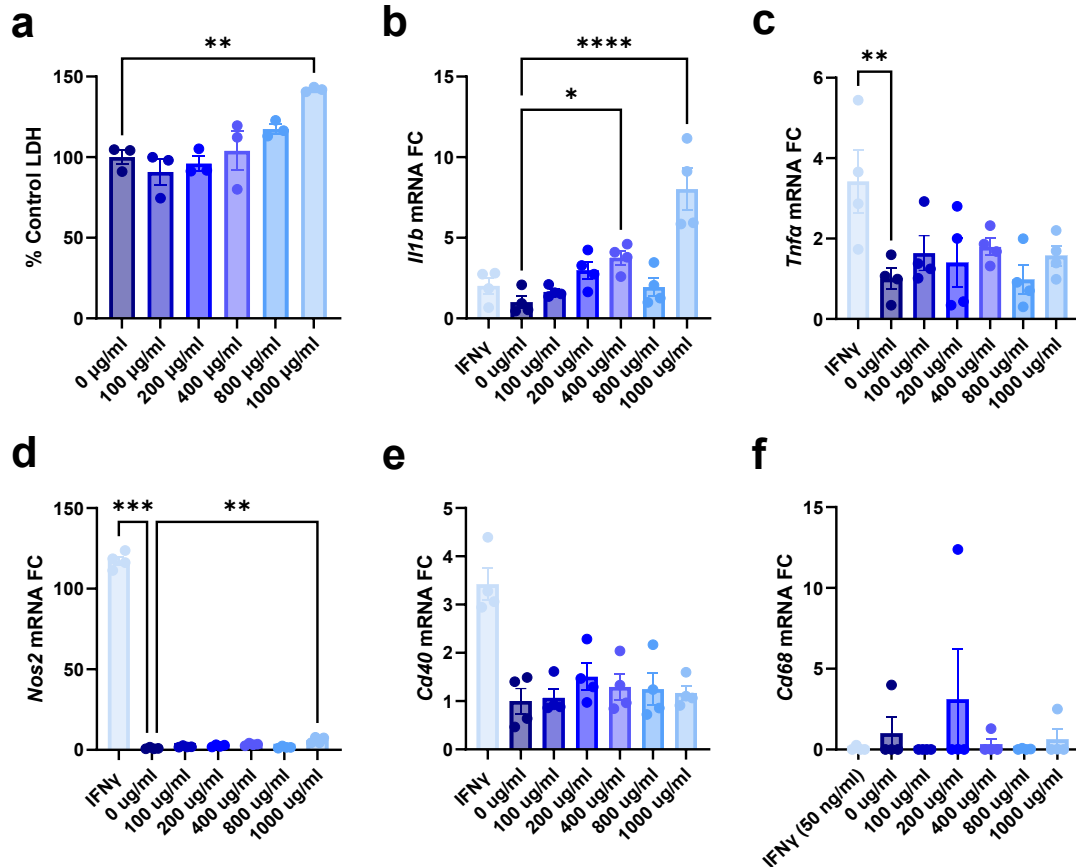


Figure 4.2. Biocompatibility of PEG-PLGA in primary enriched microglial cultures.

Enriched microglial cultures were exposed to PEG-PLGA (0-1000 µg/ml) or IFNγ (50 ng/ml) for 24 hours. (a) Extracellular LDH as a % of control and fold change (FC) in (b) *Il1b*, (c) *Tnfa*, (d) *Nos2*, (e) *Cd40* and (f) *Cd68* mRNA. Data were analysed by one-way ANOVA followed by Dunnett's multiple comparisons (a-c) or Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons (d-f) and are expressed as the mean ± SEM, where N=3-4. Significant differences between groups are denoted by* where ** P<0.01, *** P<0.001 and **** P<0.0001.

4.4.3. PEG-PLGA are biocompatible with primary rat neuronal cultures

One-way ANOVA of extracellular LDH, the number of primary neurites, the number of branches and neurite length showed no effect of PEG-PLGA (**Fig.4.3a-d**).

Two-way repeated measures of the Sholl profile showed an effect of distance [$F_{(25, 300)} = 1157$, $P < 0.0001$] (**Fig.4.3e**). Tukey's multiple comparisons did not reveal any relevant differences in the number of intersections at any distance from the soma between groups.

4.4.4. Conditioned media from glia exposed to PEG-PLGA transferred to primary rat neuronal cultures does not affect neuronal complexity

One-way ANOVA of extracellular LDH, the number of primary neurites, the number of branches and neurite length showed no effect of PEG-PLGA CM (**Fig.4.4a-d**).

Two-way repeated measures of the Sholl profile showed an effect of distance [$F_{(25, 300)} = 323.7$, $P < 0.0001$] and a distance x PEG-PLGA CM interaction [$F_{(125, 300)} = 1.37$, $P < 0.05$] (**Fig.4.4e**). Tukey's multiple comparisons did not reveal any relevant differences in the number of intersections at any distance from the soma between groups.

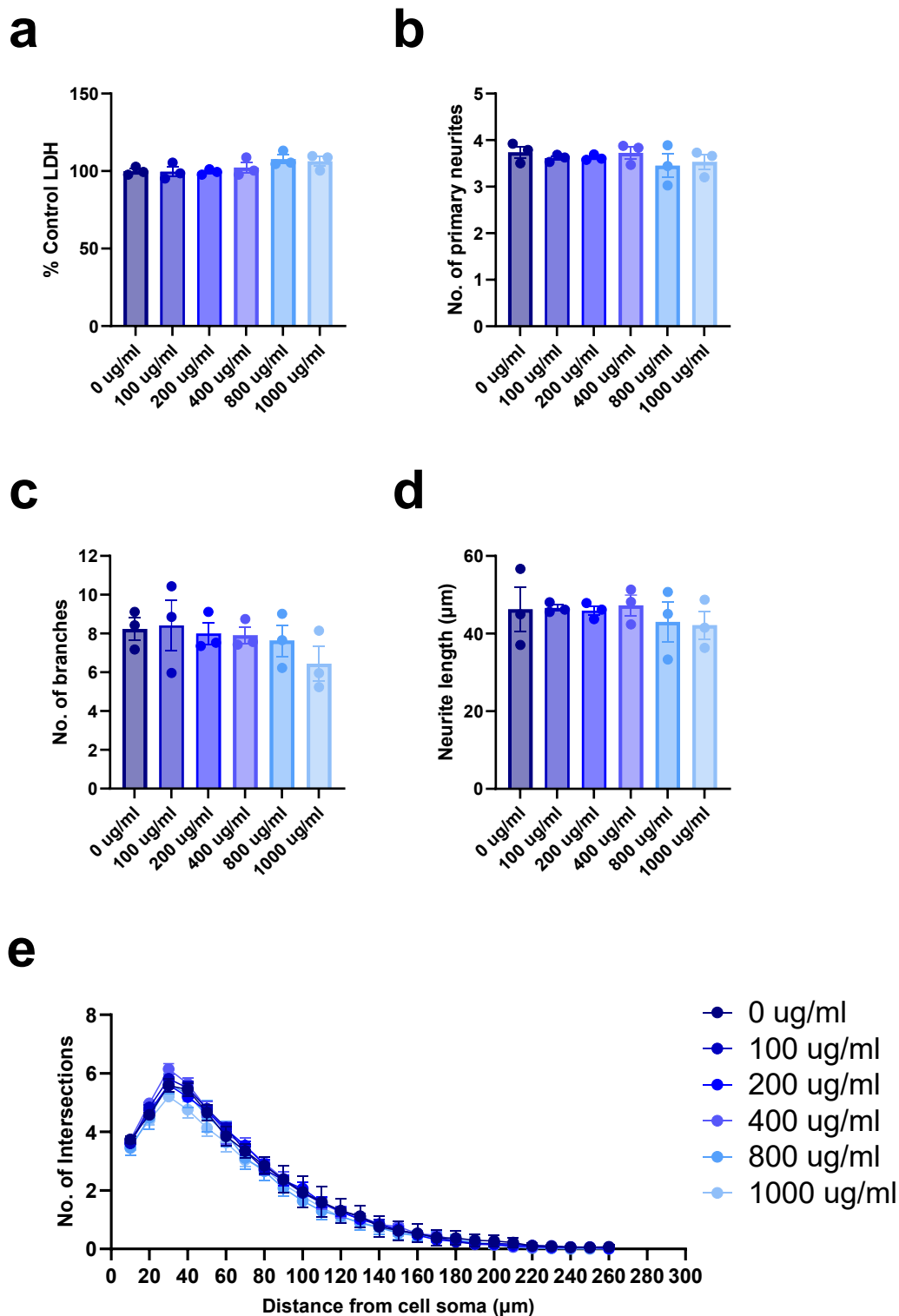


Figure 4.3. Biocompatibility of PEG-PLGA in primary cortical neuronal cultures.

Primary neuronal cultures were exposed to PEG-PLGA (0-1000 μg/ml) for 24 hours. (a) Extracellular LDH as a % of control, (b) the number of primary neurites, (c) the number of branches, (d) neurite length and (e) the Sholl profile of neuronal complexity. Data were analysed by one-way ANOVA (a-d) or two-way repeated measures ANOVA followed by Tukey's multiple comparisons (e) and are expressed as the mean ± SEM, where N=3.

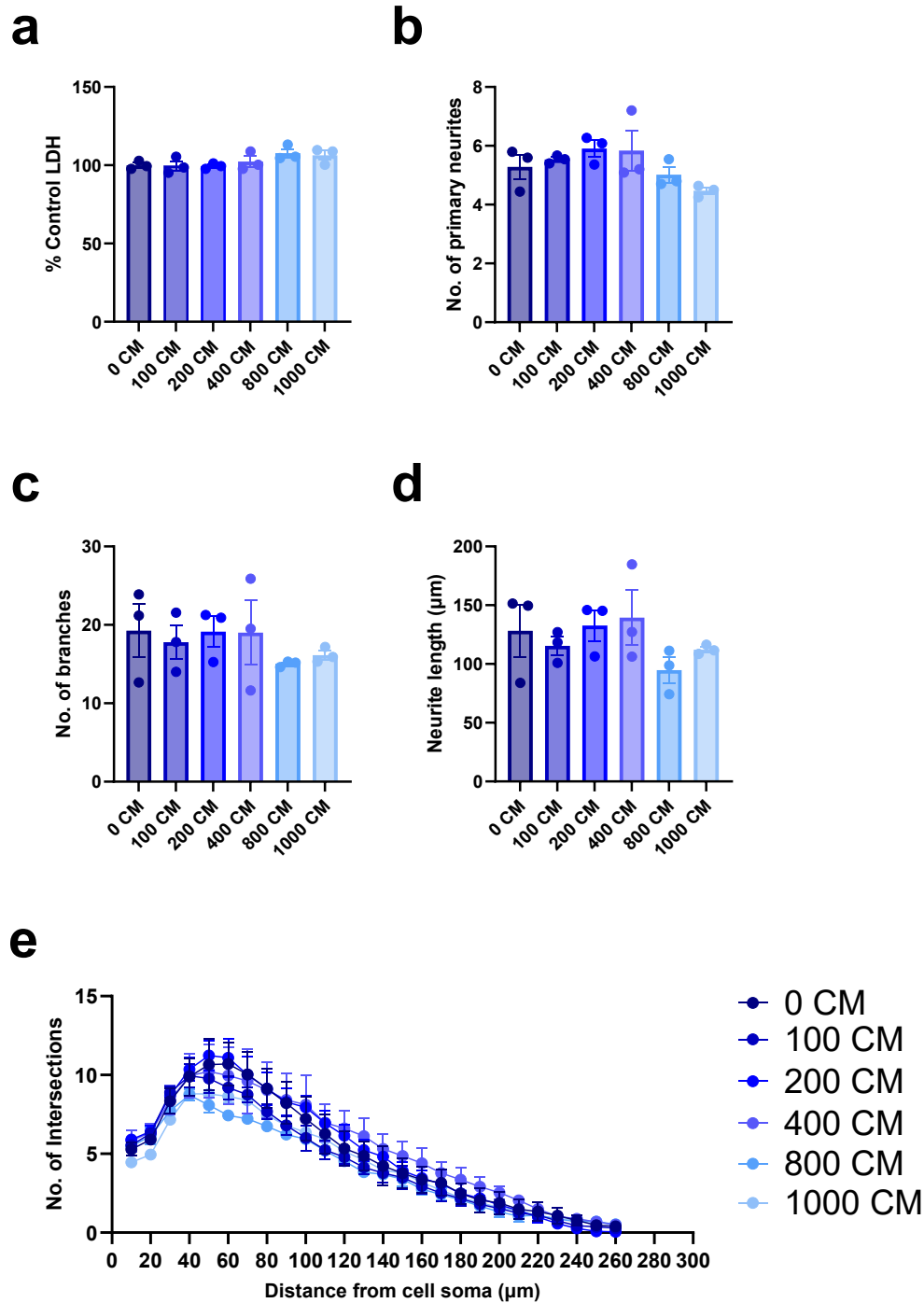


Figure 4.4. CM from glia treated with PEG-PLGA does not affect neuronal complexity.

CM from glia exposed to PEG-PLGA (0-1000 µg/ml) for 24 hours was transferred to primary neuronal cultures for a further 24 hours. (a) Extracellular LDH as a % of control, (b) the number of primary neurites, (c) the number of branches, (d) neurite length and (e) the Sholl profile of neuronal complexity. Data were analysed by one-way ANOVA (a-d) or two-way repeated measures ANOVA followed by Tukey's multiple comparisons (e) and are expressed as the mean $\pm$ SEM, where N=3.

4.5. Discussion

4.5.1. Considerations for PLGA nanoparticle synthesis

Nanoparticles were prepared using a nanoprecipitation method whereby PLGA is dissolved in a water-miscible solvent and rapidly mixed with water, so the polymer loses solubility and self-assembles into nanoparticles (de Oliveira et al., 2013). This method is advantageous for several reasons; it is fast, simple, reproducible and generally produces smaller nanoparticles than emulsions, salting-out or spray-drying. Minor adjustments to the polymer concentration, rate of injection of the organic phase, the agitation rate of the aqueous phase and the ratio of organic to aqueous phase can alter the properties of the resulting nanomaterials, allowing the particle properties to be easily tuned (Rao & Geckeler, 2011).

A 20kDa PEG-b-PLGA diblock copolymer was used to prepare nanoparticles in this study, where PEG is covalently linked to PLGA (**Fig. 4.5**). PEG is hydrophilic, so these chains are orientated toward the surface during assembly in water. The PLGA copolymer used has a 50:50 polylactic acid (PLA) to polyglycolic acid (PGA) ratio, rendering it relatively hydrophobic. However, PGA is more hydrophilic, so adjusting the ratio of PLA or PGA can alter the hydrophobicity and degradation rate of the nanoparticle core, with higher PLA content leading to slower degradation (Makadia & Siegel, 2011). PEG in the copolymer used in this study is terminally capped with a carboxylic acid group, which can be used to further conjugate surface moieties.

The PEG-PLGA nanoparticles synthesised here were under 100 nm when measured by DLS, with a Pdl of 0.097, indicating that samples were highly uniform and monodisperse. DLS measures fluctuations in scattered light due to Brownian motion to give a hydrodynamic size, where smaller particles diffuse faster than larger particles, producing more rapid fluctuations in scattered light intensity. However, particle size visualized by TEM were ~70 nm. Nanoparticle sizes obtained by DLS are often much larger than TEM (Filippov et al., 2023). This is especially true when a hydrophilic surface coating like PEG is used, as PEG attracts water molecules and forms a hydrated layer around the particle, inflating the DLS size measurement. PEGylated nanoparticles have better colloidal stability and can overcome van der Waals forces, preventing aggregate formation (Suk et al., 2016), so the larger diameter measured by DLS is indicative of the presence of PEG on the surface of the nanoparticle. TEM images revealed the spherical and relatively uniform PLGA core, and a faint outer corona consistent with PEG was visible on some particles. Generally, PEG is difficult to see under standard TEM conditions where samples are dried and the shell structure collapses. PEG is made from carbon, hydrogen and oxygen atoms and has low electron contrast, especially compared to the dense PLGA core (Pustulka et al., 2013).

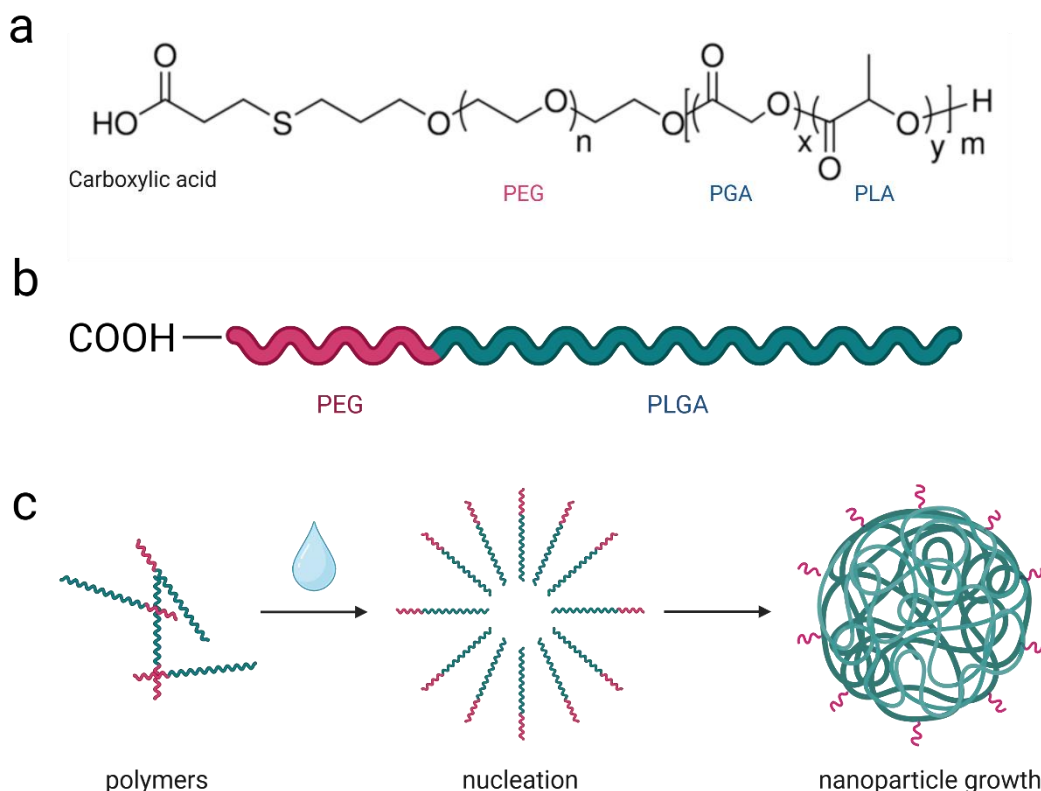


Figure 4.5. PEG-PLGA nanoparticle formation.

(a) PEG-PLGA chemical structure, which contains ester bonds that are hydrolysed during degradation, occurring along the PLGA backbone between lactic and glycolic acid monomers, and also at the junction between PEG and PLGA blocks, and (b) schematic of PEG-PLGA polymer. (c) Schematic of PEG-PLGA nanoparticle formation; the copolymers spontaneously self-assemble as the hydrophilic PEG chains interact with water, while the hydrophobic PLGA segments aggregate to form a dense polymeric core. This process produces nanoparticles with a hydrophobic PLGA core and a hydrophilic PEG corona.

The average surface charge of PEG-PLGA synthesised in this study was -24 mV. PLGA is a negatively charged polymer, while PEG carries a neutral charge. However, the carboxylic acid group attached to PEG gives it a slight negative charge. Although positively charged nanoparticles can facilitate cellular uptake in *in vitro* BBB models, *in vivo* they readily adsorb negatively charged plasma proteins, forming a protein corona that shifts their overall surface potential toward negative values (Jo et al., 2015). Therefore, weakly negative PEGylated nanoparticles may be more beneficial in drug delivery systems, especially as PEG provides steric hindrance of electrostatic and hydrophobic interactions which reduces protein binding and immune recognition, leading to longer circulation times (Kamaly et al., 2016).

PEGylation is a broad term used to describe nanoparticles that have PEG in their structure, but these can be bulk PEGylated or surface modified PEGylated nanoparticles. In this study,

PLGA nanoparticles were bulk PEGylated as PEG was covalently linked to the PLGA backbone prior to nanoparticle formation, which differs from surface PEGylated nanoparticles, in which PEG is adsorbed or grafted onto the surface of the particle after synthesis. Bulk PEGylated PLGA nanoparticles are reported to have higher drug encapsulation efficiency, enhanced solubility, controlled drug release, better stability and are more biocompatible (Alghareeb et al., 2024).

Direct comparisons with PEG-PLGA nanoparticles in the literature are difficult to make due to the use of both high and low molecular weight polymers with varying PLA:PGA ratios, different synthesis methods and a broad range of surface chemistry as well as a lack of reporting on non-drug loaded particle properties. However, in a few studies that synthesised PEG-PLGA nanoparticles using nanoprecipitation methods, hydrodynamic sizes ranged between 100-176 nm with Pdl values of 0.1-0.2 and surface charge from -15 to -30 mV (de Oliveira et al., 2013; Marinelli et al., 2022; Mohan et al., 2020; Zhang et al., 2023a), which aligns with the data described here.

4.5.2. Biocompatibility of PEG-PLGA nanoparticles in neuronal and glial cultures

PLGA is an FDA and EMA approved polymer and its biocompatibility is well established. PEG is also an FDA and EMA approved medicine for the treatment of constipation and many PEGylated therapeutics are available globally. For example, PEGylated lipid nanoparticles were used to deliver mRNA-based COVID-19 vaccines during the pandemic (Gao et al., 2024). In PEG-PLGA copolymers, PEG is mostly excreted from the body unchanged in urine following hydrolysis of the ester bond that binds it to PLGA (Webster et al., 2007).

In this study, PEG-PLGA did not induce an immunogenic response in enriched microglial cultures or affect viability at biologically relevant concentrations. The transfer of CM to neurons was performed to assess if factors secreted into glial culture medium in response to PEG-PLGA are neurotoxic. Neither CM transfer nor application of PEG-PLGA directly to neurons compromised cell viability or reduced neuronal complexity. This aligns with previous studies showing that BV2 microglia secrete IL-6 and TNF α in response to PLGA nanoparticles, but not 23 nm PLGA-PEG nanoparticles with a surface charge of -33.9 mV (Morshed et al., 2024).

These findings are in line with many other studies that reported on the biocompatibility of PLGA nanoparticles with human and rodent CNS cells *in vitro* (Cano et al., 2020; Chatzitaki et al., 2020; Garcia et al., 2023; Grabrucker et al., 2011; Jeon et al., 2019; Katila et al., 2022; Kaur et al., 2022; Meng et al., 2018; Sánchez-López et al., 2018; Vanden-Hehir et al., 2019; Wang et al., 2022). Furthermore, biomaterials are being employed as scaffolds for human iPSC and organoid growth in the field of nerve tissue engineering and PLGA was shown to have no effect on stem cell proliferation (Zhou et al., 2018). PLGA nanoparticles that were

93 nm with a zeta potential of -29 mV were tested in human iPSC-based BBB models, where they were found to permeate cells without evidence of toxicity (Onyema et al., 2021).

BV2 microglia have been shown to phagocytose PLGA nanoparticles, although if nanoparticles are less than 100 nm this activity is reduced (Vanden-Hehir et al., 2019). Moreover, PEGylation significantly reduced nanoparticle uptake by endothelial, astrocyte, microglia and neuronal rodent cell lines compared to uncoated PLGA nanoparticles (Morshed et al., 2024). Here, we have shown no effect of PEG-PLGA on *Cd68* expression, which is a common marker for phagocytosis in microglia. Future experiments may investigate whether the PEG-PLGA produced in this study are phagocytosed by cells in primary cultures using tagged polymers for nanoparticle synthesis followed by imaging or flow cytometry to quantify uptake (Lamparelli et al., 2024; Mehrizi et al., 2021).

Assuming adequate drug loading, approximately 100 µg/ml will be required in future *in vivo* studies to deliver drug to animal models of neuroinflammation. Here, we have demonstrated that 100 µg/ml of PEG-PLGA nanoparticles is biocompatible in enriched microglial cultures and neuronal cultures *in vitro* and is suitable for use in subsequent experiments.

4.5.3. Future directions

While freshly synthesised nanoparticles were used in this study, the stability of PEG-PLGA overtime and in various media warrants further investigation. PLGA nanoparticles are unstable in water at high temperatures as they begin to breakdown by hydrolysis but are reportedly stable at 4°C for up to 5 weeks (Swetledge et al., 2021). For long-term storage, PLGA nanoparticles are typically lyophilised and stored between 2-8 °C for months, however this introduces further variables such as the drying method and the type of cryoprotectant used. This process can also alter the physicochemical properties of nanoparticles so re-characterisation of particles and biocompatibility testing upon reconstitution is recommended. Future experiments may involve repeating nanoparticle characterisation at different time intervals over a period of 6 months to a year and testing the compatibility of 'old' batches *in vitro*.

Overall, the PEG-PLGA prepared in this study were biocompatible with primary neuronal and glial cells, with desirable size, shape and surface properties for potential drug loading and CNS drug delivery.

Chapter 5: PEG-PLGA drug loading, release and *in vitro* anti-inflammatory effects

5.1. Introduction

Because of their favourable properties for CNS drug delivery, PLGA nanoparticles have been tested as carriers for experimental therapies in *in vitro* models of neurodegeneration and neuroinflammation.

Nanocarrier-based gene therapies are favourable due to the protection, stability and enhanced brain bioavailability they provide. In BV2 microglial cultures, PLGA nanoparticles loaded with small interfering RNA improved intracellular delivery and successfully altered gene expression to reduce inflammatory responses to LPS (Noh et al., 2020; Phạm et al., 2020) and enhanced phagocytosis in an amyloid β cell-based model of AD (Shin et al., 2024).

Several dietary supplements show promise in a variety of neurological disorders due to their antioxidant and anti-inflammatory properties. However, poor solubility, peripheral metabolism and low bioavailability have hindered the clinical use of such compounds for the treatment of CNS diseases. Loading such therapies in PLGA nanoparticles offers solutions to overcome these challenges and enhance therapeutic potential. Indeed, PLGA nanoparticles loaded with fucoxanthin, a natural carotenoid pigment with antioxidant properties found mainly in brown seaweeds, prevented A β -induced neurotoxicity, the production of reactive oxygen species, and the release of pro-inflammatory cytokines in SH-SY5Y neurons and BV2 microglia cells (Yang et al., 2021). Similarly, pre-treatment with PLGA nanoparticles loaded with resveratrol, a natural polyphenolic antioxidant and anti-inflammatory compound found in berries and certain plants, reduced hydrogen peroxide induced ROS production and protected SH-SY5Y cells against mitochondrial dysfunction and cytotoxicity caused by the PD-associated toxin 1-methyl-4-phenylpyridinium (MPP⁺) (Katila et al., 2022).

The serotonin and noradrenaline reuptake inhibitors duloxetine and amitriptyline, both approved antidepressants, were encapsulated in PLGA nanoparticles to reduce side effects and support their repurposing for the treatment of neuropathic pain. Both duloxetine-PLGA and amitriptyline-PLGA nanoparticles significantly reduced *Tnfa* and *Il1b* expression in BV2 microglia up to 48 hours post-LPS stimulation, compared to negative control and free drug (Kim et al., 2021; Kim et al., 2024).

Upon addition of loaded PLGA nanoparticles to cell culture media, an initial burst release occurs by diffusion of drug from the particle surface. This process can be gradient driven, where drug diffuses out of the particle through water filled pores, or convection driven, where water entering the nanoparticle essentially acts as an osmotic pump and drug is

pushed out as water molecules move in (Kamaly et al., 2016). Burst release is followed by a second phase of gradual dissolution as water penetrates the nanoparticle core, leading to bulk erosion of the polymer matrix as hydrolysis proceeds throughout the particle (Makadia & Siegel, 2011). Several factors in nanoparticle design can be adjusted to alter both release mechanisms, such as the molecular weight of the polymer used, polymer crystallinity, ratio of PLA: PGA, nanoparticle size, surface PEGylation, molecular weight of cargo and the amount of drug loaded in the nanoparticle core (Kamaly et al., 2016).

Cellular uptake of nanoparticles can also affect the rate of drug release. Within minutes, a corona can form as serum proteins bind to the outer surface of nanoparticles, where the presence or absence of a protein corona can determine how immune cells recognise nanoparticles (Rampado et al., 2020). Evidence indicates that macrophages and microglia can internalise PLGA nanoparticles through phagocytosis and endocytosis *in vitro*. PEGylation, however, improves particle stability in biological fluids and reduces nonspecific protein adsorption, leading to a thinner or more weakly bound protein that favours uptake via endocytosis (Manzanares & Ceña, 2020; Marecki et al., 2024; Sousa de Almeida et al., 2021). Drug and nanoparticles likely undergo exocytosis during endosomal cycling where drug release and cell surface receptor activation ensue. Although PLGA hydrolyses slowly at neutral pH, the acidic environment of late endosomes and lysosomes accelerates this process and may potentially destabilise the released drug cargo.

Several drug candidates have been proposed for repurposing in neurodegenerative diseases including PD. In particular, drugs that enhance noradrenergic tone show translational potential, as they have demonstrated both anti-inflammatory and neuroprotective effects. The Harkin lab has previously demonstrated that treatment of primary glial cultures with the β_2 AR agonist salmeterol increases the expression of negative regulators of pro-inflammatory IL-1 β signalling (McNamee et al., 2010b). Furthermore, salmeterol treatment attenuated pro-inflammatory signalling in BV2 microglia (Sharma et al., 2019; Sharma & Flood, 2018), while formoterol, another β_2 AR agonist, attenuated LPS-mediated NLRP3 inflammasome activation and pyroptosis in the N9 microglial cell (Erdem et al., 2024). Activation of astrocytic β_2 ARs by salmeterol and clenbuterol stimulates the release of neurotrophic factors that promote neuritic growth *in vitro* (Day et al., 2014; Junker et al., 2002; Semkova et al., 1996). Indeed, pre-treatment with low doses of salbutamol, bambuterol, clenbuterol, formoterol and salmeterol protected against LPS-induced neurotoxicity in primary mesencephalic neuron-glia cultures (Qian et al., 2011). In particular, formoterol is advantageous over other β_2 AR agonists as it is FDA and EMA approved, crosses the BBB, has a long duration of action with rapid onset and is a full β_2 AR agonist with higher efficacy than partial agonists (Wang et al., 2025). Despite growing evidence of the therapeutic potential of these β_2 AR agonists, their use is limited by off-target effects from systemic adrenergic receptor activation, which causes tachycardia, muscle tremor, nausea and headache. Encapsulation in PLGA nanoparticles may help overcome these limitations by enabling targeted delivery to the CNS.

To further enhance CNS drug delivery, the intranasal route can be used to bypass both the periphery and the BBB, with drugs likely reaching the brain via the olfactory or trigeminal nerves (Jeong et al., 2023). However, intranasal drug delivery is associated with unique challenges including mucociliary clearance, enzymatic degradation and swallowing or inhalation of the formulation. To overcome these limitations, mucoadhesive and thermosensitive *in situ* hydrogels have been developed for drug delivery and can be loaded with nanoparticles (Kaur et al., 2016). Such hydrogels increase residence time in the nasal cavity and prevent swallowing or inhalation, enhancing drug or nanoparticle absorption across the nasal mucosa and into the olfactory bulb compared to drug in solution (Agrawal et al., 2020).

Mucosal irritation and toxicity have been reported for some intranasal formulations, likely linked to the materials used or a lack of sterility (Agrawal et al., 2020). Chitosan is a mucoadhesive gelling agent with an excellent safety profile. Chitosan is also reported to promote drug absorption in the nasal cavity by transiently opening tight junctions in the nasal epithelium (Wu et al., 2007). Typically, thermosensitive *in situ* gels are liquid at low temperatures because electrostatic and hydrogen bonding interactions maintain the polymer chains in solution. However, as temperature rises, dehydration of hydrophobic segments induces gelation (Kaur et al., 2016). Like PLGA nanoparticles, drug or particle release from the gel follows a biphasic mechanism involving both diffusion and erosion of the matrix (Rahmanian-Devin et al., 2021).

Given their controlled biphasic release profile and successful use in delivering experimental therapeutics in cell culture models of CNS disease and injury, PLGA nanoparticles represent a promising strategy to support drug repositioning for neurological disorders. PLGA nanoparticles are an excellent candidate for loading with formoterol, which is reported to attenuate neuroinflammation and has neuroprotective effects *in vitro*. Furthermore, incorporation of PLGA nanoparticles into a chitosan-based thermosensitive hydrogel may enhance intranasal delivery by increasing mucosal residence time and absorption.

5.2. Aims

The aims of this chapter were as follows:

1. Load PEG-PLGA nanoparticles with formoterol (experiment 1)
2. Develop a biocompatible chitosan-based hydrogel suitable for intranasal delivery of Form-PEG-PLGA in subsequent studies (experiment 2)
3. Determine the release profile of formoterol from Form-PEG-PLGA and Form-PEG-PLGA embedded in the chitosan-based hydrogel (experiment 3)
4. Investigate the anti-inflammatory properties of Form-PEG-PLGA in a cell culture model of neuroinflammation (experiment 4)
5. Assess the biocompatibility of PEG-PLGA and the chitosan-based hydrogel with human PBMCs (experiment 5)

5.3. Experimental design

Experiment 1 and 2: Form-PEG-PLGA nanoparticles were prepared using a nanoprecipitation method and assessed for hydrodynamic size and ζ -potential (**Fig. 5.1a**). Drug loading was assessed by separating free drug from Form-PEG-PLGA nanoparticles and using HPLC coupled to UV detection to quantify unencapsulated drug (**Fig. 5.1b**). A mucoadhesive and thermosensitive chitosan hydrogel was prepared and loaded with Form-PEG-PLGA (**Fig. 5.1c**)(see methods sections 2.2.1-2.2.4).

Experiment 3: *in vitro* drug release from particles at body temperature was assessed by loading a 50kDa dialysis membrane with Form-PEG-PLGA (100 $\mu\text{g/ml}$ in ultrapure water) and the amount of drug in the release media (aCSF; pH 7.4) at defined intervals over 24 hours. Drug release from Form-PEG-PLGA-hydrogel at 37°C was examined by solidifying the gel and sampling the release media (PBS; pH 6.5) at defined intervals over 72 hours. Samples were measured by HPLC coupled to UV detection and compared to unencapsulated (free) drug (**Fig. 5.1d**) (see methods section 2.2.5).

Experiment 4: mature primary enriched microglial cultures were stimulated with IFN γ (50 ng/ml) or LPS (100 ng/ml) for 30 mins prior to treatment with formoterol (10 μM), Form-PEG-PLGA (equivalent to 10 μM of drug) or unloaded PEG-PLGA for a further 6 or 24 hours. Cells were lysed, RNA was isolated and cDNA was synthesised for PCR gene expression analysis. CM was transferred to neuronal cultures for a further 24 hours before cells were fixed, stained for MAP2, imaged using a fluorescent microscope and the complexity assessed by Sholl analysis (**Fig. 5.1e**).

Experiment 5: unloaded PEG-PLGA alone or in hydrogel (100 $\mu\text{g /ml}$) were applied to human PBMCs for 24 hours, cells were lysed, RNA was isolated and cDNA was synthesised for PCR gene expression analysis(**Fig. 5.1f**).

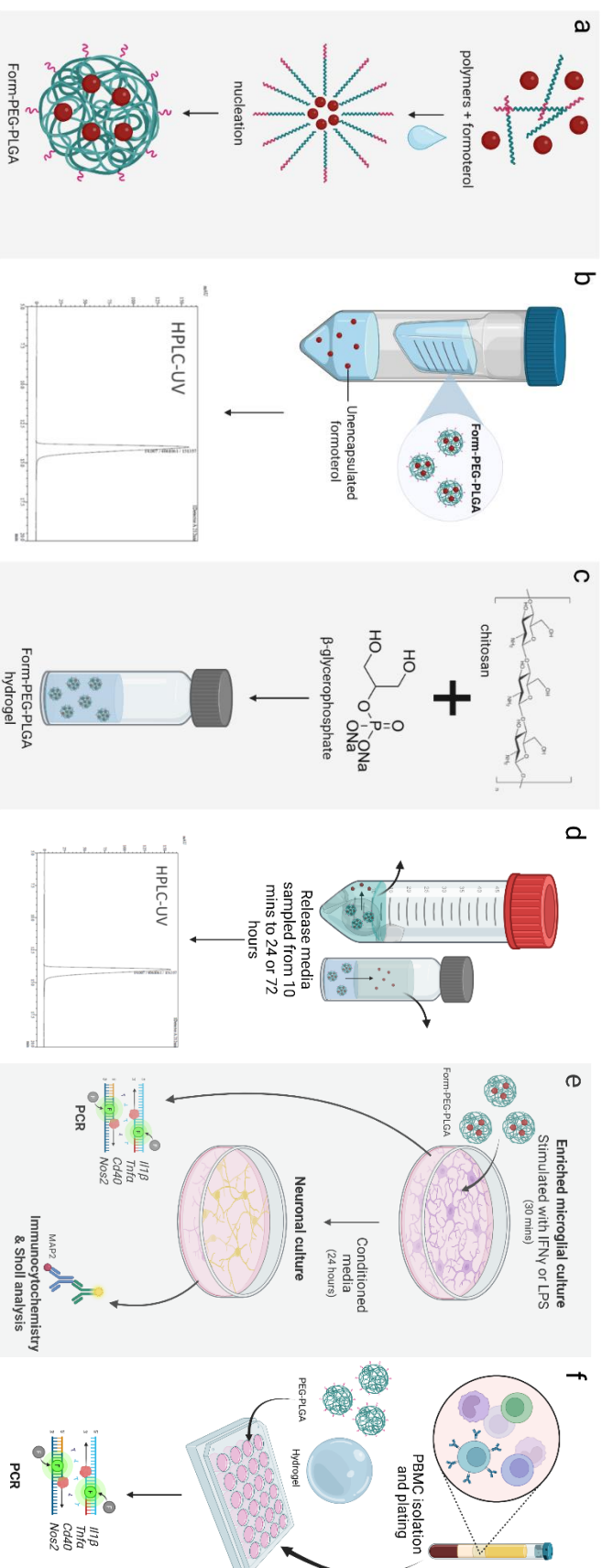


Figure 5.1 Experimental design.

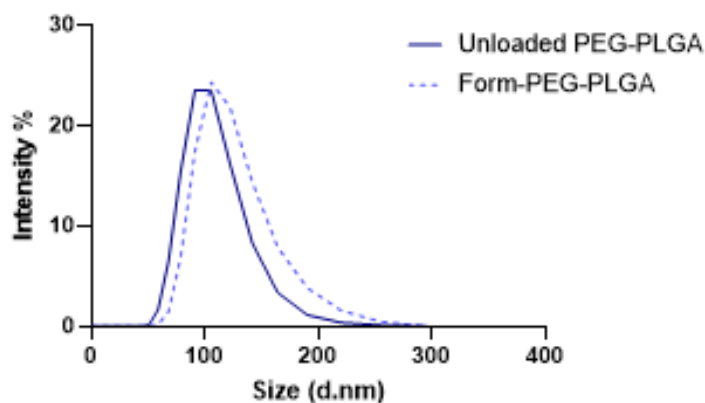
5.4. Results

5.4.1. Characterisation of Form-PEG-PLGA

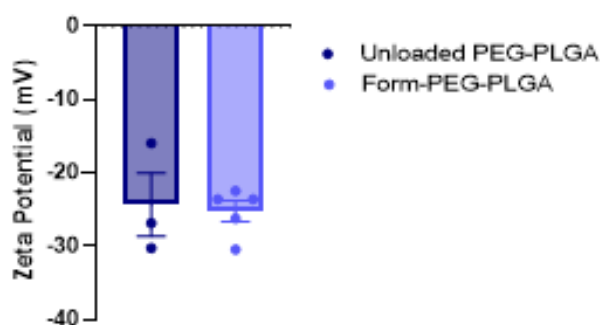
The average hydrodynamic size of Form-PEG-PLGA was 106 ± 5 nm (**Fig. 5.2a**) with a Pdl of 0.084 ± 0.03 . ζ -potential measurements showed that Form-PEG-PLGA had a surface charge of -25 ± 1 mV (**Fig. 5.2b**). The encapsulation efficiency (EE) and loading capacity (LC) of Form-PEG-PLGA was $40\% \pm 11$ and $26\% \pm 7$ (**Fig. 5.2c**).

Unpaired t-test showed no difference between the average size, Pdl and ζ -potential of unloaded PEG-PLGA and Form-PEG-PLGA.

a



b



c

	Size (nm)	ζ -potential (mV)	Pdl	EE	LC
Unloaded	96 ± 5	-24 ± 4	0.097 ± 0.01	N/A	N/A
Form-PEG-PLGA	106 ± 5	-25 ± 1	0.084 ± 0.03	$40\% \pm 11$	$26\% \pm 7$

Figure 5.2. Form-PEG-PLGA characterisation.

(a) Size distribution and (b) surface charge of Form-PEG-PLGA nanoparticles. (c) Summary of Form-PEG-PLGA nanoparticles. Data were analysed by unpaired t-test and represent the average size frequency (a) and the mean $\pm$ SEM (b), where N=3-5 separate batches of nanoparticles. Pdl: polydispersity index; EE: encapsulation efficiency; LC: loading capacity.

5.4.2. Formoterol release from Form-PEG-PLGA is sustained over 24 hours

Two-way repeated measures ANOVA of % drug release showed an effect of the formulation [$F_{(1, 6)} = 50.26$, $P < 0.001$], time [$F_{(17, 102)} = 286.2$, $P < 0.0001$] and a formulation x time interaction [$F_{(17, 102)} = 4.683$, $P < 0.0001$] (**Fig.5.3a**). Šídák's multiple comparisons revealed that unencapsulated formoterol was present in the external compartment at higher concentrations than drug released from Form-PEG-PLGA at each time point measured from 0.5 to 24 hours ($P < 0.0001$).

Within the first hour, % release of free formoterol and formoterol from Form-PEG-PLGA displayed good correlation with the Korsmeyer-Peppas model (equation 4, methods pg. 44) [free formoterol: $R^2 = 0.811$; Form-PEG-PLGA: $R^2 = 0.870$] (**Fig.5.3b**). The release exponent (n) values were 0.3865 and 0.4967 for free formoterol and Form-PEG-PLGA, respectively.

The complete release profiles for free formoterol and Form-PEG-PLGA were fitted to the Weibull release model (equation 5, methods pg. 44) (**Fig.5.3c**). Dissolution of free formoterol displayed high correlation with the Weibull model ($R^2 = 0.996$), with a scale parameter $\alpha = 0.3888$, shape parameter $\beta = 0.7102$ and Akaike's information criterion corrected (AICc) of 10.44. Drug release from Form-PEG-PLGA also displayed high correlation with the Weibull model ($R^2 = 0.999$), with $\alpha = 0.7389$, $\beta = 0.7098$ and AICc of -37.27. The resulting residuals ranged from -0.19 to 1.734 for free drug and -0.39 to 0.64 for Form-PEG-PLGA (**Fig.5.3d**).

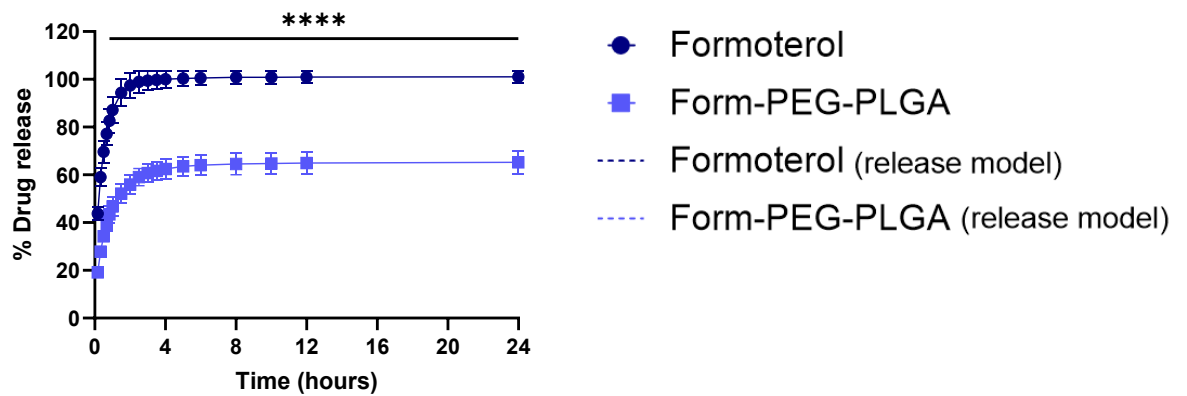
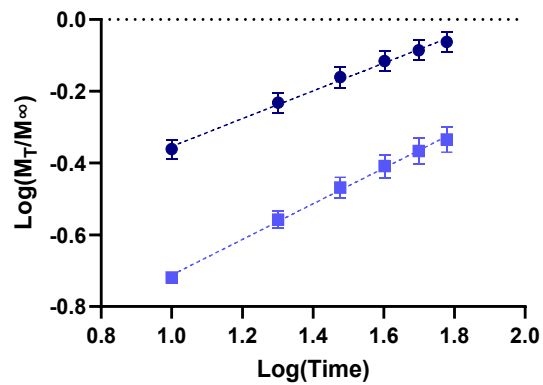
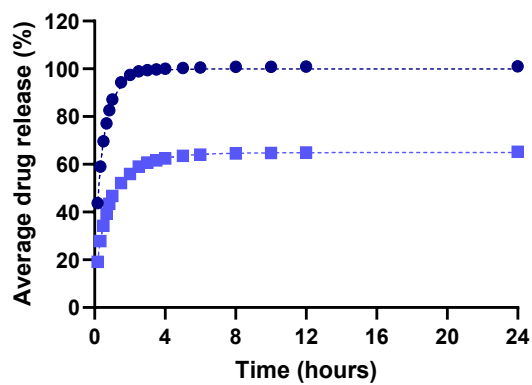
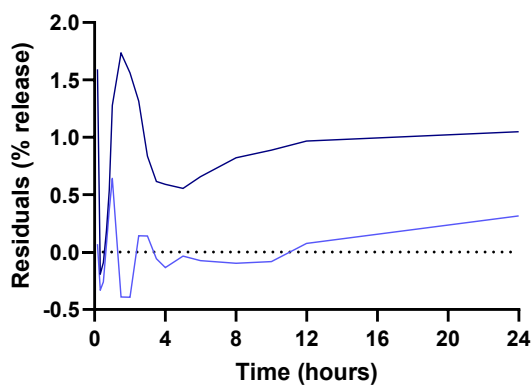
a**b****c****d**

Figure 5.3. Sustained drug release from Form-PEG-PLGA over 24 hours *in vitro*.

(a) % Drug release over 24 hours, (b) drug release in the first hour was fitted to the Korsmeyer-Peppas model (dotted line), (c) average drug release over 24 hours was fitted to the Weibull model (dotted line) and (d) average drug release residuals represent the difference between the observed and Weibull model-predicted drug release versus time, where random scatter of residuals around zero indicates appropriateness of fit. Data are represented as (a) the mean $\pm$ SEM and were analysed by two-way repeated measures ANOVA followed by Šídák's multiple comparisons (b) log transform of time and fraction of drug release at time t (M_t/M_∞) $\pm$ SEM, (c) as average % drug release and (d) % release minus Weibull model-predicted cumulative % release, where $N=3$ for all data sets. Significant differences between groups are denoted by **** $P<0.0001$ (a).

5.4.3. Formoterol release from Form-PEG-PLGA-gel is sustained over 72 hours

Two-way repeated measures ANOVA of % drug release showed an effect of the formulation [$F_{(2, 38)} = 78.17$, $P<0.0001$], time [$F_{(19, 38)} = 137.9$, $P<0.0001$] and a formulation $\times$ time interaction [$F_{(19, 38)} = 4.683$, $P<0.0001$] (**Fig.5.4a**). Šídák's multiple comparisons revealed that formoterol was released at higher concentrations from Form-gel than from Form-PEG-PLGA-gel at each time point between 0.5 to 72 hours (0.5-1.5 hours: $P<0.05$; 2-2.5 hours: $P<0.01$; 3-3.5 hours: $P<0.001$; 4-72 hours $P<0.0001$).

Within the first hour, % drug release from Form-gel but not Form-PEG-PLGA-gel displayed good correlation with the Korsmeyer-Peppas model (equation 4, pg. 44) [form-gel: $R^2 = 0.964$; Form-PEG-PLGA-gel: $R^2 = 0.4794$] (**Fig.5.4b**). The release exponent n was 0.5835 and 0.4502 for Form-gel and Form-PEG-PLGA-gel, respectively.

The complete release profiles for Form-gel and Form-PEG-PLGA-gel were fitted to a double Weibull model (equation 6, methods pg. 45) (**Fig.5.4c**). Non-linear regression analysis showed that release from Form-gel displayed high correlation with the double Weibull model ($R^2 = 0.999$), with scale parameters $\alpha_1 = 0.9633$ and $\alpha_2 = 1.688$, shape parameters $\beta_1 = 0.7984$ and $\beta_2 = 0.452$ and AICc of -1.47. Drug release from Form-PEG-PLGA-gel also displayed high correlation ($R^2 = 0.999$), with $\alpha_1 = 0.4217$, $\alpha_2 = 4.882$, $\beta_1 = 0.6886$, $\beta_2 = 0.5145$ and AICc of -13.09. The resulting residuals ranged from -0.88 to 1.322 for Form-gel and -0.79 to 0.74 for Form-PEG-PLGA-gel (**Fig.5.4d**).

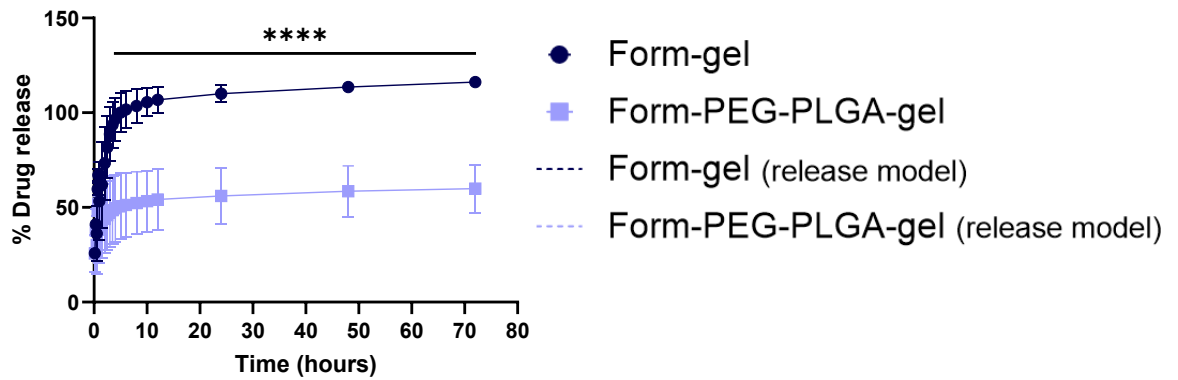
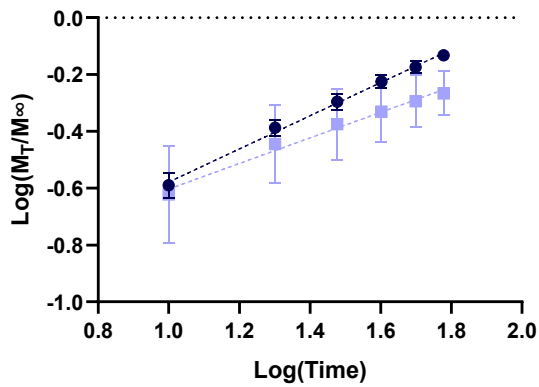
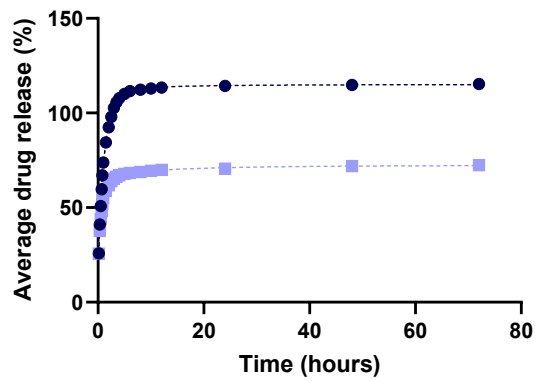
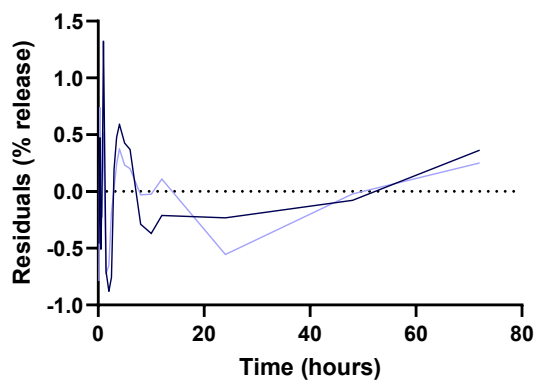
a**b****c****d**

Figure 5.4. Sustained drug release from Form-PEG-PLGA-gel over 72 hours *in vitro*.

(a) % Drug release over 72 hours, (b) drug release in the first hour was fitted to the Korsmeyer-Peppas model (dotted line), (c) average drug release over 72 hours was fitted to the Weibull model (dotted line) and (d) average drug release residuals, where random scatter of residuals around zero indicates appropriateness of fit. Data are represented as (a) the mean $\pm$ SEM and were analysed by two-way repeated measures ANOVA followed by Šídák's multiple comparisons (b) log transform of time and fraction of drug release at time t (M_t/M_∞) $\pm$ SEM, (c) as average % drug release and (d) average % release minus Weibull model-predicted drug release, where $N=3$ for all data sets. Significant differences between groups are denoted by **** $P<0.0001$ (a).

5.4.4. Form-PEG-PLGA has anti-inflammatory effects in IFN γ stimulated enriched microglia

Two-way ANOVA of fold change in *Il1b* mRNA showed main effects of IFN γ [$F_{(1, 18)} = 5.63$, $P<0.05$] and treatment [$F_{(2, 18)} = 9.119$, $P<0.01$] (**Fig.5.5a**). Tukey's multiple comparisons revealed a reduction in *Il1b* mRNA in enriched microglia treated with formoterol and Form-PEG-PLGA compared to control ($P<0.05$ and $P<0.01$, respectively). IFN γ treatment did not affect *Il1b* mRNA levels when compared to control.

Two-way ANOVA of fold change in *Tnfa* mRNA showed main effects of IFN γ [$F_{(1, 18)} = 301.7$, $P<0.0001$], treatment [$F_{(2, 18)} = 24.07$, $P<0.0001$] and an IFN γ x treatment interaction [$F_{(2, 18)} = 11.43$, $P<0.001$] (**Fig.5.5b**). Tukey's multiple comparisons revealed an increase in *Tnfa* mRNA in enriched microglia stimulated with IFN γ compared to control ($P<0.0001$). The IFN γ mediated increase in *Tnfa* mRNA was attenuated by formoterol ($P<0.05$) and Form-PEG-PLGA treatment ($P<0.0001$) compared to vehicle treated control. Form-PEG-PLGA treatment reduced *Tnfa* mRNA to a greater extent than free formoterol ($P<0.001$, not shown).

Two-way ANOVA of fold change in *Nos2* mRNA showed main effects of IFN γ [$F_{(1, 18)} = 421.6$, $P<0.0001$], treatment [$F_{(2, 18)} = 16.28$, $P<0.0001$] and an IFN γ x treatment interaction [$F_{(2, 18)} = 15.61$, $P<0.001$] (**Fig.5.5c**). Tukey's multiple comparisons revealed an increase in *Nos2* mRNA in enriched microglia stimulated with IFN γ compared to control ($P<0.0001$). Free formoterol and Form-PEG-PLGA attenuated the IFN γ -induced increase in *Nos2* mRNA ($P<0.01$ and $P<0.0001$, respectively). Form-PEG-PLGA treatment reduced *Nos2* mRNA to a greater extent than free formoterol ($P<0.01$, not shown).

Two-way ANOVA of fold change in *Cd40* mRNA showed main effects of IFN γ [$F_{(1, 18)} = 383.4$, $P<0.0001$], treatment [$F_{(2, 18)} = 46.90$, $P<0.0001$] and an IFN γ x treatment interaction [$F_{(2, 18)} = 16.51$, $P<0.001$] (**Fig.5.5d**). Tukey's multiple comparisons revealed a reduction in *Cd40* mRNA in enriched microglia treated with Form-PEG-PLGA compared to vehicle treated control ($P<0.05$). Tukey's multiple comparisons also revealed an increase in *Cd40* mRNA in glia stimulated with IFN γ compared to control ($P<0.0001$). The IFN γ mediated increase in *Cd40* mRNA was attenuated by formoterol ($P<0.01$) and Form-PEG-PLGA treatment ($P<0.0001$) compared to vehicle treated control. Form-PEG-PLGA treatment reduced *Cd40* mRNA to a greater extent than free formoterol ($P<0.0001$, not shown).

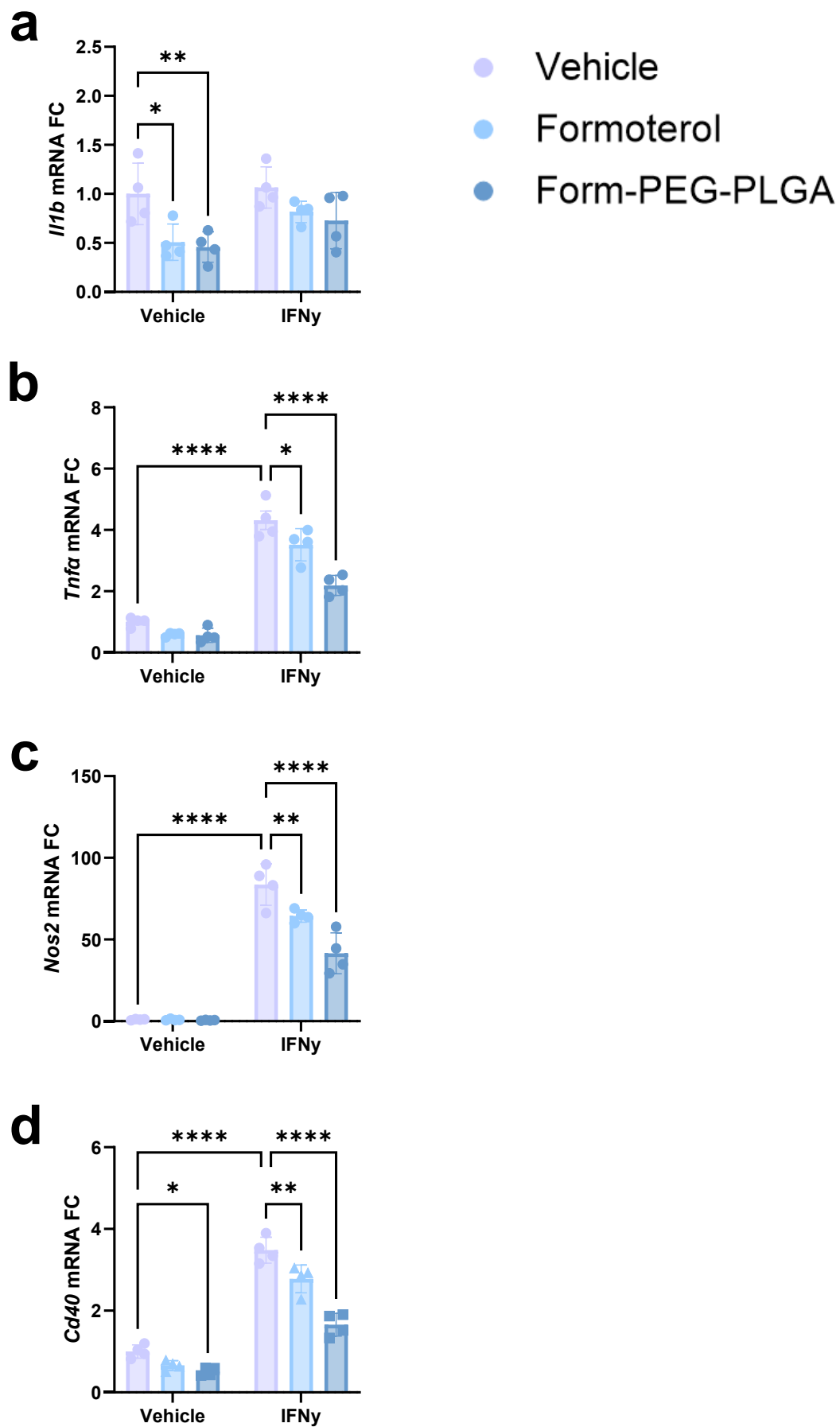


Figure 5.5. Form-PEG-PLGA attenuates IFN γ mediated pro-inflammatory responses *in vitro*.

Enriched microglial cultures were stimulated with IFN γ (50 ng/ml) for 30 mins prior to treatment with Form-PEG-PLGA (equivalent to 10 μ M of drug) or formoterol (10 μ M). Fold change (FC) in (a) *Il1b*, (b) *Tnfa*, (c) *Nos2* and (d) *Cd40* mRNA was measured after 24 hours. Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=4. Significant differences between groups are denoted by * where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** where $P < 0.0001$.

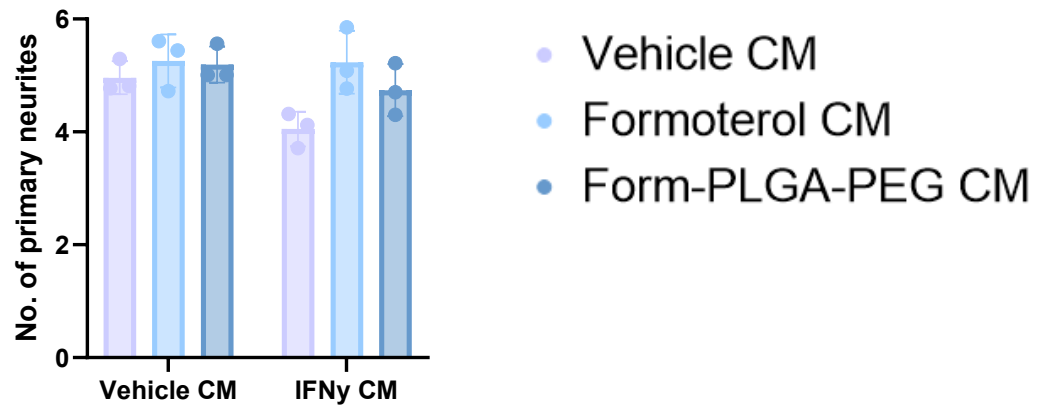
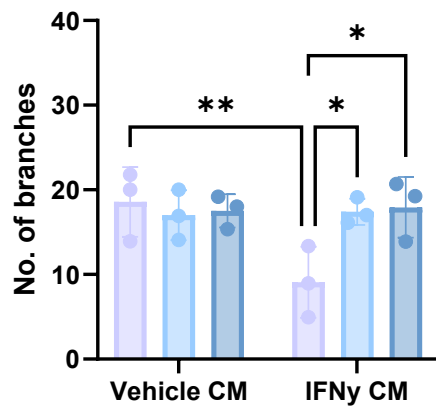
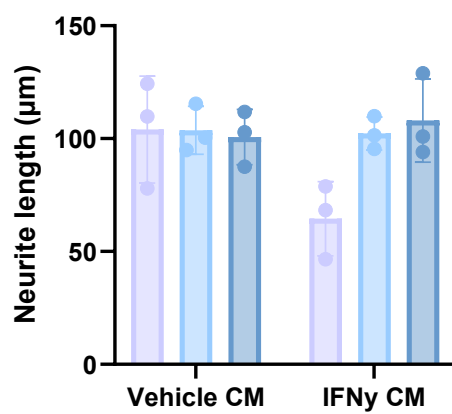
5.4.5. Form-PEG-PLGA CM protects against IFN γ CM-mediated reductions in neuronal complexity

Two-way ANOVA of the number of primary neurites after CM transfer showed main effects of IFN γ [$F_{(1, 12)} = 5.622$, $P < 0.05$] and treatment [$F_{(2, 12)} = 4.919$, $P < 0.05$] (**Fig.5.6a**). Tukey's multiple comparisons did not reveal any relevant differences between groups treated with glial CM.

Two-way ANOVA of the number of branches after CM transfer showed a main IFN γ x treatment interaction [$F_{(1, 12)} = 4.703$, $P < 0.05$] (**Fig.5.6b**). Tukey's multiple comparisons revealed a reduction in the number of branches in neurons treated with CM from IFN γ treated glia compared to neurons treated with CM from vehicle treated glia ($P < 0.0001$). This effect was rescued by CM from formoterol treated glia ($P < 0.05$) and Form-PEG-PLGA treated glia ($P < 0.05$) compared to CM from vehicle treated glia.

Two-way ANOVA of neurite length after CM transfer showed no effects of IFN γ or treatment (**Fig.5.6c**).

Two-way repeated measures ANOVA of the Sholl profile of neuronal complexity after CM transfer showed main effects of treatment [$F_{(5, 12)} = 3.169$, $P < 0.05$], distance [$F_{(25, 300)} = 628.2$, $P < 0.0001$] and a treatment x distance interaction [$F_{(125, 300)} = 2.574$, $P < 0.0001$] (**Fig.5.6d**). Tukey's multiple comparisons revealed a reduction in the number of intersections at 40 to 150 μ m from the cell soma in neurons treated with CM from IFN γ treated glia compared to CM from vehicle treated glia (40-60 μ m: $P < 0.0001$, 70-90 μ m: $P < 0.001$, 100-140 μ m: $P < 0.01$, 150 μ m: $P < 0.05$). This reduction in complexity was rescued by CM from formoterol treated glia at 40 to 150 μ m from the soma (40-90 μ m: $P < 0.01$, 100-150 μ m: $P < 0.05$, not shown) and CM from Form-PEG-PLGA treated glia at 40 to 150 μ m from the soma (40-50 μ m: $P < 0.001$, 60-90 μ m: $P < 0.0001$, 100-130 μ m $P < 0.01$, 130-150 μ m: $P < 0.05$). Statistical significance is displayed in fig. 5.5d as the minimum threshold reached ($P < 0.05$).

a**b****c**

d

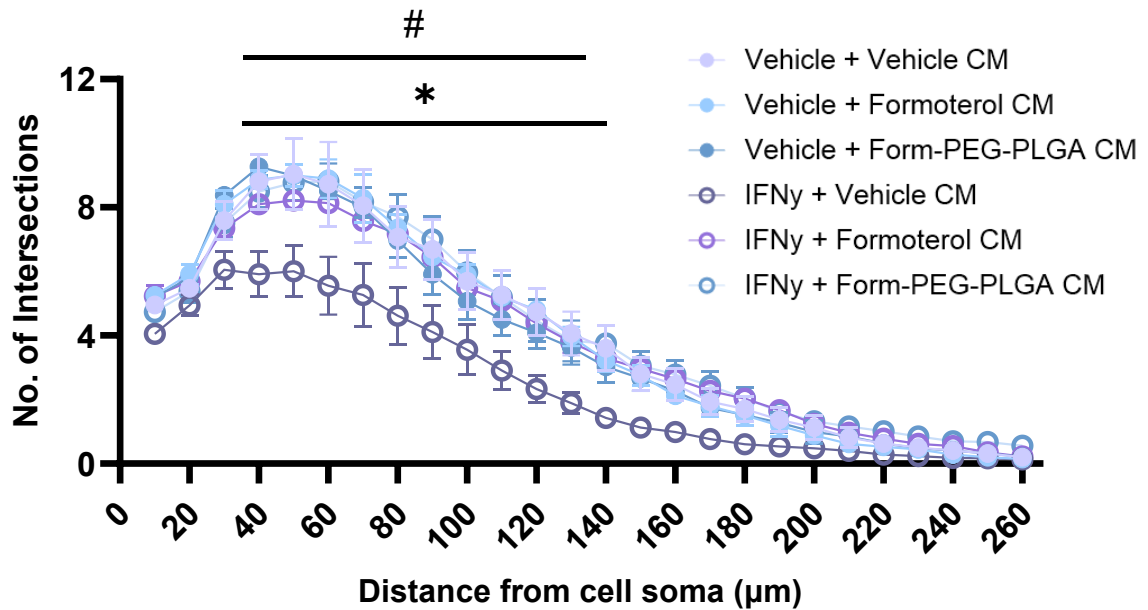


Figure 5.6. Form-PEG-PLGA rescues CM from IFN γ treated glia mediated reductions in neuronal complexity.

Effects of CM from IFN γ (50 ng/ml)-stimulated glia treated with formoterol or Form-PEG-PLGA (10uM) on (a) the number of primary neurites, (b) the number of branches, (c) neurite length and (d) the Sholl profile of neuronal complexity. Data was analysed by two-way ANOVA (a-c) or two-way repeated measures ANOVA (d) followed by Tukey's multiple comparisons and is represented as the mean $\pm$ SEM, where N=3. Significant differences between groups are denoted by * where * $P < 0.05$ and ** $P < 0.01$ (b). Significant differences in (d) are denoted by * for IFN γ + vehicle CM compared to vehicle + vehicle CM and # for IFN γ + Form-PEG-PLGA CM compared to IFN γ + vehicle CM where $P < 0.05$.

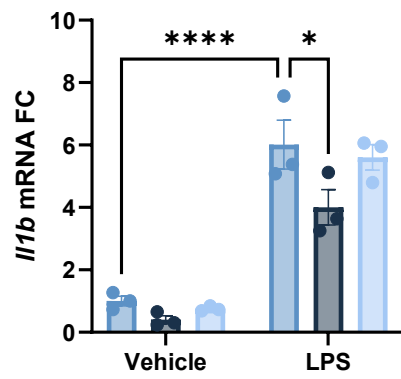
5.4.6. Form-PEG-PLGA has anti-inflammatory effects in LPS stimulated enriched microglia after 6 hours

Two-way ANOVA of fold change in *Il1b* mRNA showed main effects of LPS [$F_{(1, 12)} = 158.1$, $P < 0.0001$] and treatment [$F_{(2, 12)} = 4.814$, $P < 0.05$] (**Fig.5.7a**). Tukey's multiple comparisons revealed an increase in *Il1b* mRNA in enriched microglia stimulated with LPS for 6 hours compared to control ($P < 0.0001$). The LPS mediated increase in *Il1b* mRNA was attenuated by formoterol but not by Form-PEG-PLGA compared to vehicle treated control ($P < 0.05$).

Two-way ANOVA of fold change in *Tnfα* mRNA showed a main effect of LPS [$F_{(1, 12)} = 76.42$, $P < 0.0001$] (**Fig.5.7b**). Tukey's multiple comparisons revealed an increase in *Tnfα* mRNA in enriched microglia stimulated with LPS for 6 hours compared to control ($P < 0.01$). Treatment with formoterol or Form-PEG-PLGA did not affect *Tnfα* mRNA levels.

Two-way ANOVA of fold change in *Nos2* mRNA showed main effects of LPS [$F_{(1, 12)} = 470.5$, $P < 0.0001$], treatment [$F_{(2, 12)} = 7.753$, $P < 0.0001$] and an LPS x treatment interaction [$F_{(2, 12)} = 10.62$, $P < 0.01$] (**Fig.5.7c**). Tukey's multiple comparisons revealed an increase in *Nos2* mRNA in enriched microglia stimulated with LPS for 6 hours compared to control ($P < 0.0001$). The LPS mediated increase in *Nos2* mRNA was attenuated by formoterol ($P < 0.01$) and Form-PEG-PLGA treatment ($P < 0.001$) compared to vehicle treated control.

Two-way ANOVA of fold change in *Cd40* mRNA showed main effects of LPS [$F_{(1, 12)} = 518.5$, $P < 0.0001$], treatment [$F_{(2, 12)} = 5.609$, $P < 0.05$] and an LPS x treatment interaction [$F_{(2, 12)} = 3.997$, $P < 0.05$] (**Fig.5.7d**). Tukey's multiple comparisons revealed an increase in *Cd40* mRNA in enriched microglia stimulated with LPS 6 for hours compared to control ($P < 0.0001$). The LPS mediated increase in *Cd40* mRNA was attenuated by Form-PEG-PLGA treatment ($P < 0.01$) but not by formoterol when compared to vehicle treated control.

a

- Vehicle
- Formoterol
- Form-PEG-PLGA

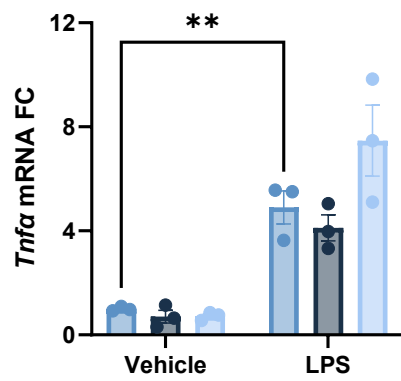
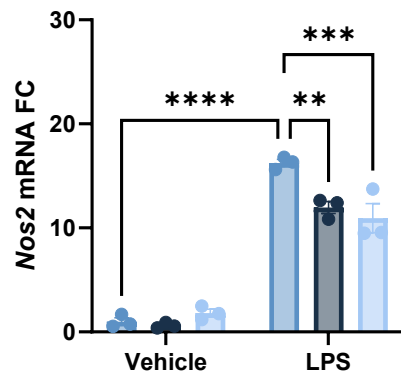
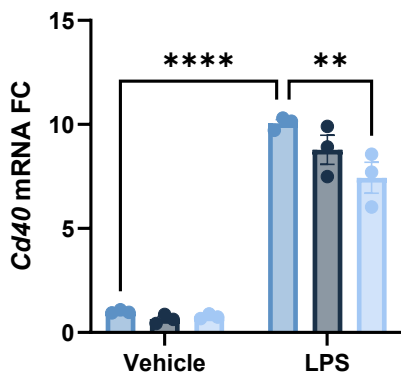
b**c****d**

Figure 5.7. Form-PEG-PLGA attenuates *Nos2* and *Cd40* mRNA expression 6 hours post LPS stimulation *in vitro*.

Enriched microglial cultures were stimulated with LPS (100 ng/ml) for 30 mins prior to treatment with Form-PEG-PLGA (equivalent to 10 μ M of drug) or formoterol (10 μ M). Fold change (FC) in (a) *Il1b*, (b) *Tnfa*, (c) *Nos2* and (d) *Cd40* mRNA was measured after 6 hours. Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=3. Significant differences between groups are denoted by * where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** where $P < 0.0001$.

5.4.7. Form-PEG-PLGA has anti-inflammatory effects in LPS stimulated enriched microglia after 24 hours

Two-way ANOVA of fold change in *Il1b* mRNA showed main effects of LPS [$F_{(1, 12)} = 57.80$, $P < 0.0001$], treatment [$F_{(2, 12)} = 20.52$, $P < 0.001$] and an LPS x treatment interaction [$F_{(2, 12)} = 11.50$, $P < 0.01$] (**Fig.5.8a**). Tukey's multiple comparisons revealed an increase in *Il1b* mRNA in enriched microglia stimulated with LPS for 24 hours compared to control ($P < 0.0001$). The LPS mediated increase in *Il1b* mRNA was attenuated by formoterol ($P < 0.01$) and Form-PEG-PLGA ($P < 0.0001$) treatment compared to vehicle treated control. Form-PEG-PLGA treatment reduced *Il1b* mRNA to a greater extent than formoterol ($P < 0.01$, not shown).

Two-way ANOVA of fold change in *Tnfa* mRNA showed main effects of LPS [$F_{(1, 12)} = 84.59$, $P < 0.0001$], treatment [$F_{(2, 12)} = 24.90$, $P < 0.0001$] and an LPS x treatment interaction [$F_{(2, 12)} = 13.89$, $P < 0.001$] (**Fig.5.8b**). Tukey's multiple comparisons revealed an increase in *Tnfa* mRNA in enriched microglia stimulated with LPS for 24 hours compared to control ($P < 0.001$). The LPS mediated increase in *Tnfa* mRNA was attenuated by formoterol ($P < 0.05$) and Form-PEG-PLGA ($P < 0.001$) treatment compared to vehicle treated control. Form-PEG-PLGA treatment reduced *Tnfa* mRNA to a greater extent than formoterol ($P < 0.0001$, not shown).

Two-way ANOVA of fold change in *Nos2* mRNA showed main effects of LPS [$F_{(1, 12)} = 96.58$, $P < 0.0001$], treatment [$F_{(2, 12)} = 9.56$, $P < 0.01$] and an LPS x treatment interaction [$F_{(2, 12)} = 13.03$, $P < 0.001$] (**Fig.5.8c**). Tukey's multiple comparisons revealed an increase in *Nos2* mRNA in enriched microglia stimulated with LPS for 24 hours compared to control ($P < 0.0001$). The LPS mediated increase in *Nos2* mRNA was attenuated by Form-PEG-PLGA treatment compared to vehicle treated control ($P < 0.001$) but not formoterol. Form-PEG-PLGA treatment reduced *Nos2* mRNA to a greater extent than formoterol ($P < 0.001$, not shown).

Two-way ANOVA of fold change in *Cd40* mRNA showed main effects of LPS [$F_{(1, 12)} = 114.5$, $P < 0.0001$], treatment [$F_{(2, 12)} = 11.55$, $P < 0.01$] and an LPS x treatment interaction [$F_{(2, 12)} = 5.354$, $P < 0.05$] (**Fig.5.8d**). Tukey's multiple comparisons revealed an increase in *Cd40* mRNA in enriched microglia stimulated with LPS for 24 hours compared to control ($P < 0.0001$). The LPS mediated increase in *Cd40* mRNA was attenuated by Form-PEG-PLGA treatment ($P < 0.01$) but not formoterol. Form-PEG-PLGA treatment reduced *Cd40* mRNA to a greater extent than formoterol ($P < 0.001$, not shown).

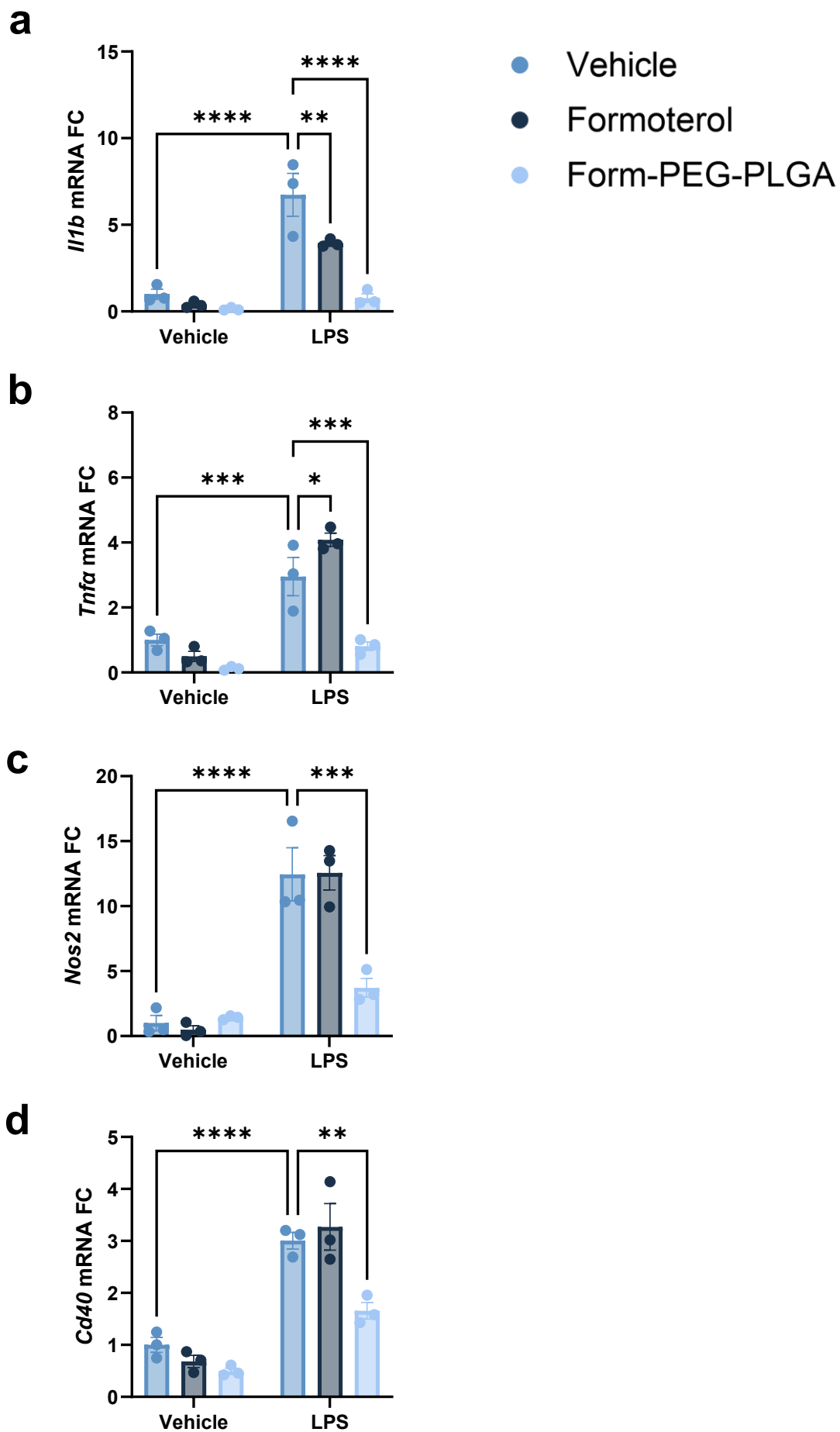


Figure 5.8. Form-PEG-PLGA attenuates LPS mediated pro-inflammatory responses after 24 hours *in vitro*.

Enriched microglial cultures were stimulated with LPS (100 ng/ml) for 30 mins prior to treatment with Form-PEG-PLGA (equivalent to 10 μ M of drug) or formoterol (10 μ M). Fold change (FC) in (a) *Il1b*, (b) *Tnfa*, (c) *Nos2* and (d) *Cd40* mRNA was measured after 24 hours. Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=4. Significant differences between groups are denoted by * where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** where $P < 0.0001$.

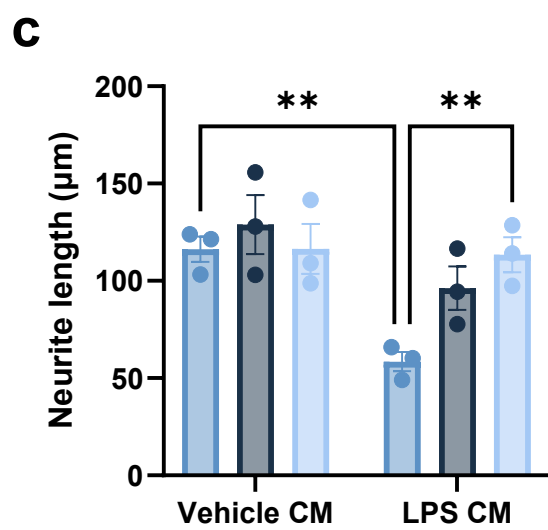
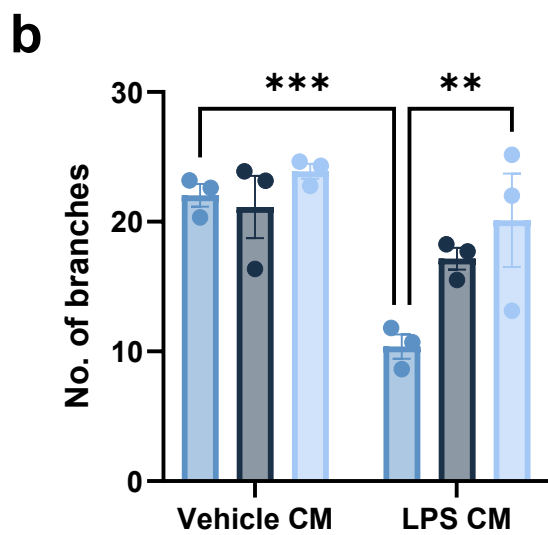
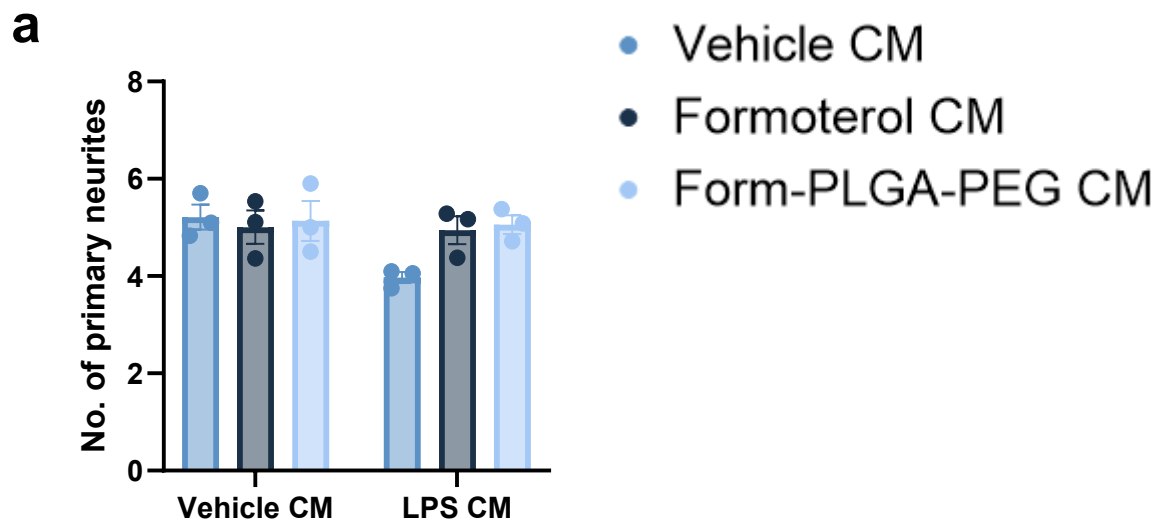
5.4.8. Form-PEG-PLGA CM protects against LPS CM (24 hour)-mediated reductions in neuronal complexity

Two-way ANOVA of the number of primary neurites after CM transfer showed no effects of LPS or treatment (**Fig.5.9a**).

Two-way ANOVA of the number of branches after CM transfer showed main effects of LPS [$F_{(1, 12)} = 17.69$, $P < 0.01$] and treatment [$F_{(2, 12)} = 4.704$, $P < 0.05$] (**Fig.5.9b**). Tukey's multiple comparisons revealed a reduction in the number of branches in neurons treated with CM from LPS treated glia compared to CM from vehicle treated glia ($P < 0.001$). This effect was rescued by CM from Form-PEG-PLGA but not formoterol treated glia compared to CM from vehicle glia ($P < 0.01$).

Two-way ANOVA of neurite length after CM transfer showed main effects of LPS [$F_{(1, 12)} = 13.05$, $P < 0.01$] and treatment [$F_{(2, 12)} = 4.182$, $P < 0.05$] (**Fig.5.9c**). Tukey's multiple comparisons revealed a reduction in neurite length in neurons treated with CM from LPS treated glia compared to CM from vehicle treated glia ($P < 0.01$). This effect was rescued by CM from Form-PEG-PLGA but not formoterol treated glia compared to CM from vehicle glia ($P < 0.01$).

Two-way repeated measures ANOVA of the Sholl profile of neuronal complexity after CM transfer showed main effects of treatment [$F_{(5, 12)} = 5.622$, $P < 0.01$], distance [$F_{(25, 300)} = 365.7$, $P < 0.0001$] and a treatment x distance interaction [$F_{(125, 300)} = 4.431$, $P < 0.0001$] (**Fig.5.9d**). Tukey's multiple comparisons revealed a reduction in the number of intersections at 40 to 110 μ m from the cell soma in neurons treated with CM from LPS treated glia compared to CM from vehicle treated glia (40-100 μ m: $P < 0.0001$, 110 μ m: $P < 0.01$). This effect was rescued by CM from formoterol treated glia at 40 to 130 μ m from the soma (40 μ m: $P < 0.0001$, 50 μ m: $P < 0.01$, 60 μ m: $P < 0.001$, 70 μ m: $P < 0.01$, 80 μ m: $P < 0.05$, not shown) and CM from Form-PEG-PLGA treated glia at 40 to 110 μ m from the soma (40 μ m: $P < 0.001$, 50-80 μ m: $P < 0.0001$, 90-100 μ m $P < 0.001$, 110 μ m: $P < 0.01$). Statistical significance is displayed in fig. 5.8d as the minimum threshold reached ($P < 0.01$).



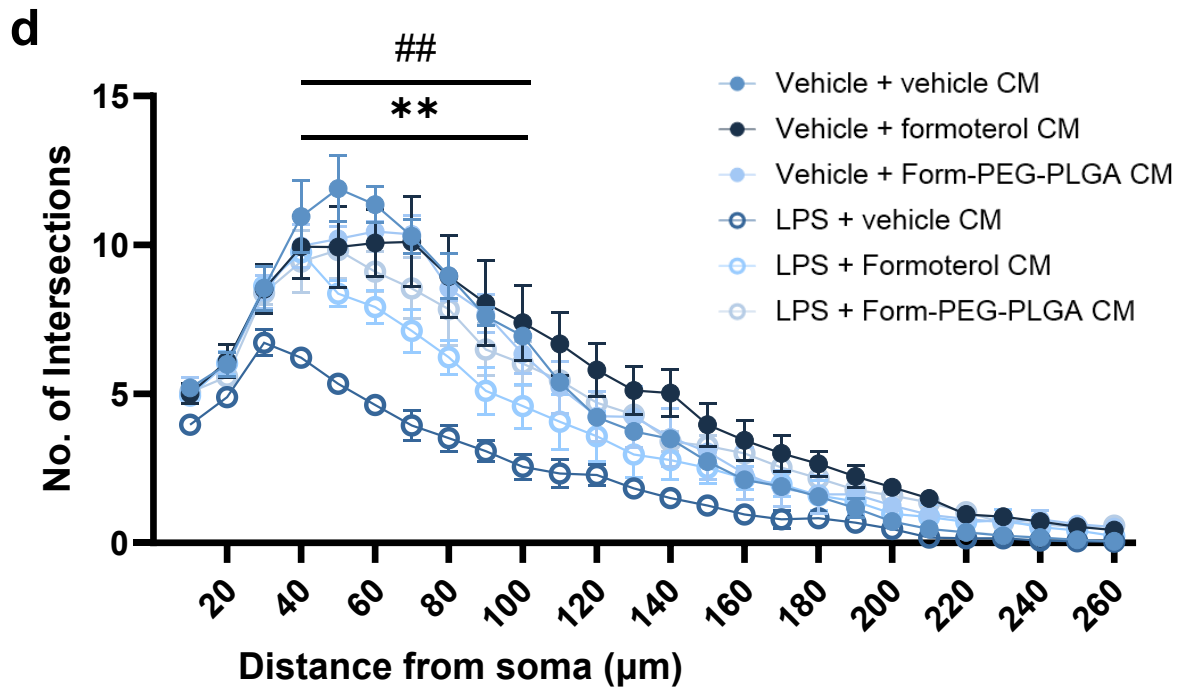


Figure 5.9. Form-PEG-PLGA rescues CM from LPS treated glia mediated changes in neuronal complexity.

Form-PEG-PLGA (10 μM) rescued CM from LPS (100 ng/ml; 24 hours) treated glia mediated changes in (a) the number of primary neurites, (b) the number of branches, (c) neurite length and (d) the Sholl profile of neuronal complexity. Data was analysed by two-way ANOVA (a-c) or two-way repeated measures ANOVA (d) followed by Tukey's multiple comparisons and is represented as the mean $\pm$ SEM, where N=3. Significant differences between groups are denoted by ** $P < 0.01$ (b and c). Significant differences in (d) are denoted by * for LPS + vehicle CM compared to vehicle + vehicle CM where ** $P < 0.01$ or **** $P < 0.0001$ and # for LPS + Form-PEG-PLGA CM compared to LPS + vehicle CM where ## $P < 0.01$, ### $P < 0.001$ or ##### $P < 0.0001$.

5.4.9. Unloaded PEG-PLGA are not anti-inflammatory in IFN γ stimulated enriched microglial cultures

Two-way ANOVA of fold change in *Il1b* mRNA showed no effect of IFN γ or PEG-PLGA (**Fig.5.10a**).

Two-way ANOVA of fold change in *Tnfa* mRNA showed a main effect of IFN γ [$F_{(1,6)} = 16.34$, $P < 0.01$] (**Fig.5.10b**). Tukey's multiple comparisons revealed an increase in *Tnfa* mRNA in enriched microglia stimulated with LPS compared to control ($P < 0.01$). Unloaded PEG-PLGA did not attenuate this increase in *Tnfa*.

Two-way ANOVA of fold change in *Nos2* mRNA showed no effect of IFN γ or PEG-PLGA (**Fig.5.10c**).

Two-way ANOVA of fold change in *Cd40* mRNA showed a main effect of IFN γ [$F_{(1,6)} = 10.74$, $P < 0.05$] (**Fig.5.10d**). Tukey's multiple comparisons revealed an increase in *Cd40* mRNA in enriched microglia stimulated with LPS compared to control ($P < 0.05$). Unloaded PEG-PLGA did not attenuate this increase in *Cd40*.

5.4.10. Unloaded PEG-PLGA are not anti-inflammatory in LPS stimulated enriched microglial cultures

Two-way ANOVA of fold change in *Il1b* mRNA showed a main effect of LPS [$F_{(1,6)} = 23.22$, $P < 0.01$] (**Fig.5.11a**). Tukey's multiple comparisons revealed an increase in *Il1b* mRNA in enriched microglia stimulated with LPS compared to control ($P < 0.05$). Unloaded PEG-PLGA did not attenuate this increase in *Il1b*.

Two-way ANOVA of fold change in *Tnfa* mRNA showed no effect of LPS or PEG-PLGA (**Fig.5.11b**).

Two-way ANOVA of fold change in *Nos2* mRNA showed a main effect of LPS [$F_{(1,6)} = 21.34$, $P < 0.01$] (**Fig.5.11c**). Tukey's multiple comparisons revealed an increase in *Nos2* mRNA in enriched microglia stimulated with LPS compared to control ($P < 0.05$). Unloaded PEG-PLGA did not attenuate this increase in *Nos2*.

Two-way ANOVA of fold change in *Cd40* mRNA showed a main effect of LPS [$F_{(1,6)} = 32.75$, $P < 0.01$] (**Fig.5.11d**). Tukey's multiple comparisons revealed an increase in *Cd40* mRNA in enriched microglia stimulated with LPS compared to control ($P < 0.01$). Unloaded PEG-PLGA did not attenuate this increase in *Cd40*.

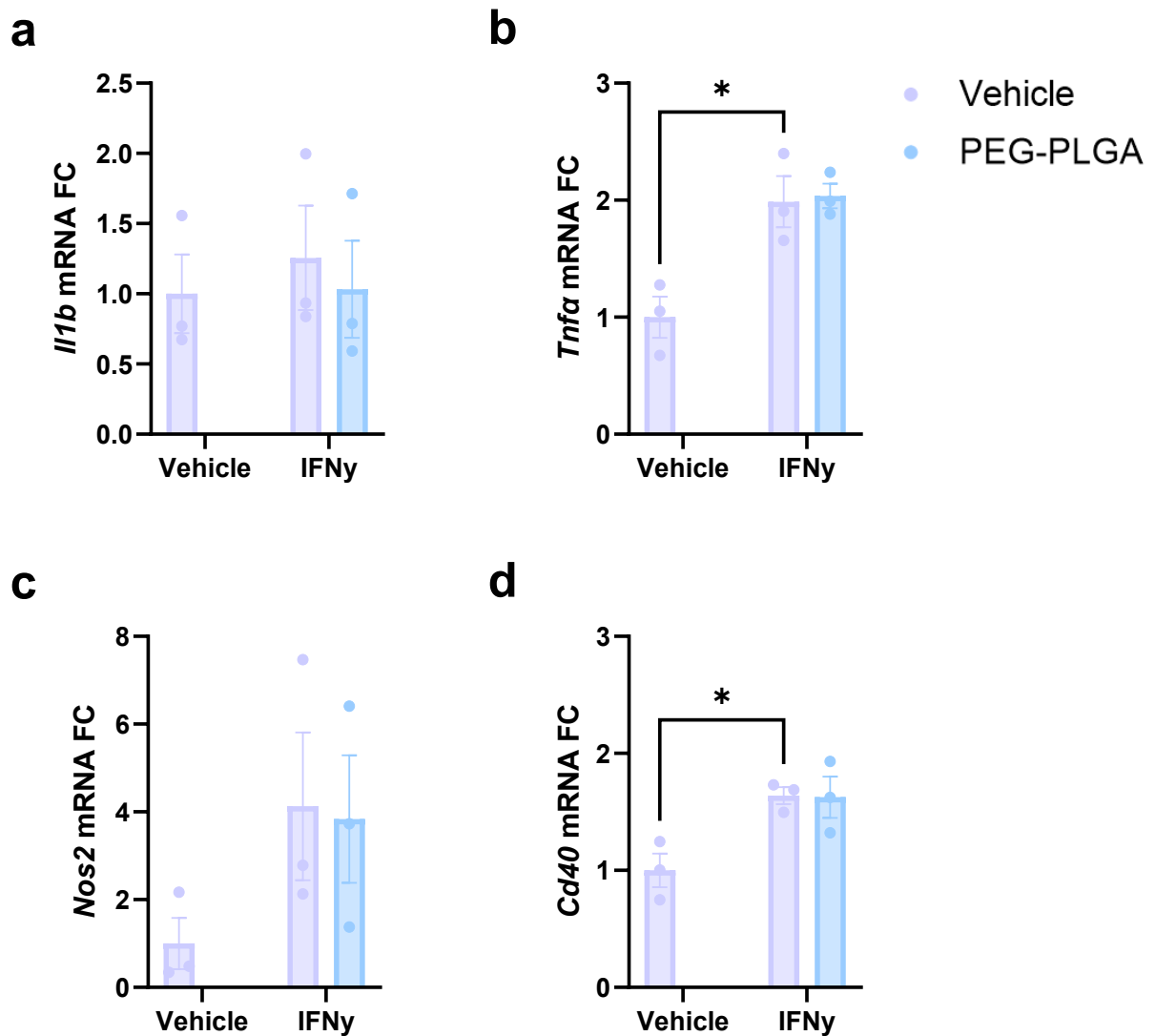


Figure 5.10. Unloaded PEG-PLGA do not attenuate IFN γ mediated pro-inflammatory responses *in vitro*.

Enriched microglial cultures were stimulated with IFN γ (50 ng/ml) for 30 mins prior to treatment with unloaded PEG-PLGA (equivalent to volume of 10 μ M Form-PEG-PLGA). Fold change (FC) in (a) *Il1b*, (b) *Tnfa*, (c) *Nos2* and (d) *Cd40* mRNA was measured after 24 hours. Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=3. Significant differences between groups are denoted by * $P < 0.05$.

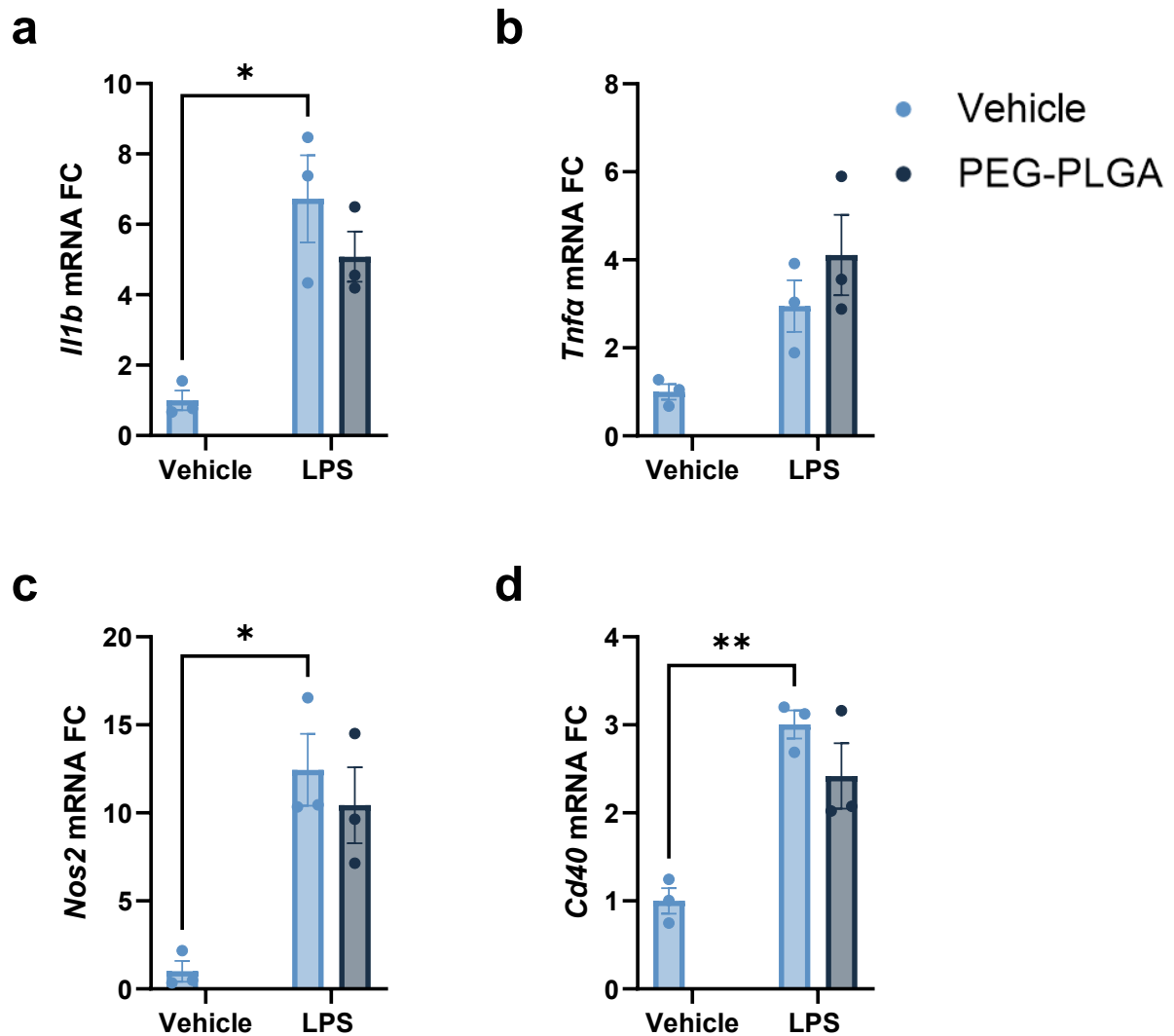


Figure 5.11. Unloaded PEG-PLGA do not attenuate pro-inflammatory responses 24 hours post LPS stimulation *in vitro*.

Enriched microglial cultures were stimulated with LPS (100 ng/ml) for 30 mins prior to treatment with unloaded PEG-PLGA (equivalent to volume of 10 μ M Form-PEG-PLGA). Fold change (FC) in (a) *I/1b*, (b) *Tnfa*, (c) *Nos2* and (d) *Cd40* mRNA was measured after 24 hours. Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=3. Significant differences between groups are denoted by * $P < 0.05$ and ** $P < 0.01$.

5.4.11. Biomaterials do not induce pro-inflammatory gene expression in human PBMCs

One-way ANOVA of extracellular LDH showed no effect of LPS, PEG-PLGA or PEG-PLGA-gel (**Fig.5.12a**).

Kruskal-Wallis ANOVA of fold change in *IL1B* mRNA showed no effect of LPS, PEG-PLGA or PEG-PLGA-gel (**Fig.5.12b**).

One-way ANOVA of fold change in *TNF α* mRNA showed no effect of LPS, PEG-PLGA or PEG-PLGA-gel (**Fig.5.12c**).

Kruskal-Wallis ANOVA of fold change in *IL6* mRNA showed no effect of LPS, PEG-PLGA or PEG-PLGA-gel (**Fig.5.12d**).

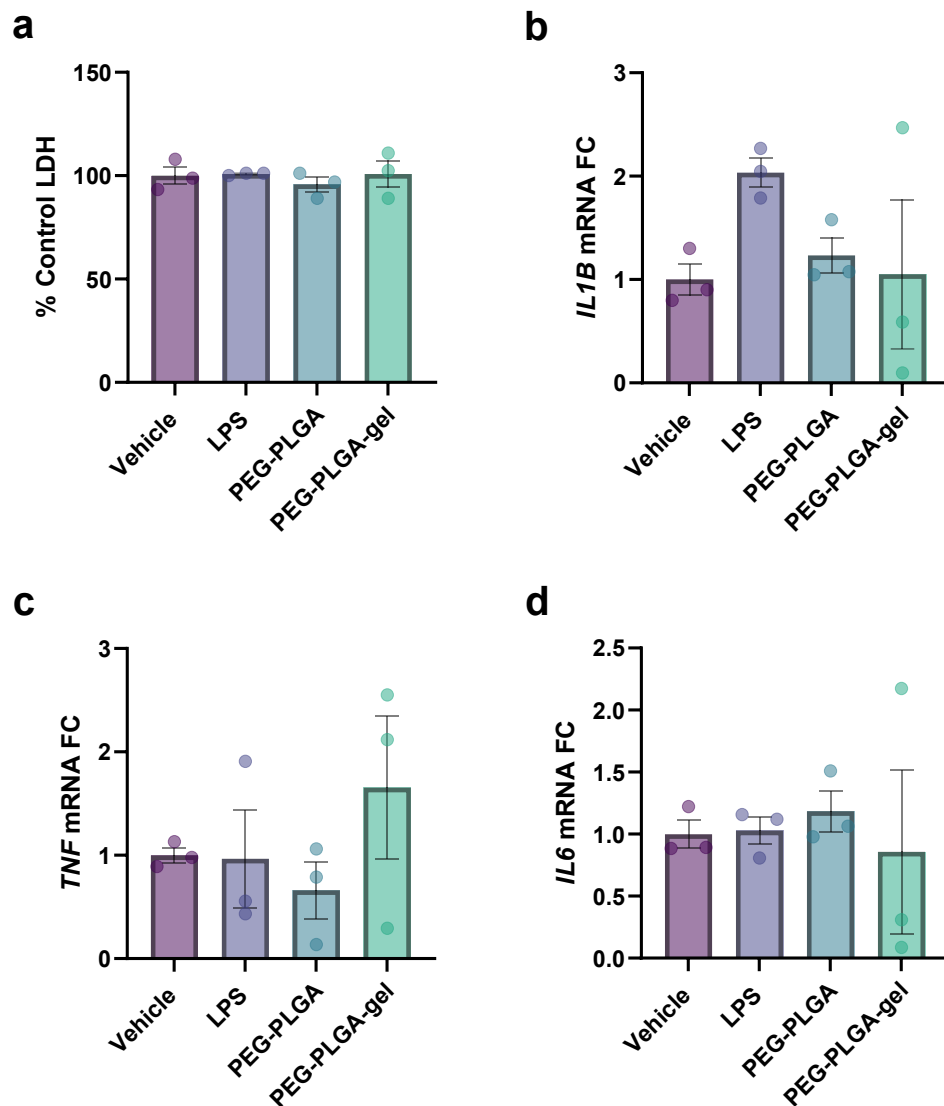


Figure 5.12. PEG-PLGA and hydrogel do not affect the viability or increase *IL1B*, *TNF α* or *IL6* mRNA in human PBMCs *in vitro*.

Human PBMCs were exposed to LPS (100 ng/ml), PEG-PLGA (100 μ g/ml) or hydrogel loaded with PEG-PLGA (30 μ l). (a) Extracellular LDH as a % of control and fold change (FC) in (b) *IL1B*, (c) *TNF α* , and (d) *IL6* mRNA after 24 hours. Data were analysed by one-way ANOVA (a, c) or Kruskal-Wallis ANOVA (b, d) and are expressed as the mean $\pm$ SEM, where N=3. Blood collection, PBMC isolation and cell preparation by Mr. Chris Sheridan (School of Medicine, Trinity College Dublin).

5.5. Discussion

In vitro analysis confirmed that Form-PEG-PLGA released formoterol in a sustained manner over time. When embedded in a hydrogel for intranasal delivery, the period of formoterol release was further extended and followed a pattern of biphasic release. Furthermore, Form-PEG-PLGA released formoterol in enriched microglial cultures pre-stimulated with IFN γ or LPS to produce anti-inflammatory effects. The transfer of the resulting CM to neuronal cultures demonstrated that Form-PEG-PLGA treatment attenuated the neurotoxic effects of CM from IFN γ and LPS treated glia. Finally, the compatibility of these biomaterials was further assessed in human PBMCs, indicating possible safety and potential for clinical translation.

5.5.1. PEG-PLGA drug loading

PLGA nanoparticle size can increase when loaded, depending on the drug and polymer used. However, since formoterol hemifumarate has balanced amphiphilic properties, exhibiting both moderate water and organic solvent solubility, it does not behave as a strongly hydrophobic drug with a dominant affinity for the PLGA polymer (Chatterjee & Chanda, 2022; Guo et al., 2023). The small, 10 nm difference in hydrodynamic size between Form-PEG-PLGA and unloaded PEG-PLGA measured by DLS is more accurately explained by formoterol's incorporation into the core of the nanoparticle rather than a strong polymer interaction preventing size change. This is further supported by the negligible change in surface charge (ζ -potential) upon drug loading, as surface adsorption of the drug would otherwise lead to a more positive nanoparticle charge.

The efficiency of drug loading in PLGA nanoparticles is highly variable depending on the physicochemical properties of the drug and polymer, preparation method, mixing time of the organic and aqueous phases, particle size and drug to polymer ratio (Kamaly et al., 2016). For a balanced amphiphilic drug like formoterol hemifumarate, successful encapsulation via the nanoprecipitation method depends on precise control over these parameters. While nanoprecipitation is conventionally favoured for highly hydrophobic drugs, it is a well-established method for encapsulating compounds with moderate solubility (Chatterjee & Chanda, 2022; Martínez Rivas et al., 2017). This is achieved by carefully selecting the solvent system and mixing conditions to promote co-precipitation of both the drug and the polymer, leading to the encapsulation of the drug within the nanoparticle core. PLGA with a higher molecular weight and higher polylactic acid content can further improve drug encapsulation by increasing the viscosity of the organic phase and reducing drug diffusion during synthesis (Wischke & Schwendeman, 2008).

The choice of organic solvent strongly influences encapsulation efficiency as it controls the rate of nanoparticle formation. During nanoprecipitation, polymer nuclei form in the aqueous phase and subsequently aggregate into 3D nanoparticles once saturation is

reached. Bovone et al. (2022) demonstrated that this process is largely determined by the solvent used. As the rate of nanoparticle growth dictates drug entrapment, the solvent used should be carefully considered. While tetrahydrofuran yields ~100 nm PLGA nanoparticles suitable for drug loading and brain delivery, nanoparticle growth in THF is slower than in N,N-Dimethylformamide or acetone. However, N,N-Dimethylformamide and acetone yield smaller particles which may in turn limit drug loading capacity (Bovone et al., 2022).

The recommended solvent for formoterol is DMSO, which presents a challenge since formoterol is not soluble in tetrahydrofuran, the solvent commonly used to dissolve PLGA. However, DMSO is highly water-miscible and can slow nanoparticle precipitation in a concentration dependent manner, thereby reducing drug encapsulation. Therefore, preparation of formoterol stock in methanol may be preferable for nanoparticle synthesis as methanol increases solution viscosity and promotes faster polymer precipitation, limiting drug loss during synthesis. Ideally, the same organic solvent would dissolve drug and polymer but, in this case, no single solvent adequately solubilises both formoterol and PLGA. Alternatively, more rapid mixing of the organic phase containing DMSO and the aqueous phase could minimise drug partitioning into water (Tao et al., 2019). However, the effects of any adjustment to the synthesis method on the resulting nanoparticle physiochemical properties and release kinetics would require careful evaluation.

The findings reported here are consistent with other studies of formoterol encapsulation, though direct comparisons are limited. Several studies conducted in Arizona State University by Professors Mansour and Schnellmann have loaded PLGA nanoparticles with formoterol for the treatment of kidney disease (summarised in **Table 5.1.**). In one study comparing the effects of PLGA molecular weight, polymer concentration and PEG terminal group modification on formoterol encapsulation, it was found that drug loading increased as PLGA molecular weight and concentration increased, although both parameters were also associated with an increase in particle size. The authors also reported that particles synthesised using PEG-PLGA with a carboxylic acid terminus achieved only 0.01% drug loading, which was marginally improved when PEG was capped with a methyl or amine group (Vallorz et al., 2022). The encapsulation efficiency and loading capacity of formoterol in PLGA nanoparticles achieved by this group were substantially lower than those reported here, due to the differences in PLGA properties, the synthesis methods and the physicochemical properties of the resulting nanoparticles.

Table 5.1. Formoterol-loaded PEG-PLGA for renal targeting.

PEG-PLGA MW	PLA:PGA	Method	Formoterol	NP properties	EE	LC	Ref
PLGA: 55 kDa PEG: 5 kDa	50:50	Oil-in-water single emulsion	Fumarate dihydrate in glacial acetic acid	442 ± 17 nm; 0.16 ± 0.04 mV	24 ± 5%	0.2 ± 0.04%	(Vallorz et al., 2021)
PLGA: 55 kDa, 30 kDa and 15 kDa PEG: 5 kDa	50:50	Oil in water (single or double emulsion)	Fumarate dihydrate in methanol	300 – 500 nm; -34.87 – 14.133 mV	Did not specify	0.01 – 1.39%	(Vallorz et al., 2022)

MW: molecular weight; NP: nanoparticle; EE: encapsulation efficiency; LC: loading capacity.

Propranolol, clenbuterol and carvedilol which are structurally similar to formoterol have been loaded into PLGA nanoparticles (de S. L. Oliveira et al., 2020; Rodgers et al., 1997; Ubrich et al., 2004). When propranolol was added at a similar concentration to that of formoterol in the present study, an encapsulation efficiency of ~25% was reported, which increased to ~80% at higher drug concentrations. Encapsulation was further enhanced when the aqueous phase pH exceeded the drugs pK_a , favouring ionisation and limiting drug loss (Ubrich et al., 2004). Conversely, the highest encapsulation efficiency of carvedilol was achieved using a 1:10 drug to polymer ratio (de S. L. Oliveira et al., 2020). Notably, emulsion methods were used to load propranolol, clenbuterol and carvedilol into PLGA nanoparticles synthesised using polymer concentrations that were over 10 times higher than those used here, and the resulting particles in each study were over 290 nm in size.

5.5.2. Controlled formoterol release from Form-PEG-PLGA and Form-PEG-PLGA-gel

In vitro drug release assays comparing free formoterol and Form-PEG-PLGA revealed that 100% of free drug diffused across the membrane within 60-90 minutes, whereas only 65% of formoterol loaded was released from Form-PEG-PLGA over 24 hours, reaching a plateau after 4 hours. These results reflect the controlled drug release properties of PEG-PLGA described in the literature, suggesting a portion of drug remains entrapped until polymer degradation occurs.

The Korsmeyer-Peppas model, commonly used to model the early burst phase of polymeric drug release (Ritger & Peppas, 1987), indicates Fickian-type diffusion when $n < 0.45$ and non-Fickian kinetics when $n > 0.45$ (Lopez-Mitjavila et al., 2025). Based on this model, the results described here show that free formoterol diffused across the membrane in the first hour of release, whereas drug release from Form-PEG-PLGA was not solely governed by

diffusion. The early phase of drug release from polymeric nanoparticles is classically described as an initial burst release of drug that is attached to or close to the surface of the particle, which then slows and is controlled by matrix degradation (Kamaly et al., 2016). This likely describes the release mechanisms involved in the early phase of drug release from Form-PEG-PLGA.

The complete release profile was fitted to the Weibull model which describes drug release when multi-mechanistic kinetics contribute to a single release phase (Papadopoulou et al., 2006). Although free formoterol was highly correlated with this model, the distribution of the residuals indicates a poor fit. This is further supported by the α (scale) parameter, which describes the time that it takes to reach 63% of the total drug release. In the case of free formoterol, 100% of the loaded drug was released during this assay and it took approximately 18 minutes for 63% of the total load to move to the external compartment. Formoterol released from Form-PEG-PLGA reached 63% of maximum release at 42 minutes. However, as only 65% of the initial load was released during the assay, this means that only 41% of the total load was released in 42 minutes (i.e. 63% of 65% total release). The β (shape) parameter that accompanied these functions was less than 1, indicating an initial diffusion process followed by deceleration due to polymer degradation in the case of Form-PEG-PLGA. Together, these mathematical models show that free formoterol diffused across the membrane more rapidly than drug released from particles, where both diffusion and polymer erosion contributed to a sustained and controlled release profile.

A mucoadhesive, thermo-responsive chitosan-based hydrogel was generated for future *in vivo* studies using the intranasal administration route and drug release from this system was also assessed. When free formoterol was encapsulated in hydrogel, 100% of free drug was released from the gel within approximately 3 hours. Similarly to nanoparticles alone, drug release from Form-PEG-PLGA-gel only reached a maximum of 60%, peaking after 8 hours. The nanoparticle-hydrogel dual release system provides sustained release kinetics, enabling less frequent dosing *in vivo*.

The release profile from Form-gel was a good fit to the Korsmeyer-Peppas model. This function showed $n > 0.45$, suggesting that degradation of the hydrogel was involved in this early release phase. Drug release from Form-PEG-PLGA-gel was not correlated with the Korsmeyer-Peppas model, indicating that more complex mechanisms are involved in drug release from this system than those described by this equation.

A single Weibull model was fitted; however, inspection of residuals displayed a non-random pattern that was not centred around zero, implying that the model was a poor fit for the data. To capture the effects of both nanoparticle and gel on drug release, a double Weibull model was applied. The AICc value was calculated for each model to evaluate fit quality. In each case, drug release from gel had a lower AICc score when fitted with the double Weibull than with the single Weibull model, indicating a better fit. The double Weibull assumes the presence of two distinct release phases, each with separate scale and shape parameters,

where α_1 is the time taken to reach 63% of total release and α_2 is the time that it takes to release a further 23% of the total amount of drug released. 63% of the total loaded drug was released by Form-gel in 57 minutes and another 23% was released after a further 43 minutes. Therefore, in under 2 hours, 76% of the total load was released from Form-gel. Although α_1 for Form-PEG-PLGA-gel revealed that 63% of the total release occurred after 25 minutes, only 60% of the total drug loaded was released. Therefore, after 25 minutes 38% of the total load was released. Moreover, the second phase of release from Form-PEG-PLGA-gel occurred much later after 4.4 hours, when a further 14% of total drug load had been released. From this, we can interpret that release from Form-gel is faster and less controlled than Form-PEG-PLGA-gel.

These results are in agreement with several others that describe PLGA nanoparticle drug release to fit well to both the Korsmeyer-Peppas and Weibull models (Aksungur et al., 2011; Garcia et al., 2023; Gupta et al., 2022; Ma et al., 2012; Öztürk et al., 2019; Rattanaseth et al., 2025; Sharaf et al., 2021). In a recent study analysing the use of the Korsmeyer-Peppas and Weibull equations to model drug release from PLGA nanoparticles using over 400 published datasets, an average n of 0.42 was calculated for the Korsmeyer-Peppas model and an average β value of 0.55 was reported using the Weibull function (Martín-Camacho et al., 2023). These values are in agreement with the present data, with slightly higher values for each model reported here. However, studies comparing frequently used models across numerous of PLGA release datasets in the literature have commented on the limited ability of mathematical expressions to accurately describe the profile of drug release from PLGA nanoparticles (Heredia et al., 2022; Pourtalebi Jahromi et al., 2020). Therefore, as nanoparticles become increasingly sophisticated, there is a need for mathematical models that can encompass all parameters associated with release.

In a study that measured the release of anticancer drug temozolomide from PLGA nanoparticles embedded in a hydrogel, the Weibull model was described as the best fit for the data (Çomoğlu, 2022) but this study did not explore the use of the recently proposed double Weibull model (Ruiz-Picazo et al., 2024), which was a better fit for our data. Çomoğlu (2022) did not report the α scale values, but the β values described are roughly half the magnitude of those obtained here to describe Form-PEG-PLGA-gel release, indicating that the release of temozolomide was primarily diffusion controlled, whereas β values closer to 1 indicate that matrix dissolution is also a contributing factor. No other examples of drug modelling from PLGA nanoparticle-hydrogel systems were found in our literature search.

For Form-PEG-PLGA and Form-PEG-PLGA-gel, 35% and 40% of loaded drug remain unreleased, respectively. It is reported that PLGA nanoparticles can take days to weeks to fully breakdown in water (Zweers et al., 2004). In earlier experiments optimising this *in vitro* release assay (not shown), no drug release from Form-PEG-PLGA was detected after 24 hours when samples were collected every 12 hours. However, it is possible that the

remaining drug is very slowly released, in quantities below the detection limit of HPLC-UV when sampled too frequently. Therefore, future experiments should include sampling up to 24 hours and again after a week to investigate the release of this remaining portion of drug.

Typical release assays from nanoparticles do not recapitulate the physiological processes and forces that contribute to nanoparticle degradation and drug release *in vivo* (Ewii et al., 2025). The dialysis method used in the present study is the most widely used approach for studying drug release from nanoparticles (Shen & Burgess, 2013). Incomplete release from the dosage form has been reported as a challenge of using this method, but new techniques are developing alongside the rapidly evolving field of nanomedicine. These include laser diffraction measurements of particle size in real time, calorimetry to assess changes in thermal properties in degrading biomaterials and differential pulse polarography for electroactive drugs (Ewii et al., 2025).

Drug release properties of PLGA nanoparticles can be modified by small adjustments to the polymer composition. PLA is more hydrophobic than PGA, so increasing the PLA content reduces water absorption and nanoparticle degradation, therefore slowing release kinetics (Makadia & Siegel, 2011). Increasing the ratio of PLA to PGA also increases nanoparticle crystallinity. As only amorphous regions are accessible to water molecules, this can further decrease the speed of drug release from PLGA nanoparticles (Müller et al., 2013).

The molecular weight of the PLGA polymer used to synthesise nanoparticles determines the rate of particle degradation and drug release. Lower molecular weight polymers are more flexible, enabling the polymer matrix to swell due to osmotic pressure, increasing pore size for drug diffusion and further influx of water molecules. However, smaller nanoparticles made from low molecular weight polymers are less likely to be taken up by macrophages (Kamaly et al., 2016).

The drug loading level can also influence release kinetics. Tightly packed cargo reduces diffusion because there is less space for water molecules to enter the particle (Kamaly et al., 2016). Indeed, when higher concentrations of propranolol (259.34 g/mol) were loaded into PLGA nanoparticles, the initial burst release was prevented and drug release steadily increased over 8 hours compared to loading with 20 mg of drug which displayed a burst release that peaked after 4 hours (Ubrich et al., 2004). Similarly, low molecular weight drugs may be able to diffuse out of the particle more easily because of increased osmotic pressure in the nanoparticle core. This may contribute to the initial burst release observed in the present study as formoterol hemifumarate has a low molecular weight (420.47 g/mol).

Predictive modelling of drug release from polymer-based systems can tell us how changing different nanoparticle parameters can influence drug release, enabling optimisation strategies with lower costs and reduced experiment times. Equations exist to model diffusion-based release, polymer dissolution, polymer degradation and combinations of

these processes to reflect the distinct phases of nanoparticle drug release (Kamaly et al., 2016). Goren et al. (2025) put together a large dataset of over 400 PLGA formulations for small-molecule delivery from the literature, which is a useful resource for AI and machine learning approaches to nanoparticle design

As the field of nanomedicine continues to develop, drug delivery systems are becoming increasingly sophisticated. Stimuli-responsive nanosystems have been developed where drug release occurs in response to internal or external stimuli. Lei et al. (2023) designed a ROS-responsive polymeric nanoparticle as ROS levels in the PD brain are elevated, and therefore, drug release is triggered in disease associated brain regions. The authors found that ROS-responsive nanoparticles were superior in rescuing behavioural deficits, dopaminergic cell loss and reducing α -synuclein aggregation in the MPTP mouse model of PD (Lei et al., 2023).

The mechanisms of release from nanoparticle alone and nanoparticle embedded in gel are distinct and require different models to describe the kinetics, so it is not possible to make direct quantitative comparisons. However, both drug encapsulation in PEG-PLGA nanoparticles and the additional loading in hydrogel slowed drug release substantially compared to free drug. Formoterol release from Form-PEG-PLGA occurred in a single phase likely governed by an initial burst and matrix erosion, while release from Form-PEG-PLGA-gel occurred more slowly over two phases, as nanoparticles were released from the gel and then drug diffused out of the particles or was released by nanoparticle breakdown.

5.5.3. Anti-inflammatory and neuroprotective effects of formoterol released from Form-PEG-PLGA in an *in vitro* model of neuroinflammation

The application of Form-PEG-PLGA to enriched microglia stimulated with IFN γ or LPS attenuated the expression of pro-inflammatory genes *Il1b*, *Tnfa*, *Nos2* and *Cd40*. Furthermore, the transfer of glial CM to neurons showed that the anti-inflammatory effects in glial cultures protected against neuronal injury. This agrees with several studies from the Harkin lab and others where modulation of noradrenergic signalling was shown to protect against neurodegeneration and neuroinflammation *in vitro* (Abdulla & Renton, 2005; Day et al., 2014; Erdem et al., 2024; Junker et al., 2002; Qian et al., 2011; Ryan et al., 2020; Semkova et al., 1996; Sharma et al., 2019; Sharma & Flood, 2018).

Overall, treatment with Form-PEG-PLGA was superior to formoterol alone at attenuating pro-inflammatory gene expression in glia stimulated with IFN γ or LPS and in protecting against the IFN γ or LPS mediated release of neurotoxic factors by glia when CM was transferred from glia to neurons. This may be linked to the slow release of the drug from nanoparticles observed in the drug release assays described in this chapter. Direct treatment with formoterol may also trigger saturation and subsequent downregulation of the β_2 AR (Ryan & Harkin, 2020), resulting in less pronounced anti-inflammatory effects

compared to the gradual receptor engagement achieved through sustained formoterol release from nanoparticles.

Time dependent effects of Form-PEG-PLGA on LPS mediated pro-inflammatory gene expression were observed in this study. Although LPS produced a comparable pro-inflammatory action in glia at 6- and 24-hours post stimulation, Form-PEG-PLGA treatment only reduced *Nos2* and *Cd40* mRNA expression at 6 hours, with no significant effect on *Il1b* or *Tnfα*. However, after 24 hours of treatment, Form-PEG-PLGA produced a robust attenuation of pro-inflammatory gene expression in LPS-stimulated glia. A temporal effect of amitriptyline release from PLGA in LPS stimulated BV2 cells was also described by Kim et al. (2024), where anti-inflammatory effects occurred later and persisted for longer than those observed with the free drug. This pattern suggests a slow and sustained release of formoterol from the PLGA nanoparticles; drug levels were initially insufficient to counter the early inflammatory response to LPS, but as the concentration increased over time, stronger and more prolonged anti-inflammatory effects emerged. Alternatively, this effect may also reflect the longer duration of drug exposure.

Limited evidence suggests that PLGA nanoparticles may exert anti-inflammatory effects in the absence of a loaded drug (Casey et al., 2019). However, the present study shows that unloaded PEG-PLGA had no effect on IFN γ or LPS induced pro-inflammatory mRNA expression. This is supported by several studies where unloaded PLGA nanoparticles did not impact LPS-induced inflammatory responses in macrophages (Shetab Boushehri et al., 2018; Simón-Vázquez et al., 2022; Udompornpitak et al., 2023).

The anti-inflammatory effects of formoterol released from Form-PEG-PLGA in this study were observed in primary cells, which are predominantly isolated from the brains of neonates. Neonatal brains are advantageous for this purpose for several reasons; cells are easily dissociated, generating high yields and, increased survival rates; adult glia, by contrast proliferate poorly and are more reactive to changes in environment (e.g. plating, media change, etc.). However, when considering *in vitro* disease models for neurodegenerative disease, region specific primary cultures from the adult brain may be more physiologically relevant. However, specialised expertise is required to establish and maintain these cultures, which carry high cost and risk due to low cell yields and technical difficulty.

While analysis of neuronal complexity can provide insights into the integrity of neurons and their connections, there are several limitations with this technique. Neuronal coverslips are full of dense neuronal networks as well as individual cells. However, to analyse single cell morphology, these networks are largely ignored as the individual branches of cells cannot be visualised for Sholl analysis even though they are more representative of neuronal behaviour *in vivo*. Future experiments may involve calcium imaging as a tool to measure neuronal function under normal and pro-inflammatory conditions and to investigate how treatments like formoterol may rescue neuronal functioning (Nietz et al., 2022).

Immunocytochemistry for pre- and post-synaptic markers PSD-95 and synaptophysin can also give insight into neuronal connectivity in extensive neuronal networks in *in vitro* models of health and disease (Verstraelen et al., 2018).

The Sholl analysis performed in this study was semi-automated and is therefore subject to experimenter bias. Blinding of treatment groups was introduced to combat this and secondary analyses were independently performed to verify results. Powerful new tools for analysing images of neurons have emerged in recent years. Of note, Neurolucida® (MBF Bioscience) facilitates neuron tracing and reconstruction to assess spine density and branching from microscope images. These processes can be done manually or in semi- or automated processes. However, this software is expensive and requires training, preventing most researchers from accessing this technology.

5.5.4. Compatibility of unloaded PEG-PLGA and gel with human PBMCs

In a previous chapter we reported that PEG-PLGA are compatible with primary rat neurons and glia *in vitro*. Here, we show that PEG-PLGA nanoparticles, hydrogel and PEG-PLGA-gel are compatible with human PBMCs. This has previously been reported by Lamparelli et al. (2024) who showed that unloaded PLGA nanoparticles did not affect viability of human PBMCs when applied for 24 or 48 hours at concentrations up to 2 mg/ml. Chitosan is also compatible with human PBMCs (Portela et al., 2022) and with human monocytes when formulated into a hydrogel (Caldera-Villalobos et al., 2025). Chitosan hydrogels have also been investigated as delivery systems for stem cells, where this system was shown to improve bone marrow-derived mesenchymal stem cell survival (Liu et al., 2020). As the intended purpose of this hydrogel is for intranasal delivery, it was unnecessary to test its compatibility with neurons and microglia as the gel would not come into direct contact with these cells. Interestingly, the PBMC response to LPS is non-existent after 24 hours, highlighting the difference between rodent and human cells and the need to progress these studies to advanced CNS models.

5.5.5. Future directions

While rudimentary tests of time to gelation and gel consistency were conducted, characterisation of the hydrogels rheological and viscosity properties are required to further understand how this gel behaves in the nasal cavity. The chitosan hydrogel used in these experiments was based on the formulation developed by Perkušić et al. (2023), who performed extensive characterisation studies and reported a sprayable formulation that formed an instant mucoadhesive gel at 34°C. Additionally, mucus binding can be examined as described by Hard et al. (2025), who incubated a hydrogel with a porcine mucus suspension and measured free mucin by UV spectrometry overtime.

Collectively, this evidence was sufficient to progress to *in vivo* testing of these materials in a rat model of neuroinflammation.

Chapter 6: Anti-inflammatory effects of intranasal Form-PEG-PLGA in the rat brain following systemic LPS challenge

6.1. Introduction

Targeting the noradrenergic system has immunoregulatory effects *in vitro* and *in vivo*. The NRI reboxetine increases noradrenergic tone and has been directly linked to enhanced expression of anti-inflammatory mediators *Il10* and *Socs3* as well as negative regulators of pro-inflammatory cytokine IL-1, *Il1ra* and *Il1r2*, in primary rat glial cultures and in the rat brain (McNamee et al., 2010a). Furthermore, i.p. administration of NRIs reboxetine, desipramine and atomoxetine suppressed the expression of pro-inflammatory cytokines IL-1 β and TNF α , and reduced increases in chemokine *Cxcl10* and *Ccl5* mRNA, cell adhesion molecules *Vcam1* and *Icam1* mRNA, *Nos2* and microglial activation markers *Cd11b* and *Cd40* in the rat brain following a sub-septic systemic LPS challenge (0.25 mg/kg; i.p.) (O'Sullivan et al., 2009; O'Sullivan et al., 2010). Direct activation of β_2 AR by long-acting agonists clenbuterol and formoterol were also shown to promote *Il10* and *Socs3* expression and regulate IL-1 signalling by upregulating *Il1ra* and *Il1r2* expression and inhibiting NF κ B activity *in vivo* (McNamee et al., 2010b; Ryan et al., 2013). Clenbuterol attenuated LPS-mediated NF κ B activity and associated increases in the expression of *Il1b*, *Tnf α* , *Il6*, and *Icam1*, and induced *Il10* and *Socs3* (McNamee et al., 2010a; Ryan et al., 2013; Ryan et al., 2016). Dexmedetomidine, an α_2 AR agonist, also exhibits anti-inflammatory effects in the CNS following systemic LPS challenge (Yeh et al., 2018), however, activation of this receptor is associated with inhibition of noradrenergic neurotransmission causing subsequent hypotension, bradycardia and sedation so its therapeutic potential is limited. Furthermore, clenbuterol (0.5 mg/kg; i.p.) not only reduced the expression of pro-inflammatory markers *Nos2* and *Cd11b* in the kainic acid rat model of excitotoxicity, but it was also neuroprotective and increased *Bdnf* and *Ngf* expression in the brain (Gleeson et al., 2010).

Formoterol, a long-acting β_2 AR agonist, is an FDA and EMA approved treatment for asthma and COPD with a well-established safety profile. Formoterol may be advantageous as an anti-inflammatory agent in the CNS as it is highly lipophilic and can penetrate the BBB (O'Neill & Harkin, 2018; O'Neill et al., 2020). Extensive pharmacokinetic data is available for inhaled formulations, but there is a paucity of information about alternative administration routes. In a 1982 pre-approval study of intravenous [^{3} H] formoterol in rats, 60% of the parent molecule was metabolised within 5 minutes of the initial injection, which further increased to 76% after one hour, suggesting rapid biodistribution and metabolism (Sasaki et al., 1982). Indeed, the authors detected formoterol in the brain, lungs, heart, liver, kidney, spleen, pancreas and muscle tissue of animals 30 minutes post-administration (Sasaki et al., 1982). This broad tissue distribution of formoterol may contribute to the associated side effect profile as the β_2 AR is found in many organs including the heart,

vasculature, skeletal muscle and intestines. While formoterol is highly selective for the β_2 AR, it can also activate other receptor subtypes such as cardiac β_1 AR at doses as low as 0.01 mg/kg (Ryall et al., 2006). Sasaki et al. (1982) isolated 2-O-glucuronide as an excreted formoterol metabolite. Formoterol undergoes peripheral phase II metabolism via hepatic uridine diphosphate (UDP)-glucuronosyltransferases (UGTs) resulting in O-glucuronidation of the phenol group (Ružena et al., 2020). Glucuronidation of formoterol makes it more polar and water soluble, limiting the ability of this metabolite to cross the BBB and likely rendering it inactive at the β_2 AR. Despite evidence of the efficacy of systemically administered formoterol against LPS related neuroinflammation, a large proportion of drug is rapidly metabolised meaning that like most CNS therapies, high doses are required to overcome peripheral metabolism to achieve therapeutically relevant concentrations in the brain.

It is proposed that entrapment of formoterol in a nanoparticle delivery system can protect and prolong the circulation time of the parent molecule, enhancing penetration across the BBB and increasing bioavailability in the CNS for glial β_2 AR binding. However, premature particle degradation following systemic administration may still limit the portion of drug that enters the brain.

The intranasal route is emerging as a promising strategy to bypass peripheral metabolism and target therapies directly to the brain. Clinical trials in healthy participants report inhalation or swallowing of the experimental intranasal drugs (Cooper et al., 2022) and mucociliary clearance can limit contact time for adequate absorption (Agrawal et al., 2020). To overcome this, intranasal therapies may be formulated into an *in situ* hydrogel system. However, the presence of peptidase enzymes in the nasal cavity and UGT expression in the nasal mucosa and olfactory epithelium mean that some drug will not likely be bioavailable (Heydel et al., 2001). A randomised controlled trial of intranasal formoterol for treatment of allergic rhinitis did not report any side effects using this route, however efficacy was limited as authors speculated that β_2 ARs in the airways became desensitised after repeated administration but the formation of inactive metabolites may have also contributed (Ahlström Emanuelsson et al., 2007). Combining both nanomedicine and intranasal delivery has the propensity to overcome these challenges of CNS drug delivery.

The exact passage from nose to brain has not been fully elucidated and is likely unique depending on the gel formulation, nanoparticle subtype and drug cargo. Upon deposition in the nasal cavity and exposure to body temperature, thermo-responsive polymers, from which most nasal hydrogels are constructed, undergo rapid physical transformation and solidify but this process varies widely depending on the polymer (Agrawal et al., 2020). Mucoadhesive polymers like chitosan form strong electrostatic bonds with mucin, cementing this newly formed gel to the nasal mucosa (Rassu et al., 2016). Therefore, the liquid state at room temperature facilitates administration whereas the solid state upon deposition in the nasal cavity prolongs residence time for enhanced drug absorption.

Initially, nanoparticles (<200 nm) diffuse through the hydrogel mesh and are released upon enzymatic hydrogel degradation that begins at the gel surface and progresses inwards (Rahmanian-Devin et al., 2021). Low molecular weight surface PEG reduces nanoparticle interactions with the mucus, essentially allowing free movement towards the nasal epithelium (Wang et al., 2008). PLGA nanoparticles are reported to traverse the rat and porcine nasal epithelium *ex vivo*, where a smaller diameter and the presence of PEG improved transport compared to unmodified and larger nanoparticles (Spindler et al., 2021; Wang et al., 2013). Chitosan transiently weakens the tight junctions in nasal epithelial cells which may facilitate paracellular transport to the brain (Rassu et al., 2016). PLGA nanoparticles have also been detected in neuronal fibres in the nasal mucosa (Spindler et al., 2021), suggesting that perineural transport is a viable route to the brain. Li et al. (2019) cleverly incorporated degradation inducing quenching probes into the design of polymeric polycaprolactone nanoparticles, where fluorescence was switched off by particle degradation, allowing only intact nanoparticles to be traced through live imaging techniques as they travelled from the nose to the brain of rats. The authors reported that PCL-PEG permeated the trigeminal nerves and were localised in the brainstem up to 8 hours post-administration and propose that this is the dominant route of polymeric nanoparticle trafficking to the brain (Li et al., 2019).

Toxicological analyses in rodents do not report evidence of tissue damage or significant levels of PLGA nanoparticles in major organs following intranasal administration (Arisoy et al., 2020; Bi et al., 2016; Cayero-Otero et al., 2019; Lu et al., 2023; Salem et al., 2023; Shah et al., 2021a; Sharma et al., 2023; Yan et al., 2018). In support of this, there is no evidence of fatalities associated with intranasal PLGA nanoparticles. One study detected uncoated PLGA nanoparticles in the lungs, likely due to inhalation, highlighting the importance of mucoadhesive formulations for absorption across the nasal mucosa (Meng et al., 2018).

Pharmacokinetic analyses have revealed the ability of intranasal PLGA nanoparticles to deliver therapeutics to the brain in concentrations higher than alternative administration routes including oral, i.p., and i.v. and formulations. This is true for FDA approved medicines including L-DOPA, paclitaxel, carmustine, lamotrigine, desvenlafaxine, experimental immunotherapies and natural products like catechin (Ahmad et al., 2022a; Ahmad et al., 2020; Ahmad et al., 2022b; Musumeci et al., 2022; Shah et al., 2021a; Tong et al., 2017; Zhang et al., 2020). Brain targeting is further improved by nanoparticle surface modifications, such as chitosan, lactoferrin, transferrin, and PEG coatings, which enhance the transport of nanoparticles from the nose to the brain (Ahmad et al., 2022a; Ahmad et al., 2020; Bi et al., 2016; Cayero-Otero et al., 2019; Meng et al., 2018). Furthermore, higher brain and lower circulating concentrations of anti-epileptic drug topiramate were achieved using an intranasal nanoparticle-in situ gelling system when compared to intranasal gel and solution (Tanna et al., 2024), emphasising the need for nanoparticle drug carriers and smart intranasal biomaterials for optimal CNS targeting.

6.2. Aims

In chapter 4, we reported that a thermosensitive chitosan-based hydrogel displayed a sustained drug-release profile *in vitro*. In the present study we aimed to show that this hydrogel could release cargo in the nasal cavity and travel to the brain *in vivo* (experiment 1). In chapter 5, we described the ability of Form-PEG-PLGA to release drug and reduce microglial reactivity in an *in vitro* model of neuroinflammation. In this chapter, our goal was to show that Form-PEG-PLGA could exert anti-inflammatory effects in an acute *in vivo* model of neuroinflammation (experiment 2).

6.3. Experimental design

Experiment 1: 3 adult Wistar rats (female) were intranasally administered vehicle (saline), Mn^{2+} in solution or Mn^{2+} incorporated into the mucoadhesive, thermosensitive hydrogel described in chapter 2 (Mn^{2+} -gel). Animals were culled 3 or 12 hours later by sodium pentobarbital overdose and transcardial perfusion. Brains were fixed with 4% PFA and a manganese enhanced MRI (MEMRI) scan was performed on the post-mortem brain using a 7T Bruker scanner. The MEMRI signal intensity in the resulting images was assessed (see methods section 2.4.2. and 2.4.3).

Experiment 2: 24 adult Wistar rats (male) were administered intranasal vehicle (unloaded PEG-PLGA), formoterol (0.1 mg/kg) or Form-PEG-PLGA (0.1 mg/kg) once daily for 7 or 3 days (**Fig. 6.1, Table 6.1**) (see methods section 2.4.2). Herein, all intranasal vehicle, formoterol and Form-PEG-PLGA were incorporated in the mucoadhesive, thermosensitive hydrogel described in chapter 5. For simplicity, Form-PEG-PLGA-gel is herein referred to as Form-PEG-PLGA in text and Form-gel is referred to as formoterol in text.

Animals received an i.p. injection of LPS (0.25 mg/kg) 90 minutes after the final drug dose. Body temperature was recorded 4 hours post-LPS injection using a rectal temperature probe. Rats were culled by CO₂ asphyxiation 4 hours post-LPS and the brains and spleen were removed. The tissue was homogenised and RNA was extracted, from which complimentary DNA strands were synthesised to perform PCR (see methods section 2.4.4). The expression of pro-inflammatory markers *Il1b*, *Tnfa*, *Nos2*, and *Cd40* in the rat cortex, striatum and spleen were analysed.

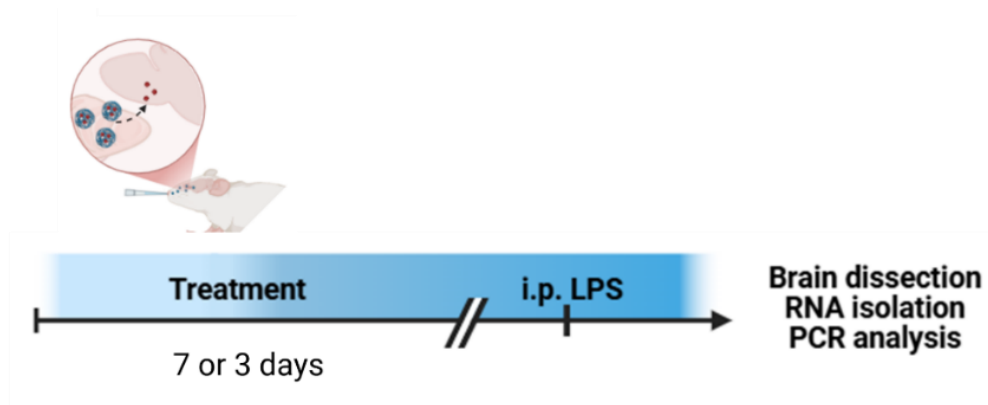


Figure 6.1. Experiment 2 timeline.

Table 6.1. Experiment 2 treatment groups.

Intranasal	No. days of dosing	i.p.
Vehicle (unloaded PEG-PLGA)	7	vehicle
Formoterol (0.1 mg/kg)	7	vehicle
Form-PEG-PLGA (0.1 mg/kg)	7	vehicle
Vehicle (unloaded PEG-PLGA)	7	LPS
Formoterol (0.1 mg/kg)	7	LPS
Form-PEG-PLGA (0.1 mg/kg)	7	LPS
Form-PEG-PLGA (0.1 mg/kg)	3	vehicle
Form-PEG-PLGA (0.1 mg/kg)	3	LPS

6.4. Results

6.4.1. Hydrogel displays slow-release properties *in vivo*

Background MEMRI signal was detected in the olfactory layer and ventricle (**Fig. 6.2.a, b**; grayscale and pseudo-coloured, respectively), ventral orbital cortex and olfactory ventricles (**Fig. 6.2.g, h**) and primary and secondary motor cortex, lateral ventricle olfactory tubercle and lateral olfactory tract (**Fig. 6.2. m, n**) of rats that were administered intranasal vehicle. MR signal was detected in the olfactory layer and ventricle (**Fig. 6.2.c, d**; grayscale and pseudo-coloured, respectively), ventral orbital cortex and olfactory ventricles (**Fig. 6.2.i, j**) and primary and secondary motor cortex, lateral ventricle, olfactory tubercle and lateral olfactory tract (**Fig. 6.2. o, p**) 3 hours post-intranasal administration of Mn^{2+} solution. Mn^{2+} was also localised in the olfactory layer and ventricle (**Fig. 6.2.e, f**; grayscale and pseudo-coloured, respectively), ventral orbital cortex and olfactory ventricles (**Fig. 6.2.k, l**) and primary and secondary motor cortex, lateral ventricle, olfactory tubercle and lateral olfactory tract (**Fig. 6.2. q, r**) of rats that were administered intranasal Mn^{2+} -hydrogel after 12 hours. Signal intensity was increased 1.26, 1.19 and 1.10-fold in the olfactory layer and ventricle, ventral orbital cortex and olfactory ventricle, and primary and secondary motor cortex, lateral ventricle, olfactory tubercle and lateral olfactory tract, respectively, in animals that were administered intranasal Mn^{2+} solution compared to vehicle treated animals (**Fig. 6.2.t**). Signal intensity was increased 1.41, 1.35 and 1.39-fold in the olfactory layer and ventricle, ventral orbital cortex and olfactory ventricle, and primary and secondary motor cortex, lateral ventricle, olfactory tubercle and lateral olfactory tract, respectively, in animals that were administered intranasal Mn^{2+} -hydrogel compared to vehicle treated animals (**Fig. 6.2.t**).

6.4.2. Daily intranasal administration of Form-PEG-PLGA does not affect bodyweight

Bodyweight did not differ between groups prior to the first intranasal dose (data not shown). Two-way repeated measures ANOVA of the % change in bodyweight of animals showed a main effect of time ($F_{(3,770, 56.55)} = 9.945$, $P < 0.0001$) and a time x treatment interaction ($F_{(12, 90)} = 2.941$, $P < 0.01$) (**Fig. 6.3**). Tukey's multiple comparisons did not reveal any relevant differences in % bodyweight change between groups across the 7-day dosing schedule.

6.4.3. Daily intranasal administration of Form-PEG-PLGA does not affect the LPS-mediated increase in body temperature

Two-way ANOVA of body temperature revealed a main effect of LPS [$F_{(1, 16)} = 34.18$, $P < 0.0001$] (**Fig. 6.4**). Tukey's multiple comparisons identified a significant increase in the body temperature of animals that were administered LPS compared to vehicle control ($P < 0.05$).

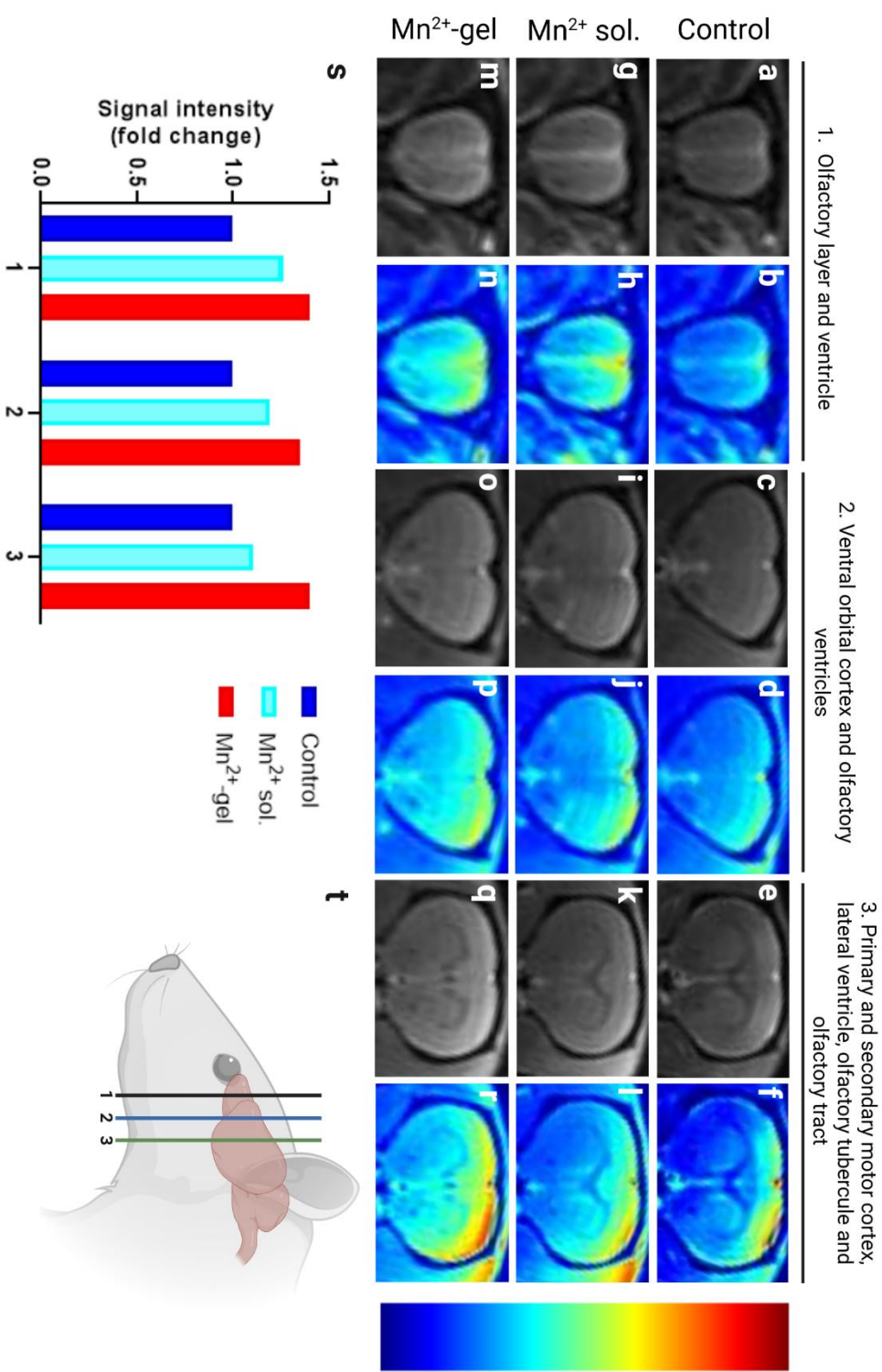


Figure 6.2. Slow release of contrast agent Mn²⁺ from chitosan-hydrogel *in vivo*. T1-weighted images of MEMRI signal 3 hours after intranasal delivery of vehicle (5 µl saline; **a-f**), Mn²⁺ in solution (5 µl, 1 M; **g-l**) or 12 hours after intranasal administration of Mn²⁺-hydrogel (5 µl, 1 M; **m-r**). (**s**) Fold change in signal intensity normalised to control in each brain region, where N=1. (**t**) Map of MRI slices in a-r, where 1 = olfactory layer and ventricle, 2 = ventral orbital cortex and olfactory ventricles and 3 = primary and secondary motor cortex, lateral ventricle olfactory tubercle and lateral olfactory tract. MR images displayed in greyscale and pseudo-coloured so that enhanced signal appears in light blue-red where an absence of signal is dark blue.

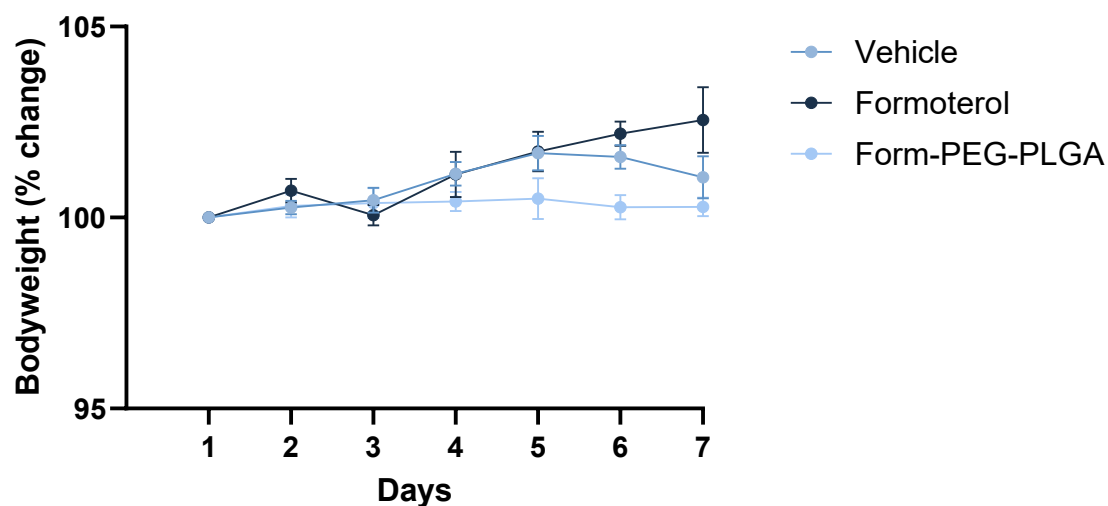


Figure 6.3. Daily intranasal administration of Form-PEG-PLGA does not affect bodyweight.

Daily intranasal administration of vehicle (30 μ l), formoterol (0.1 mg/kg; 30 μ l) or Form-PEG-PLGA (0.1 mg/kg; 30 μ l) under brief and light anaesthesia for 7 days does not affect % change in bodyweight, where 100% on day 1 represents an average bodyweight of 480.8g, 462g and 492g for animals in the vehicle, formoterol and Form-PEG-PLGA groups, respectively. Data were analysed by two-way repeated measures ANOVA followed by Tukey's multiple comparisons and are expressed as the % bodyweight change per day from baseline ($\pm$ SEM) per day where N=3.

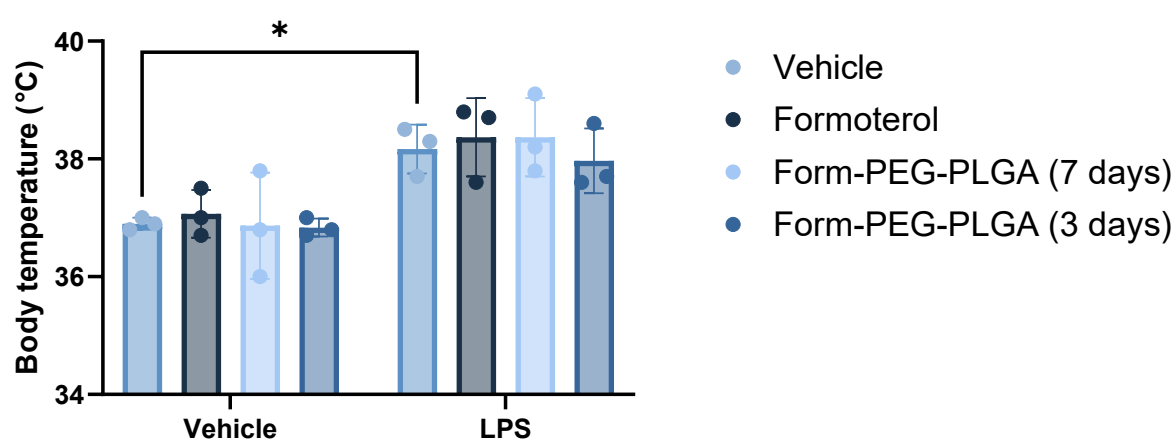


Figure 6.4. Daily intranasal administration of Form-PEG-PLGA does not affect LPS induced increase in body temperature.

Vehicle (30 μ l), formoterol (0.1 mg/kg; 30 μ l) or Form-PEG-PLGA (0.1 mg/kg; 30 μ l) were administered intranasally to rats for 7 or 3 days. 90 minutes after final drug administration, animals were challenged with LPS. Neither formoterol nor Form-PEG-PLGA affected LPS induced increase in body temperature 4 hours post LPS injection (0.25 mg/kg; i.p.). Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean ($\pm$ SEM), where N=3. Significant increases in body temperature compared to vehicle are denoted by * where $P < 0.05$.

6.4.4. Daily intranasal administration of Form-PEG-PLGA attenuates pro-inflammatory gene expression in the rat cortex following systemic LPS challenge

Two-way ANOVA of fold change *I11b* mRNA in the rat cortex showed effects of LPS [$F_{(1, 16)} = 22.18$, $P < 0.001$], treatment [$F_{(3, 16)} = 5.211$, $P < 0.05$] and an interaction between LPS and treatment [$F_{(3, 16)} = 4.981$, $P < 0.05$] (**Fig. 6.5.a**). Tukey's multiple comparisons revealed an increase in cortical *I11b* mRNA in animals that received LPS compared to animals that were injected with vehicle ($P < 0.001$). The LPS induced increase in *I11b* mRNA in the rat cortex was attenuated by 7 days of pre-dosing with formoterol ($P < 0.05$) but not attenuated by 7 days of pre-treatment with Form-PEG-PLGA. However, 3 days of pre-treatment with Form-PEG-PLGA reduced LPS-induced cortical *I11b* mRNA expression compared to vehicle treated control ($P < 0.01$).

Two-way ANOVA of fold change in *Tnf α* mRNA in the rat cortex showed effects of LPS [$F_{(1, 16)} = 8.554$, $P < 0.01$], treatment [$F_{(3, 16)} = 7.486$, $P < 0.01$] and an interaction between LPS and treatment [$F_{(3, 16)} = 4.988$, $P < 0.05$] (**Fig. 6.5.b**). Tukey's multiple comparisons revealed an increase in *Tnf α* mRNA in the cortex of animals that were administered LPS compared to the vehicle group ($P < 0.001$). The LPS mediated increase in cortical *Tnf α* mRNA expression was attenuated by 7 days of pre-treatment with formoterol ($P < 0.001$) as well as both 7- and 3-days pre-treatment with Form-PEG-PLGA compared to vehicle control (7 days: $P < 0.001$; 3 days: $P < 0.05$).

Two-way ANOVA of fold change in *Nos2* mRNA in the rat cortex showed effects of LPS [$F_{(1, 16)} = 9.387$, $P < 0.01$], treatment [$F_{(3, 16)} = 4.794$; $P < 0.05$] and an LPS x treatment interaction [$F_{(3, 16)} = 4.801$, $P < 0.05$] (**Fig. 6.5.c**). Tukey's multiple comparisons revealed an increase in cortical *Nos2* mRNA expression in rats that received LPS compared to animals that received vehicle ($P < 0.001$). The LPS induced increase in *Nos2* transcripts was attenuated by 7 days pre-treatment with formoterol ($P < 0.01$) as well as both 7- and 3-days pre-treatment with Form-PEG-PLGA compared to vehicle treated control (7 days: $P < 0.01$; 3 days: $P < 0.01$).

Two-way ANOVA of fold change in *Cd40* mRNA in the rat cortex showed effects of LPS [$F_{(1, 16)} = 9.044$, $P < 0.01$], treatment [$F_{(3, 16)} = 5.201$, $P < 0.05$] and an LPS x treatment interaction [$F_{(3, 16)} = 4.047$, $P < 0.05$] (**Fig. 6.5.d**). Tukey's multiple comparisons revealed that LPS increased *Cd40* mRNA expression in the rat cortex compared to vehicle control ($P < 0.001$). 7 days of intranasal formoterol administration attenuated the LPS-mediated upregulation in cortical *Cd40* transcription compared to vehicle treated control animals ($P < 0.05$). Both 7- and 3-days pre-treatment with intranasal Form-PEG-PLGA also attenuated the LPS-related increase in *Cd40* mRNA expression compared to vehicle control (7 days: $P < 0.01$; 3 days: $P < 0.01$).

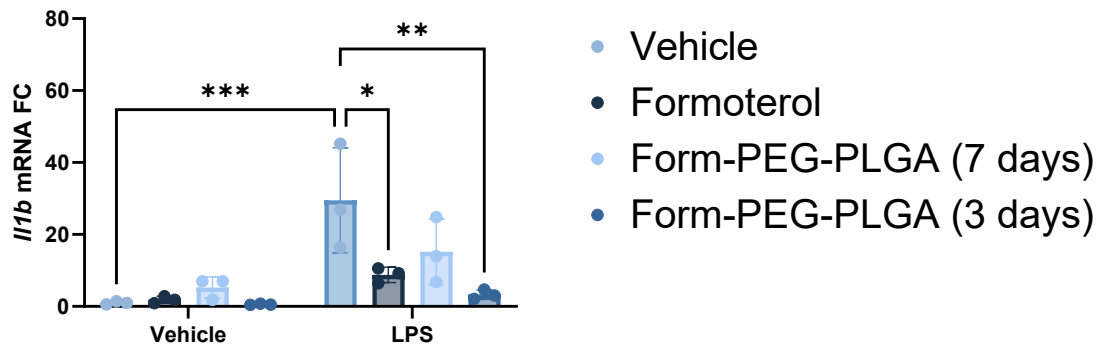
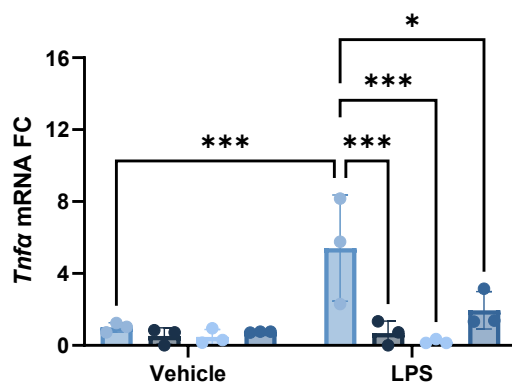
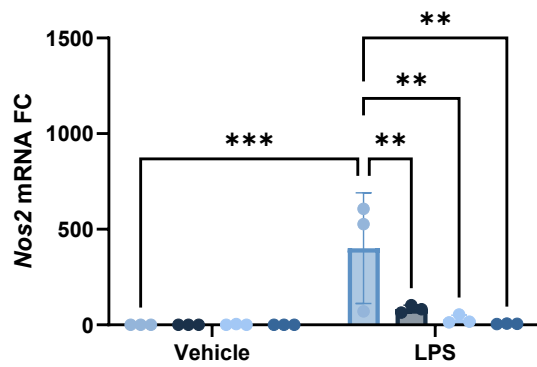
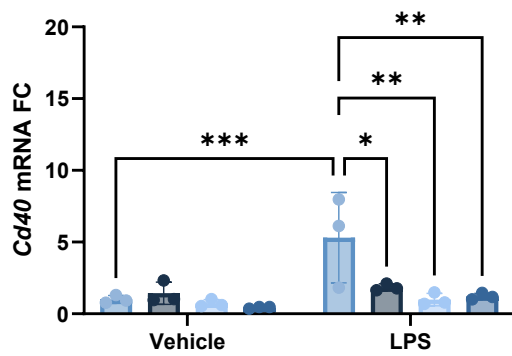
a**b****c****d**

Figure 6.5. Intranasal Form-PEG-PLGA attenuates LPS-induced pro-inflammatory gene expression in the rat cortex.

Vehicle (30 μ l), formoterol (0.1 mg/kg; 30 μ l) or Form-PEG-PLGA (0.1 mg/kg; 30 μ l) were administered intranasally to rats for 7 or 3 days. 90 minutes after final drug administration, animals were challenged with LPS (0.25 mg/kg; i.p.). (a) Fold change (FC) in *Il1b*, (b) *Tnfa*, (c) *Nos2* and (d) *Cd40* mRNA in the rat cortex 4 hours post-LPS injection was quantified. Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean fold change from control ($\pm$ SEM), where N=3. Significant differences between groups are denoted by * where * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$. FC; fold change.

6.4.5. Daily intranasal administration of Form-PEG-PLGA attenuates pro-inflammatory gene expression in the rat striatum following systemic LPS challenge

Two-way ANOVA of fold change in *Il1b* gene expression at the mRNA level in the rat striatum showed effects of LPS [$F_{(1, 16)} = 31.53$, $P<0.0001$], treatment [$F_{(3, 16)} = 10.00$, $P<0.001$] and an LPS x treatment interaction [$F_{(3, 16)} = 9.160$, $P<0.001$] (**Fig. 6.6a**). Tukey's multiple comparisons revealed an increase in striatal *Il1b* mRNA in animals that received i.p. LPS compared to vehicle ($P<0.0001$). The LPS-induced increase in *Il1b* mRNA expression was reduced when animals were pre-treated with formoterol ($P<0.01$), Form-PEG-PLGA for 7 days ($P<0.001$) or Form-PEG-PLGA for 3 days ($P<0.0001$) compared to vehicle treated controls.

Fold change in *Tnfa* mRNA in the rat striatum was not affected by LPS or treatment (**Fig. 6.6b**).

Two-way ANOVA of fold change in *Nos2* mRNA in the rat striatum showed effects of LPS [$F_{(1, 16)} = 17.69$, $P<0.001$], treatment [$F_{(3, 16)} = 6.081$, $P<0.01$] and an LPS x treatment interaction [$F_{(3, 16)} = 6.128$, $P<0.01$] (**Fig. 6.6c**). Tukey's multiple comparisons revealed an increase in striatal *Nos2* mRNA in animals that received LPS compared to vehicle control ($P<0.0001$). The LPS-mediated increase in striatal *Nos2* mRNA levels was attenuated by 7 days pre-treatment with formoterol or Form-PEG-PLGA (both $P<0.01$) or 3 days of pretreatment with Form-PEG-PLGA ($P<0.001$) compared to vehicle treated controls.

Two-way ANOVA of fold change in *Cd40* mRNA in the rat striatum showed effects of LPS [$F_{(1, 16)} = 6.868$, $P<0.05$], treatment [$F_{(3, 16)} = 5.897$, $P<0.01$] and an LPS x treatment interaction [$F_{(3, 16)} = 4.473$, $P<0.05$] (**Fig. 6.6d**). Tukey's multiple comparisons revealed an increase in *Cd40* mRNA in the striatum when animals were administered LPS compared to vehicle control ($P<0.001$). The LPS-induced increase in striatal *Cd40* expression was ameliorated by 7 days pre-treatment with formoterol or Form-PEG-PLGA (both $P<0.01$) or 3 days of pre-treatment with Form-PEG-PLGA ($P<0.01$) compared to vehicle treated controls.

Figure 6.6. Intranasal Form-PEG-PLGA attenuates LPS-induced pro-inflammatory gene expression in the rat striatum.

Vehicle (30 μ l), formoterol (0.1 mg/kg; 30 μ l) or Form-PEG-PLGA (0.1 mg/kg; 30 μ l) were administered intranasally to rats for 7 or 3 days. 90 minutes after final drug administration, animals were challenged with LPS (0.25 mg/kg; i.p.). (a) Fold change (FC) in *Il1b*, (b) *Tnfa*, (c) *Nos2* and (d) *Cd40* mRNA in the rat striatum 4 hours post-LPS injection was quantified. Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean fold change from control ($\pm$ SEM), where N=3. Significant differences between groups are denoted by * where ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$. FC; fold change.

6.4.6. Daily intranasal administration of Form-PEG-PLGA attenuates pro-inflammatory gene expression in the rat spleen following systemic LPS challenge

Two-way ANOVA of fold change in *Il1b* mRNA in the spleen showed an effect of LPS [$F_{(1, 16)} = 43.57$, $P<0.0001$] but not treatment (**Fig. 6.7a**). Tukey's multiple comparisons revealed an increase in splenic *Il1b* mRNA in animals that received LPS compared to vehicle control ($P<0.05$).

Two-way ANOVA of fold change in *Tnfa* mRNA expression in the spleen showed effects of LPS [$F_{(1, 16)} = 295.2$, $P<0.0001$], treatment [$F_{(3, 16)} = 13.34$, $P<0.001$] and an LPS x treatment interaction [$F_{(3, 16)} = 13.54$, $P<0.001$] (**Fig. 6.7b**). Tukey's multiple comparisons revealed an increase in *Tnfa* mRNA in the spleen of animals that received LPS compared to vehicle ($P<0.0001$). The LPS-induced increase in *Tnfa* mRNA in the spleen was attenuated by 7 days pre-treatment with formoterol or Form-PEG-PLGA (both $P<0.0001$) or 3 days of pre-treatment with Form-PEG-PLGA ($P<0.0001$) compared to vehicle treated control.

Two-way ANOVA of fold change in *Nos2* mRNA in the spleen showed effects of LPS [$F_{(1, 16)} = 25.81$, $P<0.001$], treatment [$F_{(3, 16)} = 3.39$, $P<0.05$] and an LPS x treatment interaction [$F_{(3, 16)} = 3.528$, $P<0.05$] (**Fig. 6.7c**). Tukey's multiple comparisons identified that LPS increased *Nos2* mRNA expression in the spleen compared to vehicle control ($P<0.0001$). Pre-treatment with intranasal formoterol for 7 days attenuated the LPS-mediated upregulation in splenic *Nos2* compared to vehicle treated control ($P<0.05$). The LPS-mediated increase in *Nos2* mRNA in the spleen was attenuated by 7 days pre-treatment with formoterol or Form-PEG-PLGA ($P<0.05$ and $P<0.01$, respectively) or 3 days of pretreatment with Form-PEG-PLGA ($P<0.01$) compared to vehicle treated control animals.

Two-way ANOVA of fold change in *Cd40* mRNA in the spleen showed effects of LPS [$F_{(1, 16)} = 28.31$, $P<0.0001$], treatment [$F_{(3, 16)} = 11.79$, $P<0.001$] and an LPS x treatment interaction [$F_{(3, 16)} = 4.323$, $P<0.05$] (**Fig. 6.7d**). Tukey's multiple comparisons revealed an increase in *Cd40* mRNA in the spleen of animals that received LPS compared to vehicle ($P<0.0001$). Pre-treatment for 7 days with intranasal formoterol or Form-PEG-PLGA or 3 days with Form-PEG-PLGA prevented the LPS-induced *Cd40* response compared to vehicle treated control (all $P<0.001$).

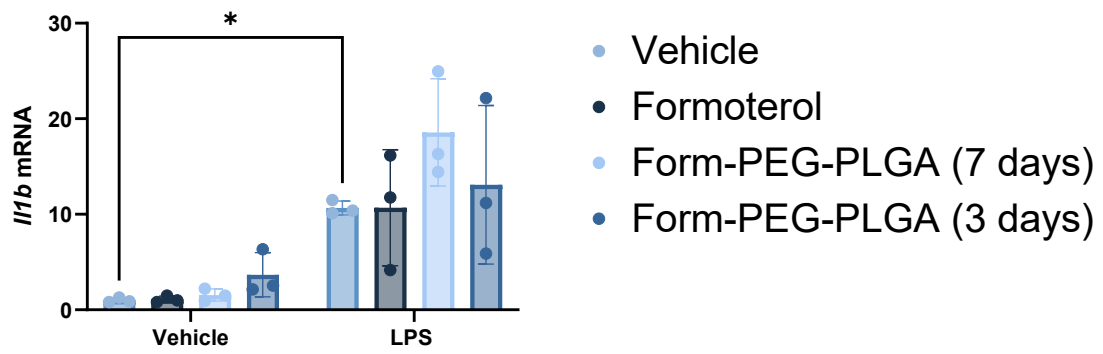
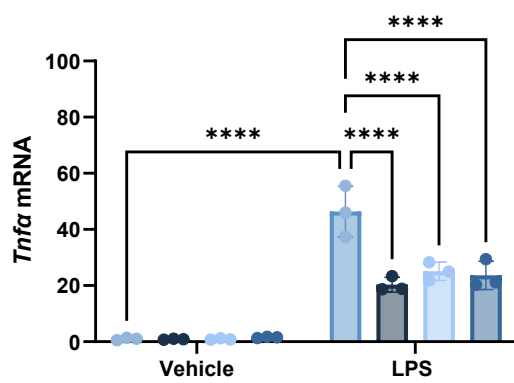
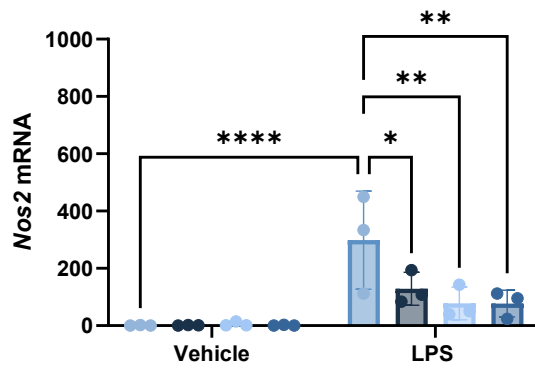
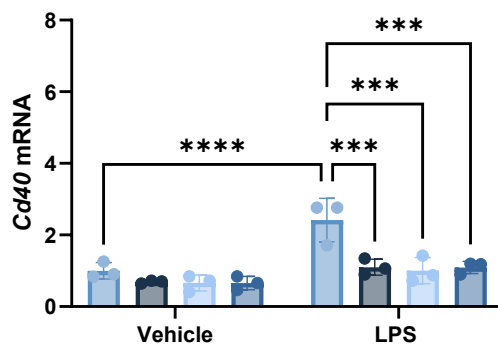
a**b****c****d**

Figure 6.7. Intranasal Form-PEG-PLGA attenuates LPS-induced pro-inflammatory gene expression in the rat spleen.

Vehicle (30 µl), formoterol (0.1 mg/kg; 30 µl) or Form-PEG-PLGA (0.1 mg/kg; 30 µl) were administered intranasally to rats for 7 or 3 days. 90 minutes after final drug administration, animals were challenged with LPS (0.25 mg/kg; i.p.). (a) Fold change in *Il1b*, (b) *Tnfa*, (c) *Nos2* and (d) *Cd40* mRNA in the rat spleen 4 hours post-LPS injection was quantified. Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean fold change from control ($\pm$ SEM), where N=3. Significant differences between groups are denoted by * where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. FC; fold change.

6.5. Discussion

6.5.1. Mn^{2+} reaches the brain via intranasal hydrogel delivery

MEMRI is a particularly useful tool for visualisation of the delivery of contrast agent to the brain following intranasal delivery. Not only is Mn^{2+} a paramagnetic ion that affects T1 relaxivity, but it also acts as a calcium Ca^{2+} analogue that enters neurons in the olfactory epithelium and is transported along their axons and synapses to the olfactory bulb and the adjacent cortices (Aoki et al., 2004; Koretsky & Silva, 2004). In T1-weighted images, signal intensity in the olfactory layer and ventricle, ventral orbital cortex and olfactory ventricle, and primary and secondary motor cortex, lateral ventricle, olfactory tubercle and lateral olfactory tract of rats that were administered an Mn^{2+} solution intranasally was increased 1.26, 1.19 and 1.11-fold, respectively, compared to the negative control, displaying a gradual dispersion of contrast agent throughout these brain regions. Previously, Gobbo et al. (2012) reported a peak in MEMRI signal in the olfactory bulbs of rats 200 minutes after intranasal Mn^{2+} administration. We hypothesised that due to the formulation of the hydrogel, it may take longer for Mn^{2+} to be released, accumulate in the olfactory bulb and for the signal intensity to peak. Here we show that MEMRI signal intensity in the olfactory layer and ventricle, ventral orbital cortex and olfactory ventricle, and primary and secondary motor cortex, lateral ventricle, olfactory tubercle and lateral olfactory tract increased 1.41, 1.35 and 1.40-fold, respectively, 12 hours after intranasal administration of Mn^{2+} in hydrogel compared to control. This signal pattern reflects the biphasic release profile observed *in vitro* in chapter 3 and may suggest a sustained release profile *in vivo*. The signal intensity in the olfactory layer 12 hours after intranasal delivery of Mn^{2+} -hydrogel is comparable to 3 hours post-intranasal Mn^{2+} solution, also pointing to a sustained release of contrast agent when incorporated into hydrogel. The biodistribution and half-life of intranasally delivered Mn^{2+} is infrequently reported but signal intensity is generally stated to peak after 3-4 hours followed by transport to the surrounding piriform and visual cortices and gradual depletion over 24-48 hours (Almahmoud et al., 2024; Gobbo et al., 2012). Therefore, it is possible that Mn^{2+} could still be detected in the olfactory layer 12 hours after

intranasal administration of Mn^{2+} solution, but the signal would likely have begun to diminish. In any case, the MEMRI signal in the olfactory layer 12 hours after intranasal Mn^{2+} -hydrogel administration is higher than that of animals that were scanned 3 hours post- Mn^{2+} solution, suggesting that Mn^{2+} was released slowly as the hydrogel broke down in the nasal cavity and accumulated in the olfactory layer over a 12-hour period.

Signal was detected near the skull in one hemisphere in control animals. Manganese is a naturally occurring trace element essential for brain homeostasis. It can cross the BBB and is stored in astrocytes (Takeda et al., 1998). However, its physiological concentrations are unlikely to be detectable by MRI. The signal observed in control animals is therefore most likely an artefact caused by coil positioning relative to the head. To account for such artefacts, a reference phantom (e.g. a water tube) can be included in the scan, allowing each region of interest to be normalised to the reference signal (Gobbo et al., 2012).

Despite these artifacts, a clear signal was visible in the olfactory bulbs of animals administered Mn^{2+} in either solution or hydrogel as well as in deeper forebrain structures where no signal was detected in controls. The aim of this experiment was to determine whether intranasal delivery of Mn^{2+} via *in situ* hydrogel could achieve brain uptake, which we have confirmed here. While the release appears to occur in a sustained manner, additional timepoints will be required to further interpret these results.

6.5.2. Intranasal Form-PEG-PLGA does not affect bodyweight or temperature

Repeated exposure to isoflurane is known to cause stress responses in mice, including increased grimace scores and reduced burrowing behaviour (Hohlbaum et al., 2017). In rats, exposure to inhalational anaesthetics increases corticosterone production, with females exhibiting higher physiological markers of stress (Bekhat et al., 2016). However, there are no reports on the direct effect of brief daily isoflurane exposure on bodyweight. In experiment 2, daily exposure to light anaesthesia did not affect bodyweight. Anaesthesia appeared to be well tolerated in this experiment as it did not affect this parameter. Neither β_2 AR agonists nor the biomaterials used in this study are reported to cause bodyweight changes in healthy rodents (Chaplin et al., 2020; De Waal et al., 1996; Venkatpurwar et al., 2015).

In this study, body temperature was increased 4 hours post-systemic LPS challenge confirming an immune and characteristic pyretic response. LPS activation of the TLR4 receptor on peripheral macrophages, monocytes, B cells and neutrophils triggers a cascade of cytokine signalling resulting in an increase in core body temperature driven by the thermoregulatory control centre in the hypothalamus (Evans et al., 2015). As exposure to anaesthesia can cause an acute stress response in rodents by activating the hypothalamic-pituitary-adrenal (HPA) axis (Bekhat et al., 2016), we allowed a 90-minute recovery period after the final intranasal administration to allow hypothalamic functioning to return to

baseline prior to LPS challenge. Previous work by the Harkin lab has shown that systemic administration of β_2 AR agonist clenbuterol reduces core body temperature (Ryan et al., 2016). Here, we show that pre-treatment with intranasal formoterol or Form-PEG-PLGA hydrogel formulations did not attenuate the LPS-mediated increase in body temperature, suggesting that the circulating concentration of formoterol was insufficient to reduce the initial pyretic response to LPS.

Formoterol has some affinity, albeit low, for the β_3 AR which may directly influence core body temperature through thermogenesis in brown adipose tissue. This effect has been observed in humans, where β_2 ARs play a dominant role in brown adipocyte thermogenesis (Ishida et al., 2024; Straat et al., 2023). β_2 ARs are also expressed on vascular smooth muscle cells, where they regulate blood vessel tone, which in turn can affect core body temperature (Chruscinski et al., 2001). In this study, daily intranasal administration of formoterol or Form-PEG-PLGA in the absence of LPS did not increase core body temperature, indicating that peripheral mechanisms regulating thermogenesis were not affected. This suggests that systemic drug exposure was limited.

6.5.3. Form-PEG-PLGA attenuates LPS-mediated neuroinflammatory responses

Systemic LPS challenge produced an upregulation of *Il1b*, *Tnfa*, *Nos2* and *Cd40* mRNA in the rat cortex and striatum. This challenge is extensively used in the field to induce neuroinflammation in the rodent brain (Hoogland et al., 2015; Skrzypczak-Wiercioch & Sałat, 2022). The Harkin lab has previously shown that i.p. administration of LPS stimulates the expression of pro-inflammatory genes—*Il1b*, *Tnfa*, *Nos2*, *Cd40*, *Cd11b*, and *Rantes*—in the rat cortex. This response can be suppressed by enhancing noradrenergic signalling through a single dose of norepinephrine reuptake inhibitors, reboxetine, desipramine and atomoxetine or the β_2 AR agonist clenbuterol prior to LPS challenge (McNamee et al., 2010a; O'Sullivan et al., 2009; O'Sullivan et al., 2010). Evans et al. (2020) also reported that daily administration of β_2 AR agonist mabuterol (s.c.) attenuated the production of various pro-inflammatory cytokines including CXCL1, CXCL2 and CCL10 in the hippocampus of APP mice that were acutely challenged with LPS. However, there are reports that the inhalational anaesthetic isoflurane can increase *Tnfa*, *Il6*, and *Il1b* expression in the brains of mice (Wu et al., 2012). This must be controlled for by ensuring control animals experience equal exposure to isoflurane as animals receiving drug treatment, as was the case for this set of experiments.

In the present study, both 7- and 3-days pre-treatment with Form-PEG-PLGA significantly reduced LPS-mediated increases in pro-inflammatory mRNA in the rat cortex and striatum, which aligns with previous findings that systemic administration of formoterol suppresses NF κ B activity in the rat cortex and hippocampus (Ryan et al., 2013). For some genes, including *Il1b* in the cortex, the anti-inflammatory effects of 3 days of Form-PEG-PLGA prior

to i.p. LPS were superior to 7 days of pre-treatment, but generally the responses to both dosing regimens were similar. Prolonged exposure to β_2 AR agonists in the CNS is linked to receptor desensitisation in the rat cortex (Frazer et al., 2007; O'Donnell & Frazer, 1985) and inflammatory stimuli like LPS have also been shown to downregulate glial expression of the β_2 AR (Ryan et al., 2020; Ryan & Harkin, 2020). However, there is no evidence of a loss of anti-inflammatory action following 7 or 3 days of intranasal formoterol or Form-PEG-PLGA administration.

Our lab has previously demonstrated robust anti-inflammatory effects of β_2 AR agonists in the rat brain following systemic administration (McNamee et al., 2010a; Ryan et al., 2013). While the responses observed are comparable in efficacy independent of the route of administration in this acute model of LPS-mediated neuroinflammation, drug entrapment in PLGA nanocarriers offers added benefits including increased brain bioavailability and reduced circulating drug, when delivered intranasally (Ahmad et al., 2022a; Ahmad et al., 2020; Ahmad et al., 2022b; Musumeci et al., 2022; Shah et al., 2021a; Tong et al., 2017; Zhang et al., 2020). Clinically, this translates to fewer side effects and the potential to administer lower drug doses.

6.5.4. Anti-inflammatory effects of Form-PEG-PLGA in the spleen

The spleen is a crucial site for peripheral immune surveillance through antigen presentation and the production of antibodies. As expected, systemic LPS challenge induced a robust pro-inflammatory response in the spleen, measured by significant increases in the expression of *Il1b*, *Tnfa*, *Nos2* and *Cd40*. In this chapter, we aimed to show that when delivered intranasally, formoterol could enter the brain and exclusively attenuate LPS-mediated neuroinflammation with little to no effect on the peripheral immune response. However, intranasal administration of formoterol and Form-PEG-PLGA also attenuated the LPS-triggered upregulation of *Tnfa*, *Nos2* and *Cd40* in the spleen, but interestingly not *Il1b*. This indicates some nanoparticles and formoterol are entering the peripheral blood circulation, likely via capillaries in the nasal epithelium or if the animal inadvertently swallowed the gel. The latter is less likely as the hydrogel was formulated to have thermosensitive and mucoadhesive properties to prevent this.

The spleen is innervated by the splenic nerve which predominantly releases noradrenaline in response to activation of the sympathetic nervous system. The activation of central β_2 ARs in the hypothalamus, brainstem or limbic system can influence the release of noradrenaline from the splenic nerve to promote peripheral anti-inflammatory signalling. Devi et al. (2021) established a direct link between noradrenaline release from splenic nerve terminals and an inhibition of leukocyte migration in response to β_2 AR-agonists independent of the expression of the β_2 AR on proximal immune cells (Devi et al., 2021). Therefore, central activation of β_2 ARs in this study following intranasal administration of formoterol or Form-

PEG-PLGA may be influencing anti-inflammatory signalling in the spleen. However, there are reports that PLGA nanoparticles can be detected in the spleen following intranasal administration (Zhang et al., 2020) so it is plausible that formoterol in the blood stream is acting on β_2 ARs locally to drive anti-inflammatory responses in the spleen. Future investigations may involve pre-administration of a peripherally acting β_2 AR antagonist such as nadolol. If the anti-inflammatory response in the spleen remains following nadolol pre-treatment, then these effects are mediated via activation of central β_2 ARs following intranasal formoterol delivery.

6.5.5. CNS bioavailability following intranasal PLGA-mediated drug delivery

Whole brain analyses have revealed higher brain concentrations of several drug classes when loaded into PLGA nanoparticles and administered via the intranasal route compared to intranasal delivery of free drug in rodent studies (Ahmad et al., 2022a; Ahmad et al., 2020; Ahmad et al., 2022b; Lu et al., 2023; Musumeci et al., 2022; Salem et al., 2023; Shah et al., 2021b; Sharma et al., 2023; Tong et al., 2017; Yan et al., 2018; Zhang et al., 2020). *In vivo* imaging has also been used to trace labelled drug entities following intranasal delivery of PLGA nanoparticles. Sharma et al., 2015 used gamma scintigraphy to show that radiolabelled diazepam had higher brain penetrance 0.5, 1, 2, 4 and 8 hours after intranasal delivery of drug-loaded PLGA nanoparticles compared to both intranasal and intravenous administration of free drug. Furthermore, over 60% of the administered dose reached the brain from the nasal mucosa using a PLGA nanocarrier compared to 1% when free drug was administered intranasally (Sharma et al., 2015). The use of fluorescently labelled PLGA nanoparticles has confirmed the ability of these carriers to deliver cargo to the brain in rodents when delivered intranasally (Meng et al., 2018; Nema & Bhargava, 2024). However, these studies trace the load rather than the nanoparticle. As the drug was the labelled entity, it is unclear if the nanoparticles are transported to the brain to release drug or if the nanoparticles release drug in the nasal cavity which then travels to the brain and periphery. Li et al. (2019) demonstrated that polymeric polycaprolactone nanoparticles reach the brain by attaching probes to the nanoparticle matrix, but this has not yet been confirmed for PLGA.

To further investigate the central and systemic anti-inflammatory effects of formoterol and Form-PEG-PLGA described here, it is necessary to quantify drug concentrations in both the brain and peripheral tissues. Throughout this study, we have attempted to use HPLC coupled to UV detection, to measure formoterol in the brain following intranasal administration. However, HPLC lacks the sensitivity required to detect the picogram-level concentrations expected in rat brain tissue post-dosing. Liquid chromatography coupled to mass spectrometry (LC-MS) is a more sensitive analytical method and may be used to quantify formoterol in blood, spleen, and brain regions such as the cortex and striatum. Although several studies have reported the detection of formoterol in human and rodent

biological samples (Kakubari et al., 2007; Lee et al., 2014; Mazzarino et al., 2013; Peterson et al., 2024), the reported lower limits of detection may still be inadequate for reliable measurement in rat brain tissue. Samples from this study were obtained for this purpose; however further optimisation of LC-MS conditions and sample preparation are required. Based on the available literature, we hypothesize that intranasal administration will result in higher concentrations of formoterol in the brain compared to the periphery, with the addition of nanoparticle carriers further enhancing this effect. A systemic formoterol injection may be used as a comparison, which would be expected to show higher concentrations peripherally compared to centrally. In this case, given the rapid metabolism of formoterol in the periphery as reported by Sasaki et al. (1982) it may be necessary to analyse both parent drug and the primary 2-O glucuronide metabolite.

6.5.6. Considerations for intranasal delivery to rats

In 2019 an intranasal formulation of esketamine, SPRAVATO®, was approved by both the FDA and EMA for the treatment of major depressive disorder, marking a seminal advancement in intranasal therapies for CNS disorders. However, when ketamine was administered via the intranasal, oral and i.p. routes in rats, highest drug concentrations in both brain and plasma were observed following i.p. administration. Intranasal delivery produced brain and plasma concentrations that were roughly equal to each other but approximately five times lower than those achieved with i.p. injection (Naidoo et al., 2019). In this study, it is possible that some of the intranasally administered ketamine was swallowed, which may help explain discrepancies between preclinical pharmacokinetic results and the robust clinical efficacy that supported the drug's approval. These results also underscore the potential advantages of using mucoadhesive gel in rodent studies to enable sustained intranasal drug delivery and controlled release.

Placement of drug in the rodent nasal cavity has major effects on brain uptake as lower depositions are more likely to be swallowed. Morin et al. (2025) studied brain uptake using different intranasal methods and found that using a pipette tip and depositing drug closer to the nostril resulted in lower brain concentrations of drug compared to intravenous administration. However, using a canula type applicator significantly enhanced brain uptake compared to pipette administered doses, showing high reproducibility and accuracy across a wide range of drug compounds (Morin et al., 2025). Furthermore, drugs are more susceptible to mucociliary clearance, nasal drip and swallowing when delivered to the lower nasal region (Gänger & Schindowski, 2018). To overcome these challenges, we dissected the nasal cavity of a rat cadaver of the same age and strain as those used in this study. We measured the length of the cavity to be approximately 9 mm, in line with other reports (Alvites et al., 2018). We then adjusted the length of a canula type applicator to 9 mm so that gel containing Form-PEG-PLGA was deposited as close to the olfactory epithelium as possible (**Fig. 6.8a**). However, standard procedures for intranasal drug

administration to rodents are required as a large portion of the literature describes the pipette method, which may lead to conflicting results.

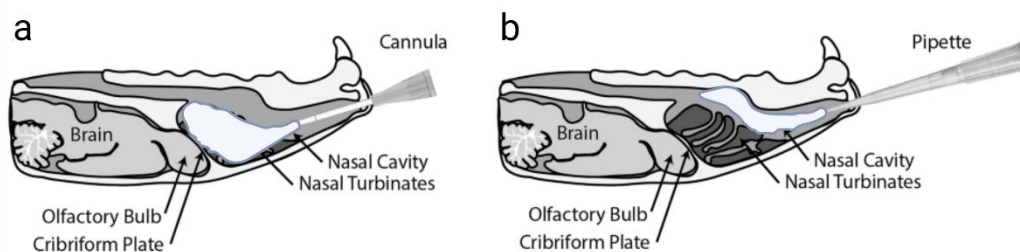


Figure 6.8. Drug placement in the rat nasal cavity affects brain uptake

(a) the use of a cannula more precisely targets the olfactory cleft in the upper nasal cavity than (b) the use of pipette tips, adapted from Morin et al. (2025).

Every effort was made in the initial biomaterial design to increase the chances of formoterol reaching the brain upon release from Form-PEG-PLGA after intranasal administration. We used a thermo-responsive hydrogel to allow for a smooth application at room temperature and a rapid transformation to a solid gel at body temperature, thereby minimising the risk of the animal swallowing the formulation or it being removed by mucociliary clearance (Agrawal et al., 2020). The use of a chitosan-based hydrogel further improved this design as it has mucoadhesive properties to prolong the time that the gel can stay in place and allow for nanoparticle absorption (Rassu et al., 2016). A pleasant scent was also introduced (apple scent) to the recovery cage to encourage animals to sniff upon waking, activating olfactory neurons to promote drug transport from the nasal cavity to the brain (Gobbo et al., 2012). Nanoparticles with a diameter of ~ 100 nm and surface low molecular weight PEG move easily through the mucus and across the nasal epithelium (Lai et al., 2009). The density of this PEG coating may impact motility but consensus in the literature is unclear as some articles report that high density PEG coatings ($>50\%$) are more favourable (Wang et al., 2008) whereas others claim that surface PEG densities as low as 16% are optimal for permeation of the mucosa and olfactory epithelium (Li et al., 2019). Based on the PEG block-copolymer used in this study as opposed to PEGylation, we would estimate surface PEG of our nanoparticles to be between 18 and 25% but further clarification of this is required using nuclear magnetic resonance or Fourier transform infrared spectroscopy techniques that are commonly used to investigate nanoparticle surface chemistry.

Drug delivery to the brain likely occurs through a combination of direct brain targeting and some drug entering local blood capillaries where it crosses the BBB and enters the brain. During administration, animals may move on rare occasions which can give rise to minor bleeding. In these incidences, the nanomedicine may have access to the general circulation. While polymeric nanoparticles have been shown to reach the brain via the trigeminal

nerve, it is likely that a combination of paracellular and perineural transport takes place. Furthermore, drug release from these nanoparticles is likely to be gradual as the nanoparticle is trafficked to the brain. Therefore, an analogy of a nanoparticle being an amazon van delivering a package (drug) to your door (target brain region), may not be completely accurate. A more appropriate analogy is of a truck transporting gravel to a building site: some of the load may spill onto the road and the scattered gravel may still roll along or be picked up by the wheels of passing cars to reach its destination, however, there may be detours on the way and a small amount of that initial cargo not reach its intended destination at all.

6.5.7. Future directions

These experiments set out to determine whether intranasal administration of Form-PEG-PLGA could reduce neuroinflammation in brain structures relevant to PD. While the systemic LPS model is widely used to study CNS immune signalling, it does not accurately represent the region-specific neuroinflammation observed in neurodegenerative diseases. It is necessary to measure brain concentrations of formoterol and compare these to circulating drug concentrations to confirm these effects.

None the less, these results provide further rationale for advancing to preclinical testing of intranasally administered Form-PEG-PLGA in an LPS inflammatory rat model of PD.

Chapter 7: Anti-inflammatory and neuroprotective effects of intranasal Form-PEG-PLGA in the LPS inflammatory rat model of Parkinson's disease

7.1. Introduction

Neuroinflammation is a key hallmark of PD pathology. Elevated serum and CSF levels of pro-inflammatory cytokines have been reported in PD patients and post-mortem examination of PD brains revealed extensive microglial reactivity in the substantia nigra (Hirsch & Hunot, 2009; McGeer et al., 1988). This is supported by a wealth of preclinical *in vitro* and *in vivo* evidence (Tansey & Goldberg, 2010).

Noradrenaline acts as an endogenous CNS immunomodulator through activation of β_2 ARs expressed by microglia and its depletion is intrinsically linked to PD-related neuroinflammation and pathology (Feinstein et al., 2002; Heneka et al., 2003). The locus coeruleus, a small brainstem nucleus, is the principal noradrenergic hub in the CNS and has a widespread projection network throughout the brain including to the substantia nigra and striatum (Paredes-Rodriguez et al., 2020). In several canonical post-mortem studies, substantial volumetric loss of the locus coeruleus was observed in PD brains (Bertrand et al., 1997; Chan-Palay & Asan, 1989; German et al., 1992; Hoogendijk et al., 1995; McMillan et al., 2011). Furthermore, both degeneration and the accumulation of Lewy bodies occurred to a higher extent in the locus coeruleus than the substantia nigra (Baloyannis et al., 2006; Zarow et al., 2003). Based on extensive histopathological analysis, locus coeruleus pathology is suggested to precede degeneration in the substantia nigra and striatum, correlating to prodromal PD symptoms like autonomic dysregulation and sleep disturbances; functions which are controlled by the locus coeruleus (Braak & Del Tredici, 2016; Braak et al., 2003; Braak et al., 2004). This was more recently corroborated by *in vivo* imaging studies that show a progressive reduction in locus coeruleus signal in the brains of patients with both idiopathic and familial forms of PD, which correlated to the development of PD-related depression (Castellanos et al., 2015; Sasaki et al., 2006; Schwarz et al., 2017; Wang et al., 2018).

Preclinical evidence also supports a role of locus coeruleus-mediated noradrenaline signalling in the pathology of PD. *PINK1*, *LRRK* and *PRKIN* knockout mouse models display non-motor symptoms of PD as well as a reduction in noradrenergic neurons and an accumulation of α -synuclein in the locus coeruleus (Cullen et al., 2018; Giaime et al., 2017; Von Coelln et al., 2004). In non-human primates, a bilateral 6-OHDA locus coeruleus lesion significantly exacerbated MPTP-mediated dopaminergic cell death in the substantia nigra, whereas animals that received a sham locus coeruleus lesion recovered from MPTP toxicity (Mavridis et al., 1991). The opposite is also true, where injection of DSP4, a neurotoxin that

selectively acts upon noradrenergic neurons, potentiates motor symptoms and substantia nigra dopamine depletion in rats previously lesioned with 6-OHDA or LPS (Jiang et al., 2015; Song et al., 2019b; Srinivasan & Schmidt, 2003; Torrente et al., 2023). Furthermore, knockout and pharmacological inhibition of the noradrenaline transporter (NET) and the resulting elevated synaptic levels of noradrenaline protected against MPTP toxicity in the dopaminergic system (Rommelfanger et al., 2004).

Noradrenergic input to the substantia nigra is vital to maintain dopaminergic neuronal integrity and has been linked to altered microglial activation. af Bjerkén et al. (2019) showed that DSP4 injection in non-lesioned healthy rats impaired motor function in the rotarod test 3 and 6 months after administration and the number of TH⁺ neurons in the substantia nigra was significantly reduced 6 months but not 3 months post-injection. IBA1⁺ microglia in the substantia nigra of DSP4 treated animals also displayed a distinct morphology, characteristic of a reactive state with large cell bodies and short processes (af Bjerkén et al., 2019). Furthermore, DSP4 administration in mice prior to a systemic LPS challenge showed that noradrenaline depletion exacerbated microglial reactivity and sequential dopaminergic cell death in the substantia nigra. The authors also reported that animals injected with DSP4 and LPS showed early signs of motor impairments and exhibited non-motor symptoms when compared to LPS alone (Song et al., 2019b). Taken together, these studies highlight the detrimental effects of reduced noradrenaline input to the substantia nigra and support the Braak theory that locus coeruleus degeneration occurs early in PD onset. Furthermore, prolonged noradrenaline depletion in the substantia nigra promotes nigro-striatal degeneration particularly in response to inflammatory triggers through suppressed anti-inflammatory signalling.

Several lines of evidence suggest that targeting the noradrenaline system can have therapeutic effects in PD. In a landmark retrospective study of over 4 million people, Mittal et al. (2017) reported that long-term use of the β_2 AR agonist salbutamol is associated with a decreased risk of developing PD. The converse is also true, where long-term use of 'beta-blockers' or β_2 AR antagonist propranolol was associated with an increased risk of PD. The authors also reported that β_2 AR agonist clenbuterol significantly reduced the expression of the α -synuclein encoding *Snc*a gene in wildtype mice (10 mg/kg; i.p.) and human iPSC-derived neuronal cultures carrying the *SNCA* triplication (20 μ M). In addition, clenbuterol protected against dopaminergic cell loss in the substantia nigra of mice administered MPTP (Mittal et al., 2017).

Our research group and others have demonstrated the efficacy of selective β_2 AR agonists in a variety of rodent PD models. Daily infusions of salmeterol (0.01 mg/kg) in mice for two weeks beginning two days prior to MPTP (15 mg/kg; s.c. for 6 days) protected against TH⁺ dopaminergic cell loss in the substantia nigra and rescued both motor function and substantia nigra TH⁺ cell integrity three months after a single LPS challenge (5 mg/kg; i.p.) in mice (Qian et al., 2011). Clenbuterol treatment reduced pro-inflammatory cytokine

expression in mice overexpressing wild-type α -synuclein (10 mg/kg; i.p. daily for 4 weeks) and when administered as a single dose prior to intra-striatal IL-1 β injection (0.5 mg/kg; i.p.) (Griffin et al., 2018; Torrente et al., 2023). Both clenbuterol and formoterol (0.1 mg/kg; i.p.) rescued motor impairments, suppressed microglial reactivity in the substantia nigra, prevented depletion of midbrain dopamine and protected against TH⁺ dopaminergic cell loss in both the substantia nigra and the striatum when administered for 7 days after intranigral LPS challenge (O'Neill et al., 2020).

CuraSen, a biopharmaceutical company based in California has been established based on the above evidence illustrating the therapeutic potential of targeting the noradrenaline system and has several therapeutics undergoing clinical trials for the treatment of neurological disorders. Of these experimental therapies, CuraCN, a fixed-dose combination oral capsule of clenbuterol and nadolol, and CuraXN, a novel brain penetrant selective β_2 AR agonist plus nadolol (also delivered orally), successfully improved cognition and mood in patients suffering from PD with cognitive impairment and patients with MCI or mild dementia due to PD or AD, respectively (Clinical trial ID: NCT04739423, NCT05104463). Notably, nadolol was incorporated into each treatment to block peripheral β_2 AR effects and vital signs were closely monitored in both trials. Mild adverse effects were reported in each instance including anxiety, dizziness, dry mouth and fatigue. In these cases, nadolol likely prevented β_2 AR-mediated tremor and increases in heart rate that have been reported after a single dose of clenbuterol (Lodeweyckx et al., 2024). However, nadolol can cause blurred vision, chest pain, confusion, dizziness and fatigue. Both trials involved a 14-day treatment period so nadolol related side effects may not have been evident during this time.

PD generally manifests later in life and the likelihood of patients having cardiovascular comorbidities increases with age. In a cross-sectional study of medical databases in the US, heart failure was found to be twice as prevalent in elderly PD patients than age matched non-PD individuals (Zesiewicz et al., 2004). The activation of skeletal muscle β_2 ARs can also cause tremor and could transiently worsen such symptoms in PD patients. This puts into question the safety of administering β_2 AR agonists like clenbuterol and formoterol for the treatment of PD.

One such strategy of mitigating these risks is to deliver β_2 AR agonists via nanoparticles. There are many advantages to the use of nanoparticles for CNS drug delivery. In particular, nanoparticles synthesised using the FDA approved polymer PLGA are gaining popularity in the field as they are biocompatible and biodegradable with tuneable physicochemical properties. PLGA nanoparticles can protect drug cargo from tissue binding and metabolism in peripheral tissues, increasing bioavailability in the CNS and improving the overall pharmacokinetic drug profile, especially when surface modifications like PEGylation or targeting ligands are incorporated into nanocarrier design (Maher et al., 2023). Separate investigations have disclosed improved efficacy of drug nanoparticulate systems compared

to standard formulations in preclinical PD rodent models (Casanova et al., 2022; Katila et al., 2022; Monge-Fuentes et al., 2021).

The periphery can be entirely bypassed by delivering therapies intranasally to further enhance CNS drug targeting. Thus far, preclinical studies of PLGA nanoparticle-mediated drug delivery via the intranasal route are limited to the encapsulation of existing PD drugs. Loading of BBB impermeable dopamine into PLGA nanoparticles and intranasal delivery rescued striatal dopamine concentrations and rotational behaviour in response to amphetamine challenge in unilateral 6-OHDA lesioned rats (Tang et al., 2019). Intranasal Levodopa-PLGA nanoparticles improved motor function to a greater extent and for a prolonged period after discontinuation of treatment compared to an intranasal levodopa solution in rats bearing a unilateral 6-OHDA lesion (Gambaryan et al., 2014). Similarly, Arisoy and colleagues reported that the positive effects of levodopa on MPTP induced motor symptoms were prolonged following intranasal administration of drug-loaded PLGA nanoparticles compared to oral and intranasal formulations of free drug in mice (Arisoy et al., 2020).

Rotigotine, which is available as a transdermal patch for PD treatment, was loaded into PEG-PLGA nanoparticles and delivered intranasally, also in the unilateral 6-OHDA rat model of PD. In this pharmacodynamic study, the authors show that this intranasal drug nanosystem conferred higher neuroprotective effects as measured by significant differences in rotational activity after one week and higher TH⁺ dopaminergic cell counts in the substantia nigra after a further two weeks of treatment, compared to standard of care L-DOPA oral formulations (Yan et al., 2018). In mice administered the dopamine receptor antagonist haloperidol daily for two weeks, intranasal delivery of bromocriptine-loaded PLGA nanoparticles (given 30 minutes after each haloperidol injection), significantly improved performance in catalepsy, akinesia, mobility and grip strength tests compared to animals that received intranasal control or oral bromocriptine. These findings were also accompanied by reduced levels of neuroinflammatory and oxidative stress markers in brain tissue (Cong et al., 2025). While several of these studies have demonstrated brain uptake of drug via the intranasal route, the incorporation of nanoparticles into in-situ hydrogels can further promote brain uptake as these formulations adhere to the nasal mucosa, preventing swallowing or inhalation of drug. This strategy improved efficacy in models of AD, epilepsy and stroke where drug bearing non-PLGA polymeric or lipid nanoparticles were administered intranasally in thermo-gels (Abou-Taleb & El-Ganainy, 2023; Wavikar et al., 2017; Zhang et al., 2023b).

Evidence suggests that combining the intranasal route with nanoparticle delivery systems may be superior for delivering anti-inflammatory therapies like the β_2 AR agonist formoterol to the brain for the treatment of PD and other neurodegenerative diseases.

7.2. Aims

The aim of this study was to determine if intranasal delivery of the β_2 AR agonist formoterol-loaded PEG-PLGA nanoparticle encapsulated in a mucoadhesive, thermosensitive hydrogel can protect against the microglial reactivity, dopaminergic cell loss and motor impairments induced by stereotactic delivery of LPS into the rat substantia nigra. From herein, all Form-PEG-PLGA formulations were incorporated into hydrogel to facilitate intranasal delivery but are referred to as Form-PEG-PLGA for simplicity.

7.3. Experimental design

30 4-month-old Wistar rats (14 male, 16 female) underwent baseline behavioural testing in the stepping, cylinder and rotarod tests (see methods section 2.4.6-2.4.8) before being randomly assigned to treatment groups (equal males and females in each group). Rats were administered vehicle (unloaded nanoparticles intranasal in $\sim 30\mu\text{l}$ hydrogel) or Form-PEG-PLGA (0.1 mg/kg; intranasal in $\sim 30\mu\text{l}$ hydrogel) once daily for 3 days (see methods section 2.4.2). On day 4 all animals underwent stereotactic surgery and received a unilateral 2 μl injection of vehicle (0.89% sterile saline) or LPS (5 $\mu\text{g}/\mu\text{l}$) to the substantia nigra (female co-ordinates from bregma: AP -5.3, ML +2.0, DV -7.5; male co-ordinates from bregma: AP -5.3, ML +2.0, DV -8.5) (see methods section 2.4.5). Animals received another vehicle or Form-PEG-PLGA intranasal dose immediately after surgery and daily for a further 3 days (7 days of dosing in total with surgery on day 4; see **fig. 7.1.**). Two weeks post-lesion the stepping, cylinder and rotarod tests were repeated along with the amphetamine-induced rotations test (see methods section 2.4.9). Four weeks post-surgery rats were euthanised by sodium pentobarbital overdose and transcardial perfusion/fixation (see methods section 2.4.10). Immunohistochemical analysis of IBA1⁺ microglia and TH⁺ neuronal cell bodies in the substantia nigra was carried out to investigate microglial reactivity and neuronal integrity (see methods section 2.4.11-2.4.13).

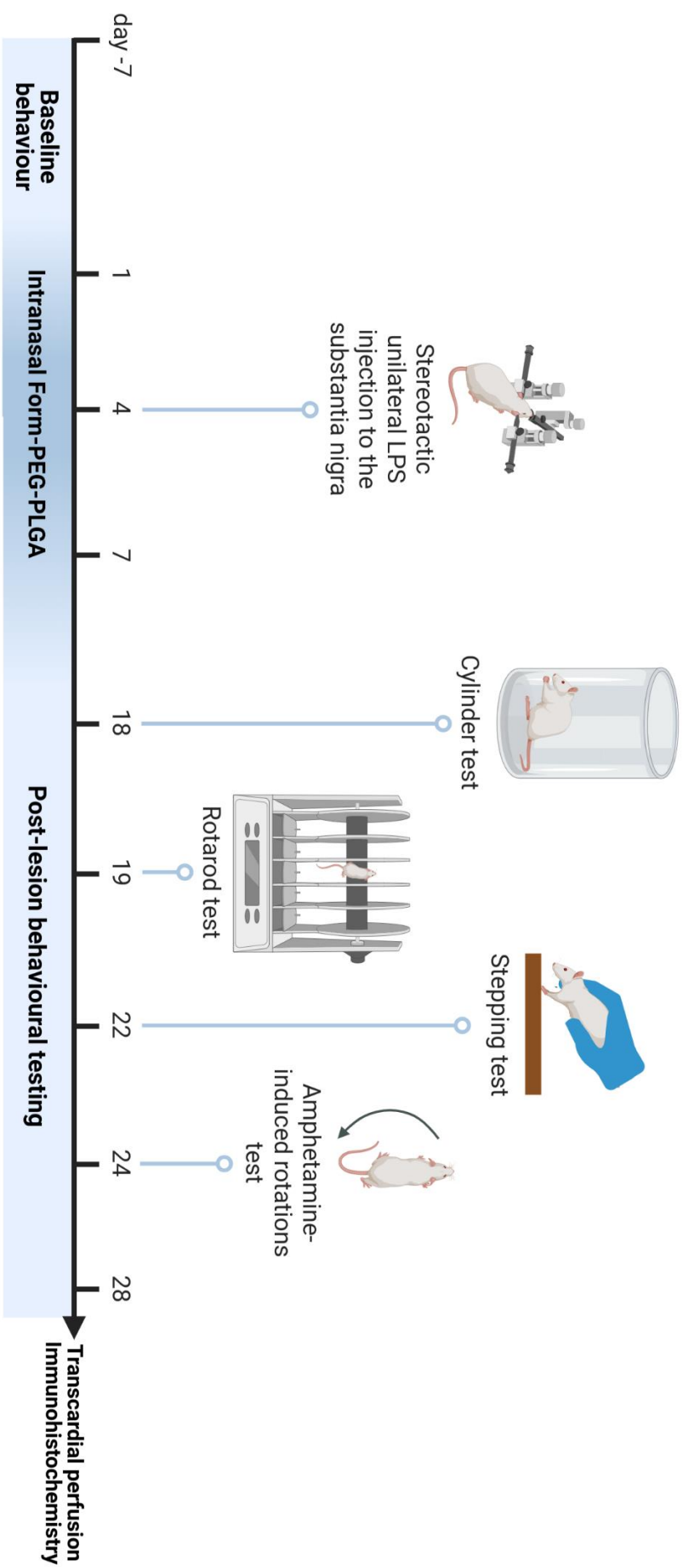


Figure 7.1 Experimental timeline.

7.4. Results

7.4.1. Form-PEG-PLGA attenuates LPS-mediated forelimb asymmetry in the cylinder test

Three-way ANOVA of the total number of contacts made with the wall and floor of the cylinder using both forelimbs at the same time showed no effect of time, LPS or treatment (**Fig. 7.2a**).

Three-way ANOVA of the total number of contacts made with the wall and floor of the cylinder using the forelimb ipsilateral to the LPS lesion showed an interaction between time and LPS [$F_{(1, 52)} = 5.084$, $P < 0.05$] (**Fig. 7.2b**). Tukey's multiple comparisons did not reveal any relevant differences between groups.

Three-way ANOVA of the total number of contacts made with the wall and floor of the cylinder using the forelimb contralateral to the LPS lesion showed an effect of time [$F_{(1, 52)} = 6.687$, $P < 0.05$] (**Fig. 7.2c**). Tukey's multiple comparisons did not reveal any relevant differences between groups.

Three-way ANOVA of contralateral forelimb use as a percentage of ipsilateral forelimb use showed an effect of time [$F_{(1, 52)} = 11.08$, $P < 0.01$], LPS [$F_{(1, 52)} = 7.049$, $P < 0.05$], a time x LPS interaction [$F_{(1, 52)} = 19.72$, $P < 0.0001$] and a time x LPS x treatment interaction [$F_{(1, 52)} = 7.001$, $P < 0.05$] (**Fig. 7.2d**). Tukey's multiple comparisons revealed that intranigral LPS decreased contralateral forelimb use compared to vehicle control ($P < 0.001$) and baseline scores in this group ($P < 0.001$; not shown). The LPS induced decrease in contralateral forelimb use was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control ($P < 0.05$).

Three-way ANOVA of the ratio of contralateral to ipsilateral forelimb use showed an effect of time [$F_{(1, 52)} = 10.32$, $P < 0.01$], LPS [$F_{(1, 52)} = 4.149$, $P < 0.05$], a time x LPS interaction [$F_{(1, 52)} = 17.95$, $P < 0.0001$] and a time x LPS x treatment interaction [$F_{(1, 52)} = 5.081$, $P < 0.05$] (**Fig. 7.2e**). Tukey's multiple comparisons revealed that the ratio of contralateral to ipsilateral forelimb use in animals that were lesioned with LPS was lower than vehicle control ($P < 0.01$) and baseline scores in this group ($P < 0.001$; not shown).

Three-way ANOVA of forelimb asymmetry showed an effect of time [$F_{(1, 52)} = 13.26$, $P < 0.001$], LPS [$F_{(1, 52)} = 11.58$, $P < 0.01$], treatment [$F_{(1, 52)} = 4.547$, $P < 0.05$], a time x LPS interaction [$F_{(1, 52)} = 25.35$, $P < 0.0001$] and a time x LPS x treatment interaction [$F_{(1, 52)} = 8.417$, $P < 0.01$] (**Fig. 7.2f**). Tukey's multiple comparisons revealed that the limb asymmetry index score was more negative in animals that received an intranigral stereotactic injection of LPS compared to vehicle control ($P < 0.0001$) and baseline scores in the same animals ($P < 0.0001$; not shown). The LPS induced negative limb asymmetry index score was rescued by intranasal Form-PEG-PLGA compared to vehicle treated control ($P < 0.01$).

Three-way ANOVA of the total number of contacts made with the wall of the cylinder using both limbs at the same time or either forelimb in isolation revealed no effect of time, LPS or treatment (data not shown).

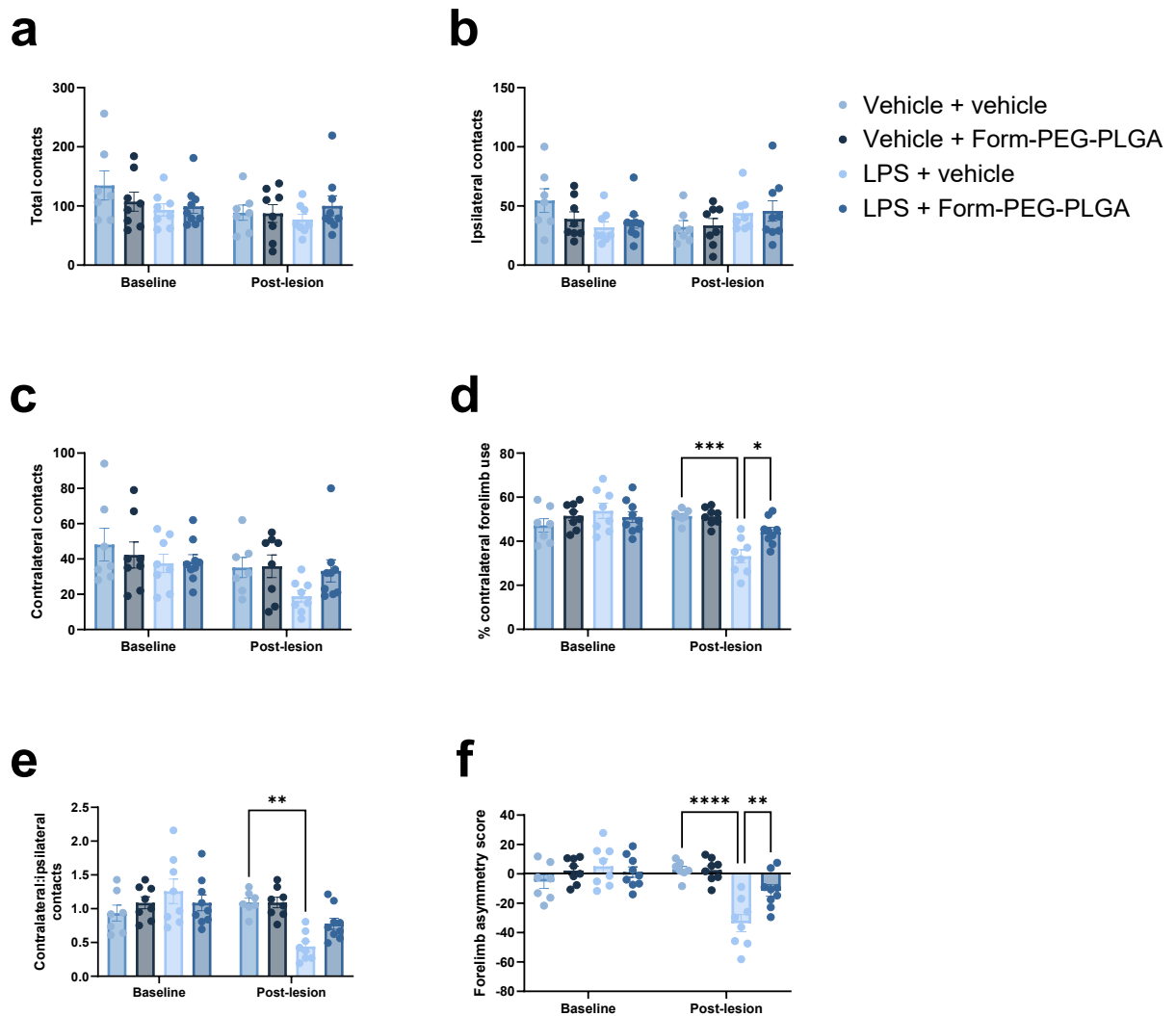


Figure 7.2. Unilateral intranigral LPS induced forelimb asymmetry in the cylinder test is attenuated by intranasal Form-PEG-PLGA.

(a) Total wall and floor contacts made with both forelimbs combined, (b) total wall and floor contacts made with the forelimb ipsilateral to intranigral LPS lesion, (c) total wall and floor contacts made with the forelimb contralateral to intranigral LPS lesion, (d) % contralateral forelimb use, (e) ratio of contralateral to ipsilateral forelimb contacts and (f) forelimb asymmetry index score at baseline and post-intranigral LPS injection (5 $\mu\text{g}/\mu\text{l}$) and intranasal Form-PEG-PLGA (0.1 mg/kg). Data were analysed by three-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=6-9. Significant differences between groups are denoted by * where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Three-way ANOVA of the total number of contacts made with the wall and floor of the cylinder post-lesion using both forelimbs at the same time showed a sex x LPS x treatment interaction [$F_{(1, 22)} = 5.084$, $P < 0.05$] (**Fig. 7.3a**). Tukey's multiple comparisons did not reveal any relevant differences between groups.

Three-way ANOVA of the total number of contacts made with the wall and floor of the cylinder post-lesion using the forelimb ipsilateral to the LPS lesion revealed a significant interaction between sex, LPS and treatment [$F_{(1, 22)} = 17.56$, $P < 0.001$] (**Fig. 7.3b**). Tukey's multiple comparisons revealed a significant difference in ipsilateral forelimb use between males and females that were lesioned with LPS and treated with intranasal Form-PEG-PLGA ($P < 0.05$).

Three-way ANOVA of the total number of contacts made with the wall and floor of the cylinder post-lesion using the forelimb contralateral to the LPS lesion showed an interaction between sex, LPS and treatment [$F_{(1, 22)} = 9.495$, $P < 0.01$] (**Fig. 7.3c**). Tukey's multiple comparisons did not reveal any relevant differences between groups.

Three-way ANOVA of contralateral forelimb use as a percentage of ipsilateral forelimb use post-lesion showed an effect of LPS [$F_{(1, 22)} = 32.78$, $P < 0.0001$], treatment [$F_{(1, 22)} = 7.649$, $P < 0.05$] and an LPS x treatment interaction [$F_{(1, 22)} = 7.3$, $P < 0.05$] (**Fig. 7.3d**). Tukey's multiple comparisons revealed that intranigral LPS decreased contralateral forelimb use compared to vehicle control in males ($P < 0.05$) and females ($P < 0.01$). The LPS induced decrease in contralateral forelimb use was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control in males ($P < 0.05$) but not females.

Three-way ANOVA of the ratio of contralateral to ipsilateral forelimb use post-lesion showed an effect of LPS [$F_{(1, 22)} = 38.03$, $P < 0.0001$], treatment [$F_{(1, 22)} = 6.123$, $P < 0.05$] and an LPS x treatment interaction [$F_{(1, 22)} = 5.522$, $P < 0.05$] (**Fig. 7.3e**). Tukey's multiple comparisons revealed that the ratio of contralateral to ipsilateral forelimb use in animals that were lesioned with LPS was lower when compared to vehicle control in males ($P < 0.05$) and females ($P < 0.01$). The LPS induced decrease in the ratio of contralateral to ipsilateral forelimb use was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control in males ($P < 0.05$) but not females.

Three-way ANOVA of forelimb asymmetry post-lesion showed an effect of LPS [$F_{(1, 22)} = 32.78$, $P < 0.0001$], treatment [$F_{(1, 22)} = 7.649$, $P < 0.05$] and an LPS x treatment interaction [$F_{(1, 22)} = 7.3$, $P < 0.05$] (**Fig. 7.3f**). Tukey's multiple comparisons revealed that the limb asymmetry index score was more negative in animals that received an intranigral stereotactic injection of LPS compared to vehicle control in males ($P < 0.05$) and females ($P < 0.01$). The LPS induced negative limb asymmetry index score was rescued by intranasal Form-PEG-PLGA compared to vehicle treated control in males ($P < 0.05$) but not females.

Three-way ANOVA of the total number of contacts made with the wall of the cylinder using both limbs at the same time, the ipsilateral forelimb and the contralateral forelimb post-lesion showed a sex x LPS x treatment interaction [total: $F_{(1, 22)} = 12.27$, $P < 0.01$; ipsilateral:

$F_{(1,22)} = 13.86$, $P < 0.01$; contralateral: $F_{(1,22)} = 10.27$, $P < 0.01$]. Tukey's multiple comparisons did not identify any relevant differences between groups (data not shown).

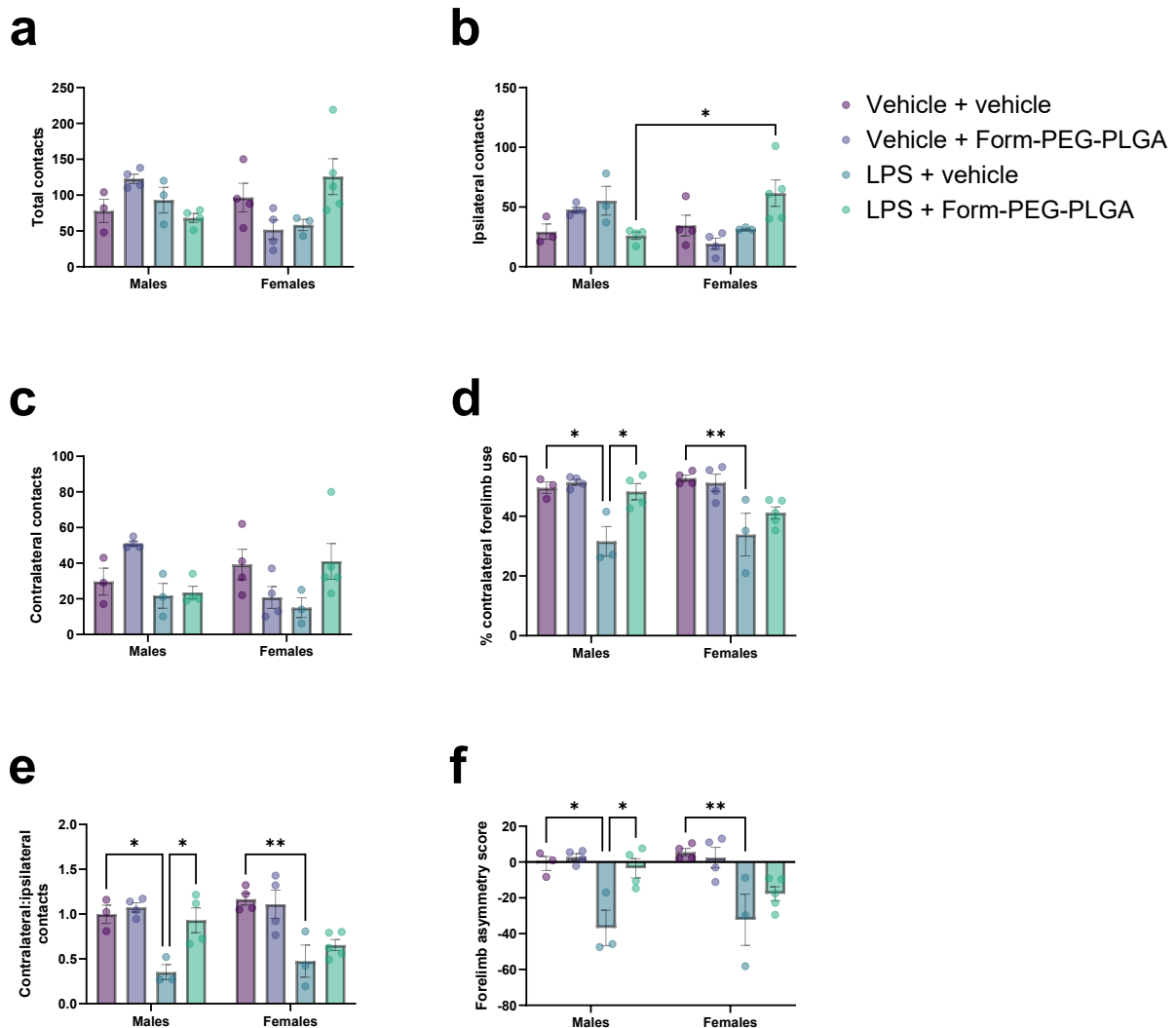


Figure 7.3. Sex differences in forelimb asymmetry in the cylinder test following unilateral intranigral LPS lesion.

(a) Total wall and floor contacts made with both forelimbs combined, (b) total wall and floor contacts made with the forelimb ipsilateral to intranigral LPS lesion, (c) total wall and floor contacts made with the forelimb contralateral to intranigral LPS lesion, (d) % contralateral forelimb use, (e) ratio of contralateral to ipsilateral forelimb contacts and (f) forelimb asymmetry index score in males and females post-intranigral LPS injection ($5 \mu\text{g}/\mu\text{l}$) and intranasal Form-PEG-PLGA (0.1 mg/kg). Data were analysed by three-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where $N=3-5$. Significant differences between groups are denoted by * where $* P < 0.05$ and $** P < 0.01$.

7.4.2. LPS did not induce forelimb akinesia in the stepping test

Three-way ANOVA of the total number of steps using either forelimb in the forward and backwards directions combined showed an interaction between LPS and treatment [$F_{(1, 52)} = 6.117$, $P < 0.05$] (**Fig. 7.4a**). Tukey's multiple comparisons did not reveal any relevant differences between groups.

Three-way ANOVA of the total number of steps using the forelimb ipsilateral to the lesion in the forward and backwards directions combined showed no effect of time, LPS or treatment (**Fig. 7.4b**).

Three-way ANOVA of the total number of steps using the forelimb contralateral to the lesion in the forward and backwards directions combined showed an interaction between LPS and treatment [$F_{(1, 52)} = 6.442$, $P < 0.05$] (**Fig. 7.4c**). Tukey's multiple comparisons did not reveal any relevant differences between groups.

Three-way ANOVA of the number of contralateral steps expressed as a percentage of ipsilateral steps showed no effect of time, LPS or treatment (**Fig. 7.4d**).

Three-way ANOVA of the ratio of contralateral to ipsilateral steps showed no effect of time, LPS or treatment (**Fig. 7.4e**).

Three-way ANOVA of the forelimb asymmetry index score showed no effect of time, LPS or treatment (**Fig. 7.4f**).

Three-way ANOVA of the total number of forward steps and forward steps made using the limb contralateral to the lesion showed an LPS x treatment interaction [total: $F_{(1, 52)} = 6.239$, $P < 0.05$; contralateral: $F_{(1, 52)} = 6.776$, $P < 0.05$]. Tukey's multiple comparisons did not reveal any relevant differences between groups. Three-way ANOVA of forward steps made using the forelimb ipsilateral to the lesion showed no effect of time, LPS or treatment (data not shown).

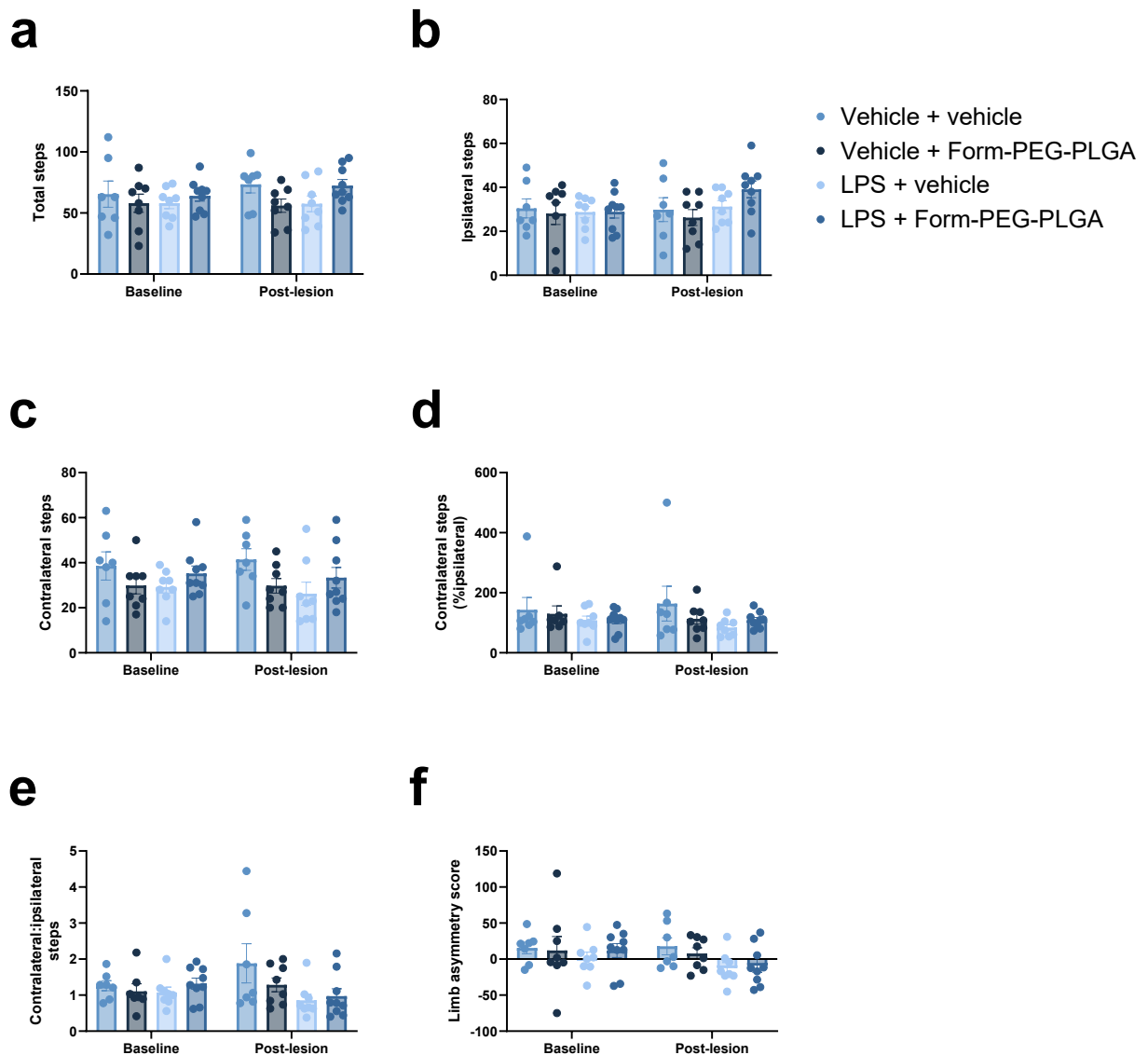


Figure 7.4. Unilateral intranigral LPS did not induce forelimb akinesia in the stepping test.

(a) Total steps, (b) total steps with limbs ipsilateral to intranigral LPS lesion, (c) total steps with limbs contralateral to intranigral LPS lesion, (d) contralateral steps as a % of ipsilateral steps, (e) ratio of contralateral to ipsilateral steps and (f) limb use asymmetry score at baseline and post-intranigral LPS injection (5 $\mu\text{g}/\mu\text{l}$) and intranasal Form-PEG-PLGA (0.1 mg/kg). Data were analysed by three-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=6-9.

Three-way ANOVA of the total number of steps using either forelimb in the forward and backwards directions combined by males or females post-lesion showed an interaction between LPS and treatment [$F_{(1, 22)} = 6.178$, $P < 0.05$] (**Fig. 7.5a**). Tukey's multiple comparisons did not reveal any relevant differences between groups.

Three-way ANOVA of the total number of steps using the forelimb ipsilateral to the lesion in the forward and backwards directions combined post-lesion showed no effect of sex, LPS or treatment (**Fig. 7.5b**).

Three-way ANOVA of the total number of steps using the forelimb contralateral to the lesion in the forward and backwards directions combined post-lesion showed no effect of sex, LPS or treatment (**Fig. 7.5c**).

Three-way ANOVA of the number of contralateral steps expressed as a percentage of ipsilateral steps post-lesion showed no effect of sex, LPS or treatment (**Fig. 7.5d**).

Three-way ANOVA of the ratio of contralateral to ipsilateral steps post lesion showed no effect of sex, LPS or treatment (**Fig. 7.5e**).

Three-way ANOVA of the forelimb asymmetry scores in males or females post-lesion showed an effect of LPS [$F_{(1, 22)} = 4.84$, $P < 0.05$] (**Fig. 7.5f**). Tukey's multiple comparisons did not reveal any relevant differences between groups.

Three-way ANOVA of forward steps made using the limb contralateral to the lesion by males or females post-lesion showed an LPS x treatment interaction [$F_{(1, 52)} = 4.323$, $P < 0.05$]. Tukey's multiple comparisons did not reveal any relevant differences between groups. Three-way ANOVA of the total number of forward steps when both forelimbs were combined or forward steps made using the forelimb ipsilateral to the lesion showed no effect of sex, LPS or treatment (data not shown).

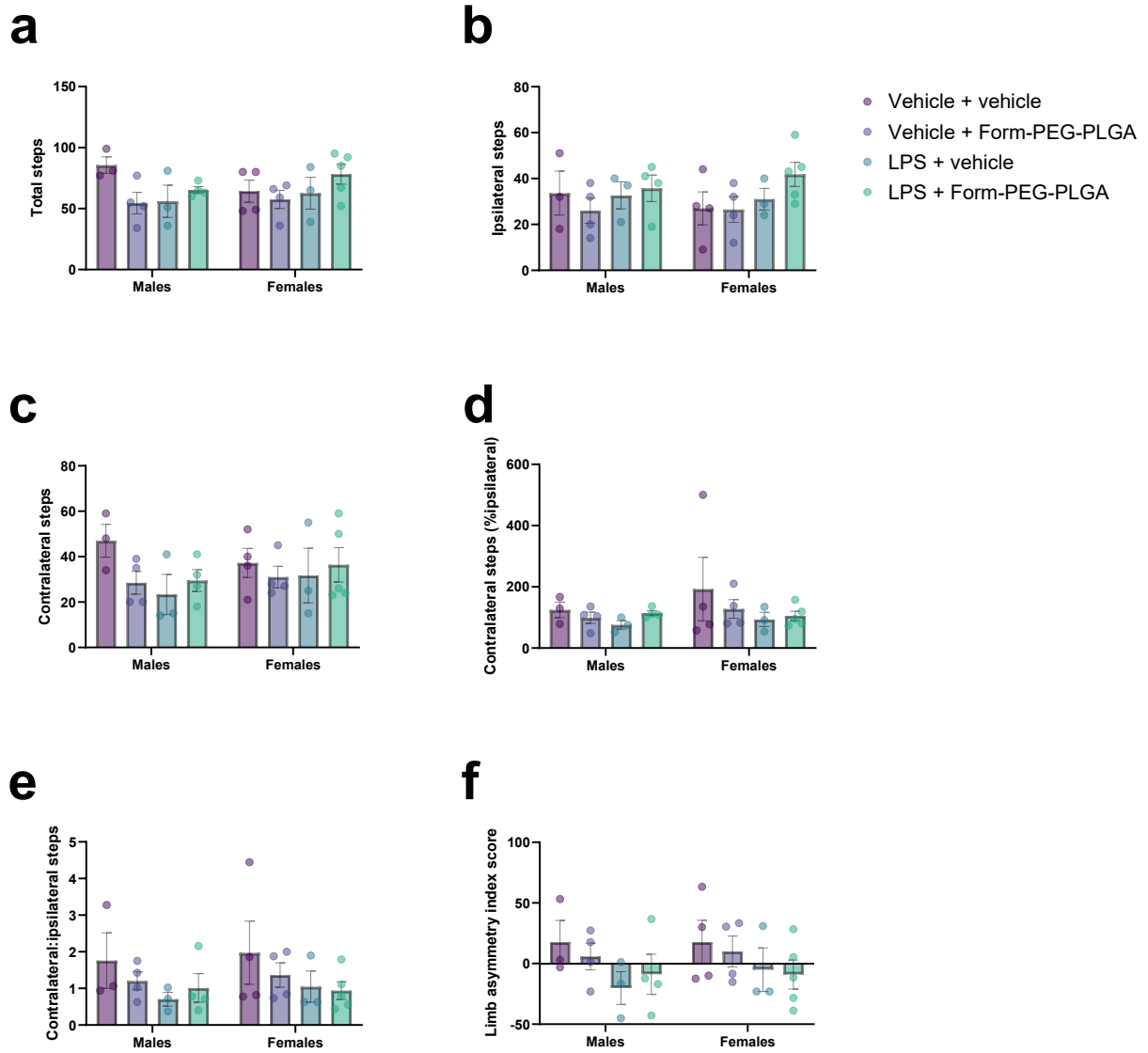


Figure 7.5. Sex did not affect performance in the stepping test following unilateral intranigral LPS lesion.

(a) Total steps, (b) total steps with limbs ipsilateral to intranigral LPS lesion, (c) total steps with limbs contralateral to intranigral LPS lesion, (d) contralateral steps as a % of ipsilateral steps, (e) ratio of contralateral to ipsilateral steps and (f) limb use asymmetry score in males and females post-intranigral LPS injection ($5 \mu\text{g}/\mu\text{l}$) and intranasal Form-PEG-PLGA (0.1 mg/kg). Data were analysed by three-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where $N=3-5$.

7.4.3. LPS did not affect latency to fall in the rotarod test

Three-way ANOVA of the latency to fall from the rotarod showed an effect of time [$F_{(1, 48)} = 7.068$, $P < 0.05$] (**Fig. 7.6**). Tukey's multiple comparisons did not reveal any relevant differences between groups. Three-way ANOVA of latency to fall showed no sex differences on rotarod performance post-unilateral intranigral LPS lesion (**Fig. 7.7**).

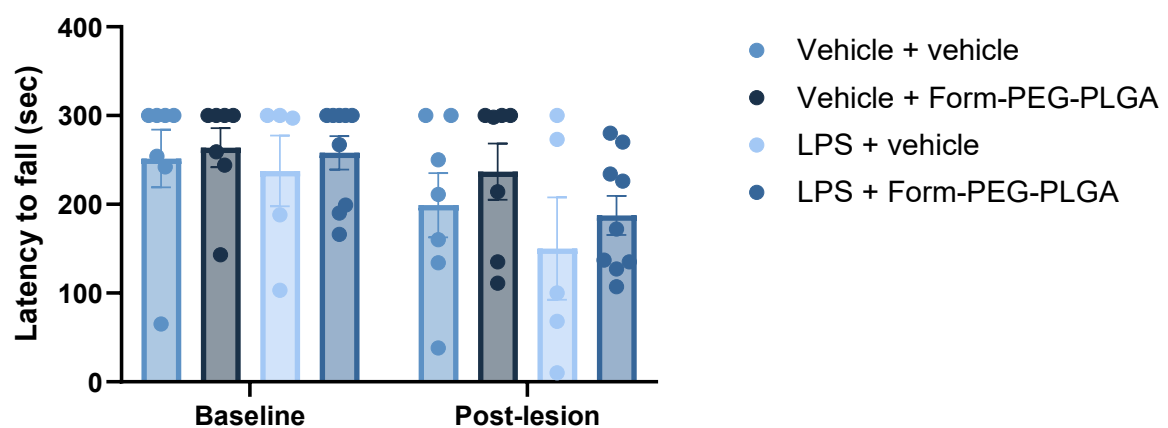


Figure 7.6. Unilateral intranigral LPS did not affect rotarod performance.

Latency to fall from rotarod was unaffected by intranigral LPS lesion ($5 \mu\text{g}/\mu\text{l}$). Data were analysed by three-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where $N=6-9$.

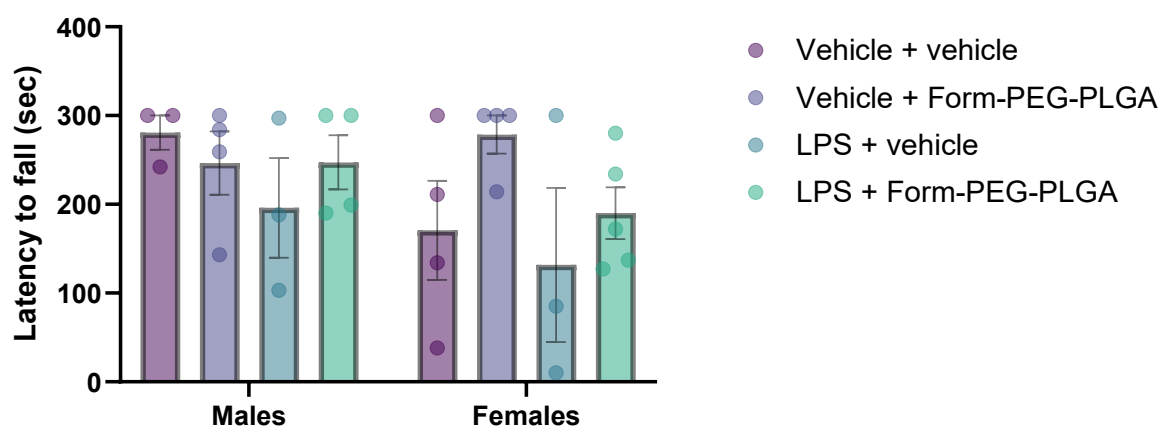


Figure 7.7. Sex did not affect performance in the rotarod test following unilateral intranigral LPS lesion.

Latency to fall from rotarod was unaffected by intranigral LPS lesion ($5 \mu\text{g}/\mu\text{l}$) in males and females. Data were analysed by three-way ANOVA and are expressed as the mean $\pm$ SEM, where $N=3-5$.

7.4.4. Form-PEG-PLGA attenuates LPS-mediated ipsilateral turning behaviour in the D-amphetamine induced rotations test

Two-way ANOVA of the total number of rotations in the D-amphetamine-induced rotations test showed an effect of LPS [$F_{(1, 26)} = 23.72$, $P < 0.0001$] and an LPS x treatment interaction [$F_{(1, 26)} = 5.47$, $P < 0.05$] (**Fig. 7.8a**). Tukey's multiple comparisons revealed that the total number of rotations made by animals that were lesioned with LPS was higher compared to vehicle control ($P < 0.001$).

Two-way ANOVA of the number of contralateral rotations showed no effect of LPS or treatment (**Fig. 7.8b**).

Two-way ANOVA of the number of ipsilateral rotations in the D-amphetamine-induced rotations test revealed an effect of LPS [$F_{(1, 26)} = 28.04$, $P < 0.0001$] and an LPS x treatment interaction [$F_{(1, 26)} = 7.491$, $P < 0.05$] (**Fig. 7.8c**). Tukey's multiple comparisons revealed that animals injected with intranigral LPS had a higher number of ipsilateral rotations compared to vehicle control ($P < 0.0001$). The LPS induced increase in ipsilateral rotations was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control ($P < 0.05$).

Two-way ANOVA of the ratio of ipsilateral to contralateral rotations in the D-amphetamine-induced rotations test revealed an effect of LPS [$F_{(1, 26)} = 6.376$, $P < 0.05$] and an LPS x treatment interaction [$F_{(1, 26)} = 9.023$, $P < 0.01$] (**Fig. 7.8d**). Tukey's multiple comparisons revealed that animals lesioned with LPS had a higher ratio of ipsilateral to contralateral rotations compared to vehicle control ($P < 0.01$). The LPS induced increase in the ratio of ipsilateral to contralateral rotations was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control ($P < 0.05$).

Two-way ANOVA of the net ipsilateral rotations in the D-amphetamine-induced rotations test revealed an effect of LPS [$F_{(1, 26)} = 20.97$, $P < 0.001$] and an LPS x treatment interaction [$F_{(1, 26)} = 6.29$, $P < 0.05$] (**Fig. 7.8e**). Tukey's multiple comparisons revealed increased net ipsilateral rotations following intranigral LPS compared to vehicle control ($P < 0.001$). The LPS induced increase in net ipsilateral rotations was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control ($P < 0.05$).

Two-way ANOVA of the number of ipsilateral rotations per minute in the D-amphetamine-induced rotations test showed an effect of LPS [$F_{(1, 26)} = 20.97$, $P < 0.001$] and an LPS x treatment interaction [$F_{(1, 26)} = 6.29$, $P < 0.05$] (**Fig. 7.8f**). Tukey's multiple comparisons revealed that the number of ipsilateral turns per minute were higher following intranigral LPS to vehicle control ($P < 0.001$). The LPS induced increase in the rate of ipsilateral rotations was significantly attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control ($P < 0.05$).

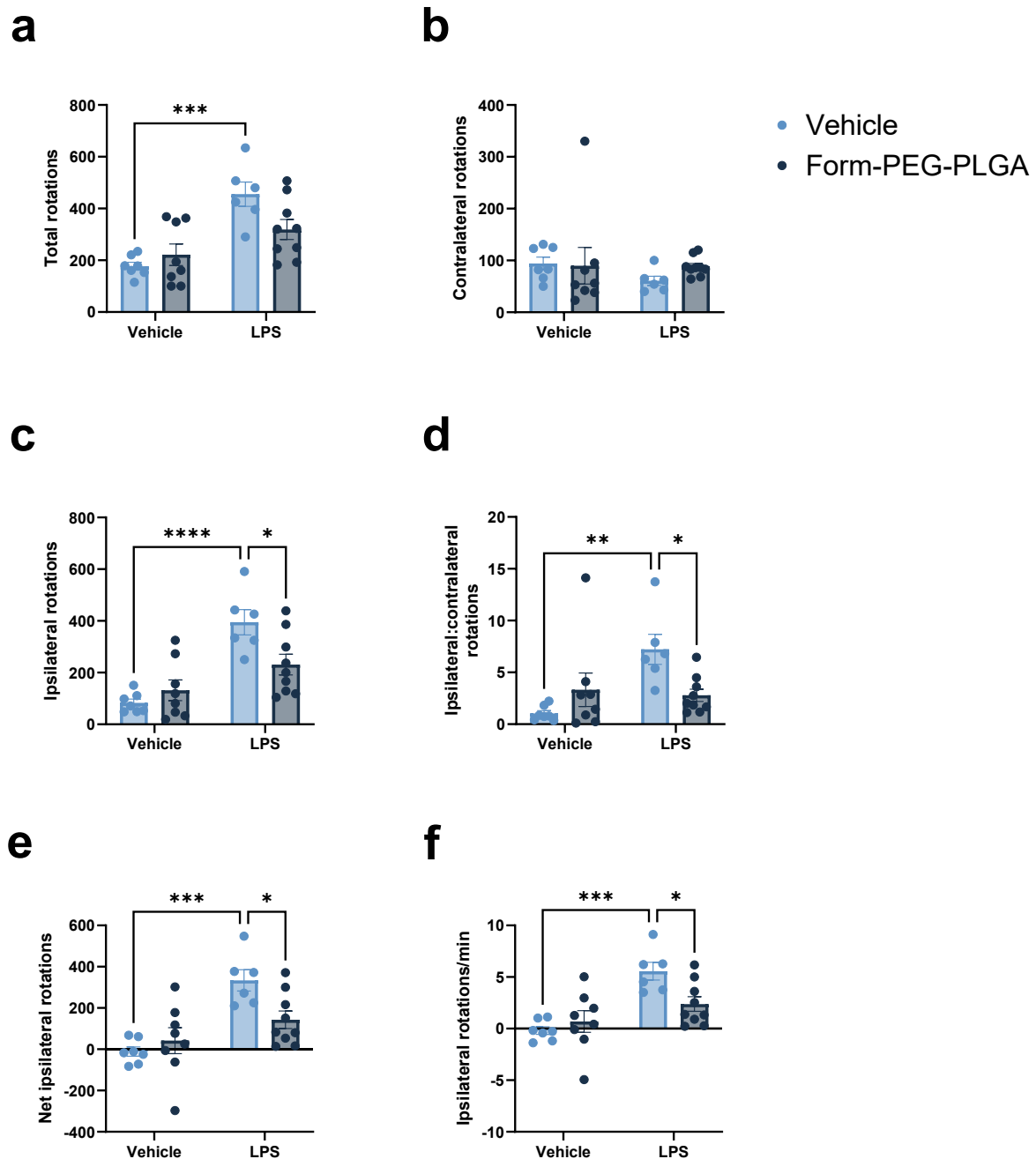


Figure 7.8. Unilateral intranigral LPS mediated increases in ipsilateral rotations in the D-amphetamine rotations test is attenuated by intranasal Form-PEG-PLGA.

(a) Total rotations, (b) rotations contralateral to intranigral LPS lesion, (c) rotations ipsilateral to intranigral LPS lesion, (d) ratio of ipsilateral to contralateral rotations, (e) net ipsilateral rotations and (f) ipsilateral rotations per minute in the D-amphetamine induced rotations test following intranigral LPS injection (5 $\mu\text{g}/\mu\text{l}$) and intranasal Form-PEG-PLGA (0.1 mg/kg). Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=6-9. Significant differences between groups are denoted by * where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Three-way ANOVA of the total number of rotations in the D-amphetamine-induced rotations test when males and females were separated showed an effect of LPS [$F_{(1, 22)} = 22.28$, $P < 0.001$] and an LPS x treatment interaction [$F_{(1, 22)} = 5.404$, $P < 0.05$] (**Fig. 7.9a**). Tukey's multiple comparisons revealed that females, but not males, injected with intranigral LPS had a higher total number of rotations compared to vehicle control ($P < 0.0001$).

Three-way ANOVA of the number of contralateral rotations showed no effect of LPS or treatment when males and females were separated (**Fig. 7.9b**).

Three-way ANOVA of the number of ipsilateral rotations in the D-amphetamine-induced rotations test when males and females were separated showed an effect of LPS [$F_{(1, 22)} = 24.37$, $P < 0.0001$] and an LPS x treatment interaction [$F_{(1, 26)} = 6.806$, $P < 0.05$] (**Fig. 7.9c**). Tukey's multiple comparisons revealed that animals injected with intranigral LPS had a higher number of ipsilateral rotations compared to vehicle control in males ($P < 0.05$) and females ($P < 0.05$).

Three-way ANOVA of the ratio of ipsilateral to contralateral rotations in the D-amphetamine-induced rotations test when males and females were separated showed an effect of LPS [$F_{(1, 22)} = 6.126$, $P < 0.05$] and an LPS x treatment interaction [$F_{(1, 22)} = 8.902$, $P < 0.01$] (**Fig. 7.9d**). Tukey's multiple comparisons did not reveal any relevant differences between groups.

Three-way ANOVA of the net ipsilateral rotations in the D-amphetamine-induced rotations test when males and females were separated showed an effect of LPS [$F_{(1, 22)} = 18.25$, $P < 0.001$] and an LPS x treatment interaction [$F_{(1, 22)} = 5.689$, $P < 0.05$] (**Fig. 7.9e**). Tukey's multiple comparisons revealed that LPS lesion increased net ipsilateral rotations compared to vehicle control in males ($P < 0.05$) but not females.

Three-way ANOVA of the number of ipsilateral rotations per minute during the D-amphetamine-induced rotations test when males and females were separated showed an effect of LPS [$F_{(1, 22)} = 18.25$, $P < 0.001$] and an LPS x treatment interaction [$F_{(1, 22)} = 5.689$, $P < 0.05$] (**Fig. 7.9f**). Tukey's multiple comparisons revealed a higher rate of ipsilateral rotations per minute following intranigral LPS compared to vehicle control in males ($P < 0.05$) but not females.

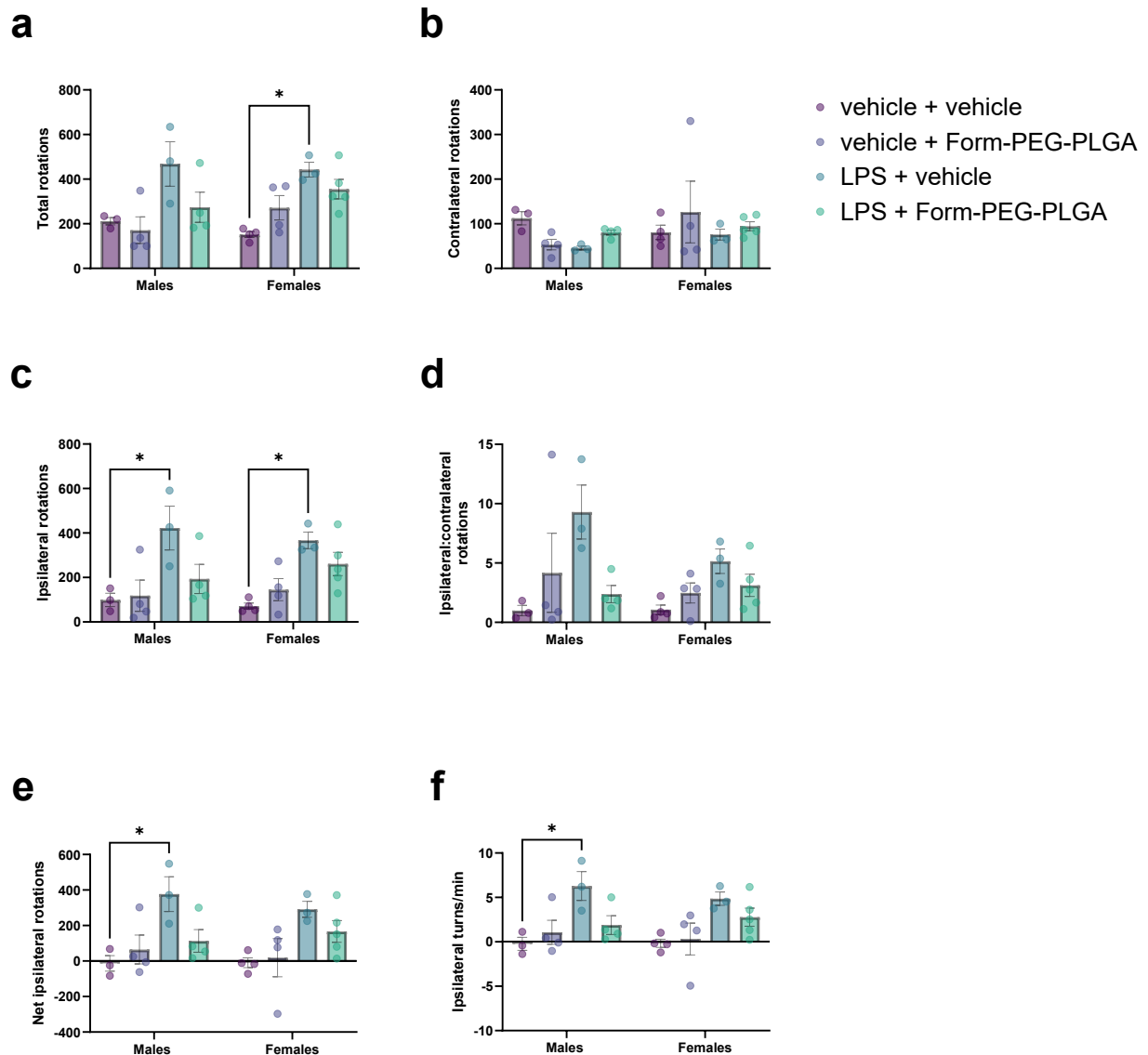


Figure 7.9. Sex differences in rotational behaviour in the D-amphetamine induced rotations test following unilateral intranigral LPS.

(a) total rotations, (b) rotations contralateral to intranigral LPS lesion, (c) rotations ipsilateral to intranigral LPS lesion, (d) ratio of ipsilateral to contralateral rotations, (e) net ipsilateral rotations and (f) ipsilateral rotations per minute in males and females post-intranigral LPS injection (5 $\mu\text{g}/\mu\text{l}$) and intranasal Form-PEG-PLGA (0.1 mg/kg). Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons and are expressed as the mean $\pm$ SEM, where N=3-5. Significant differences between groups are denoted by * where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

7.4.5. LPS induced microgliosis in the substantia nigra is attenuated by Form-PEG-PLGA

Two-way ANOVA of the number of IBA1⁺ cells in the substantia nigra (**Fig. 7.10**) showed an effect of LPS [$F_{(1, 26)} = 70.88$, $P < 0.0001$], treatment [$F_{(1, 26)} = 11.71$, $P < 0.01$] and an LPS x treatment interaction [$F_{(1, 26)} = 18.49$, $P < 0.001$] (**Fig. 7.11a**). Tukey's multiple comparisons revealed an increase in IBA1⁺ cells in the substantia nigra of animals lesioned with LPS compared to vehicle control ($P < 0.0001$). The LPS induced increase in IBA1⁺ cells in the substantia nigra was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control ($P < 0.0001$). No changes in IBA1⁺ cell density was observed in the uninjected hemisphere (**Fig. 12**).

Two-way ANOVA of IBA1⁺ cell area showed an effect of LPS [$F_{(1, 26)} = 114.4$, $P < 0.0001$], treatment [$F_{(1, 26)} = 4.972$, $P < 0.05$] and an LPS x treatment interaction [$F_{(1, 26)} = 4.837$, $P < 0.05$] (**Fig. 7.11b**). Tukey's multiple comparisons revealed an increase in IBA1⁺ cell area in animals lesioned with LPS compared to vehicle control ($P < 0.0001$). The LPS induced increase IBA1⁺ cells in the substantia nigra was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control ($P < 0.05$).

Three-way ANOVA of the number of IBA1⁺ cells the substantia nigra in males or females showed an effect of sex [$F_{(1, 22)} = 7.284$, $P < 0.05$], LPS [$F_{(1, 22)} = 97.19$, $P < 0.0001$], treatment [$F_{(1, 22)} = 17.62$, $P < 0.001$] and an LPS x treatment interaction [$F_{(1, 22)} = 28.4$, $P < 0.0001$] (**Fig. 7.13a**). Tukey's multiple comparisons revealed an increase in IBA1⁺ cells in the substantia nigra of animals lesioned with LPS compared to vehicle control in males ($P < 0.0001$) and females ($P < 0.0001$). The LPS induced increase IBA1⁺ cells in the substantia nigra was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control in males ($P < 0.001$) and females ($P < 0.05$).

Three-way ANOVA of IBA1⁺ cell area in males or females showed an effect of LPS [$F_{(1, 22)} = 213.5$, $P < 0.0001$], treatment [$F_{(1, 22)} = 11.33$, $P < 0.01$] and a sex x LPS [$F_{(1, 22)} = 4.691$, $P < 0.05$], sex x treatment [$F_{(1, 22)} = 6.773$, $P < 0.05$], LPS x treatment [$F_{(1, 22)} = 11.07$, $P < 0.01$] and sex x LPS x treatment [$F_{(1, 22)} = 8.754$, $P < 0.01$] interactions (**Fig. 7.13b**). Tukey's multiple comparisons revealed an increase in IBA1⁺ cells in the substantia nigra of animals lesioned with LPS compared to vehicle control in males ($P < 0.0001$) and females ($P < 0.0001$). The LPS induced increase IBA1⁺ cells in the substantia nigra was attenuated by intranasal Form-PEG-PLGA compared to vehicle treated control in males ($P < 0.001$) but not females.

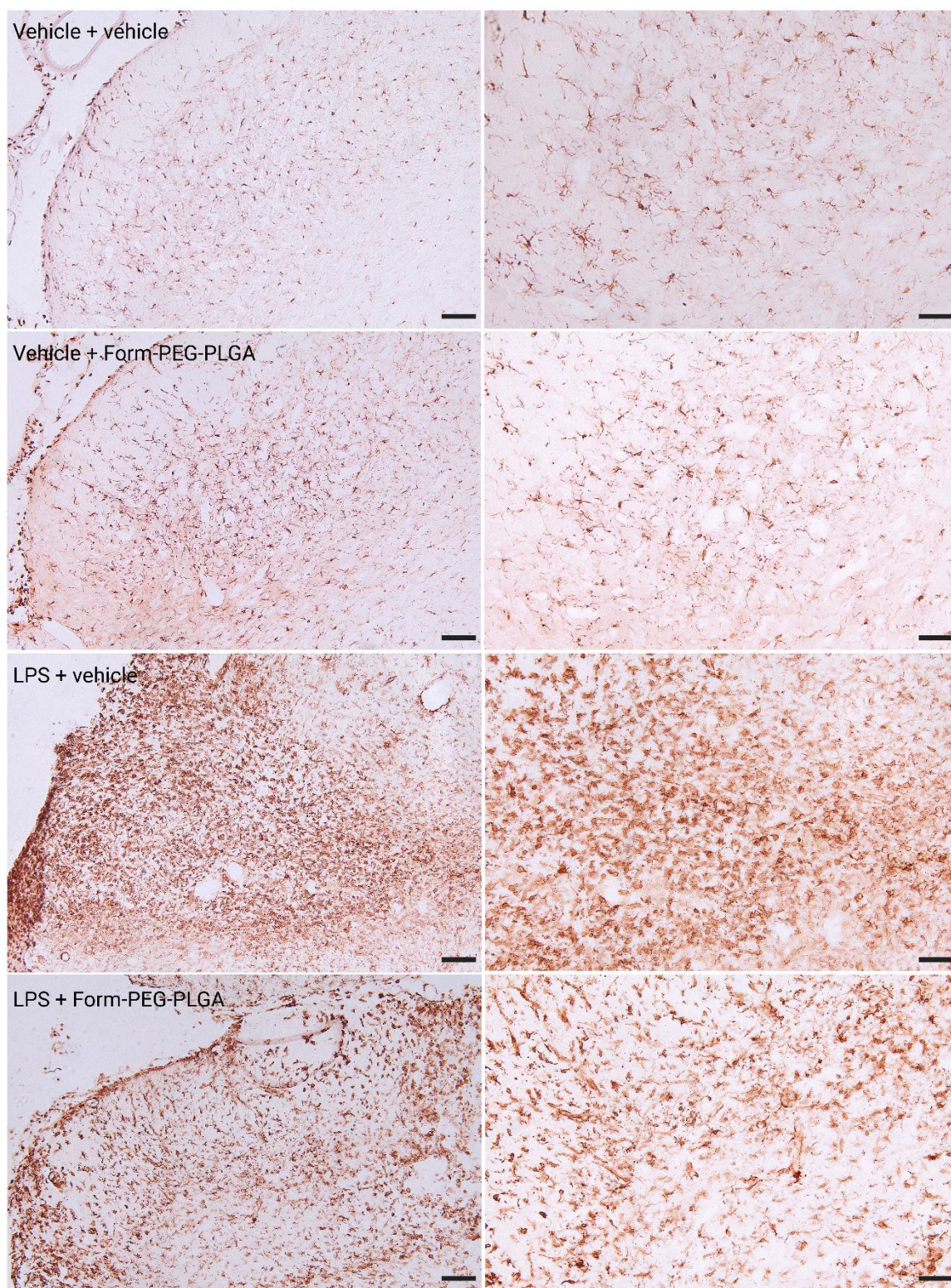


Figure 7.10. Unilateral intranigral LPS induced increases in microglial cell density is attenuated by intranasal Form-PEG-PLGA.

Representative photomicrographs of IBA1 DAB-stained substantia nigra tissue displaying LPS (5 $\mu\text{g}/\mu\text{l}$) induced increases in microglial cell density and morphological changes that are rescued by intranasal Form-PEG-PLGA (0.1 mg/kg). Left panel: magnification 10X, scale bar = 100 μm , right panel: magnification 20X, scale bar = 50 μm .

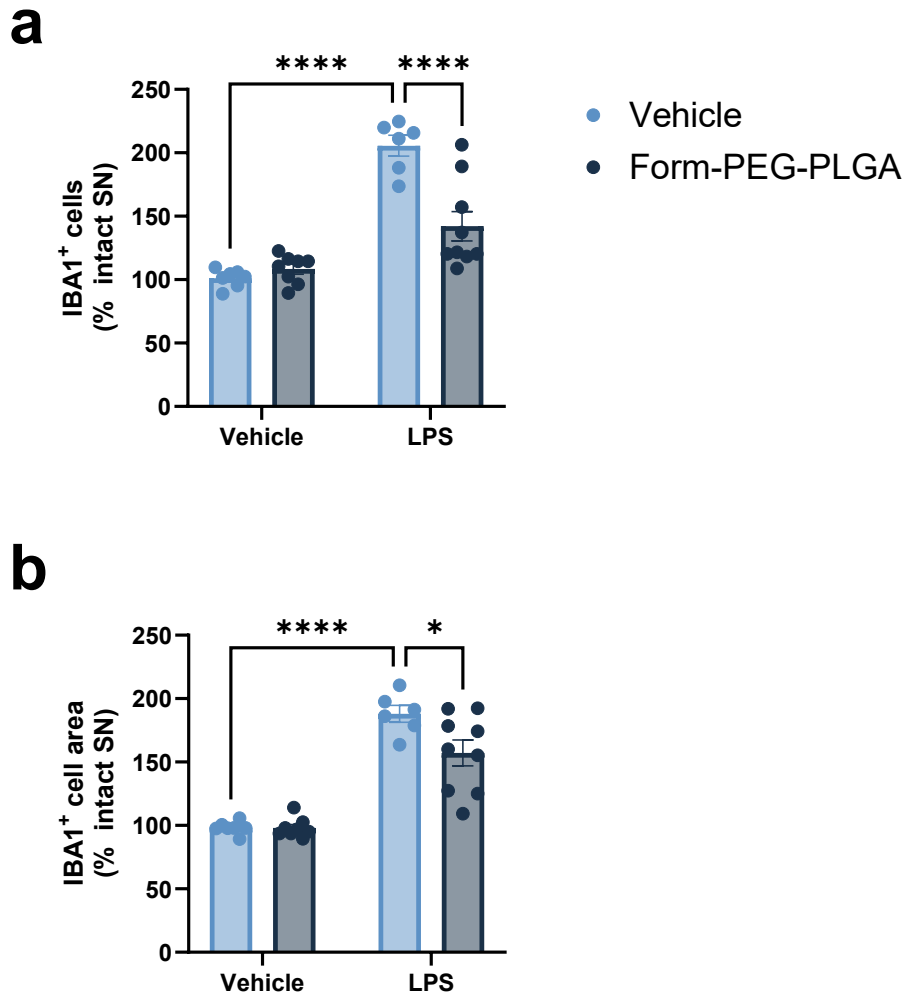


Figure 7.11. Unilateral intranigral LPS induced microgliosis is attenuated by intranasal Form-PEG-PLGA.

(a) IBA1⁺ cell counts and (b) IBA1⁺ cell area following intranigral LPS lesion (5 µg/µl) and intranasal Form-PEG-PLGA (0.1 mg/kg). Data were analysed by Two-way ANOVA followed by Tukey's multiple comparisons and are expressed as mean percentage of IBA1⁺ cells in the intact substantia nigra (SN) ± SEM, where N=6-9. Significant differences between groups are denoted by * where * P<0.05 and **** P<0.0001.

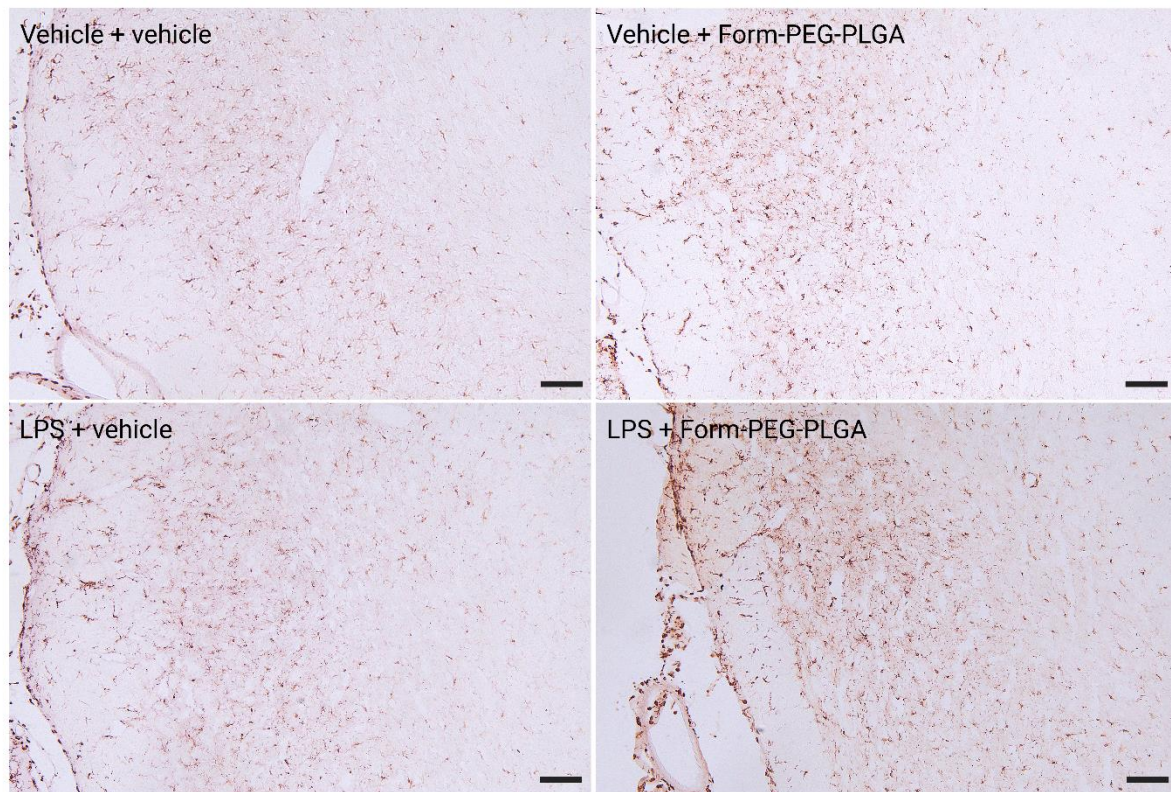


Figure 7.12. Unilateral intranigral LPS does not increase microglial cell density in the uninjected hemisphere.

Representative photomicrographs of IBA1 DAB-stained substantia nigra tissue from the uninjected hemisphere displaying no changes in microglial cell density. Magnification 10X, scale bar = 100 μ m.

7.4.6. LPS mediated TH⁺ cell loss is rescued by Form-PEG-PLGA

Two-way ANOVA of the number of TH⁺ cells in the substantia nigra (**Fig. 7.14**) showed an effect of LPS [$F_{(1, 26)} = 72.14$, $P < 0.0001$], treatment [$F_{(1, 26)} = 16.17$, $P < 0.001$] and an LPS x treatment interaction [$F_{(1, 26)} = 50.11$, $P < 0.0001$] (**Fig. 7.15a**). Tukey's multiple comparisons revealed a decrease in TH⁺ cells in the substantia nigra of animals lesioned with LPS compared to vehicle control ($P < 0.0001$). The LPS induced loss of TH⁺ cells in the substantia nigra was rescued by intranasal Form-PEG-PLGA compared to vehicle treated control ($P < 0.0001$).

Pearson's correlation plot shows a negative correlation between the number of IBA1⁺ and TH⁺ cells in the substantia nigra [$P < 0.0001$, $R^2 = 0.6532$] (**Fig. 7.15b**). Pearson's correlation plot also shows a moderate negative correlation between the number of TH⁺ cells in the substantia nigra and ipsilateral rotation behaviour following D-amphetamine challenge [$P < 0.0004$, $R^2 = 0.6532$] (**Fig. 7.15c**).

Three-way ANOVA of the number of TH⁺ cells in the substantia nigra in males and females showed an effect of LPS [$F_{(1, 22)} = 63.61$, $P < 0.0001$], treatment [$F_{(1, 22)} = 13.79$, $P < 0.01$] and an LPS x treatment interaction [$F_{(1, 22)} = 44.35$, $P < 0.0001$] (**Fig. 7.16**). Tukey's multiple comparisons revealed a decrease in TH⁺ cells in the substantia nigra of animals lesioned with LPS compared to vehicle control in males ($P < 0.0001$) and females ($P < 0.0001$). The LPS mediated TH⁺ cell loss in the substantia nigra was rescued by intranasal Form-PEG-PLGA compared to vehicle treated control in males ($P < 0.01$) and females ($P < 0.001$).

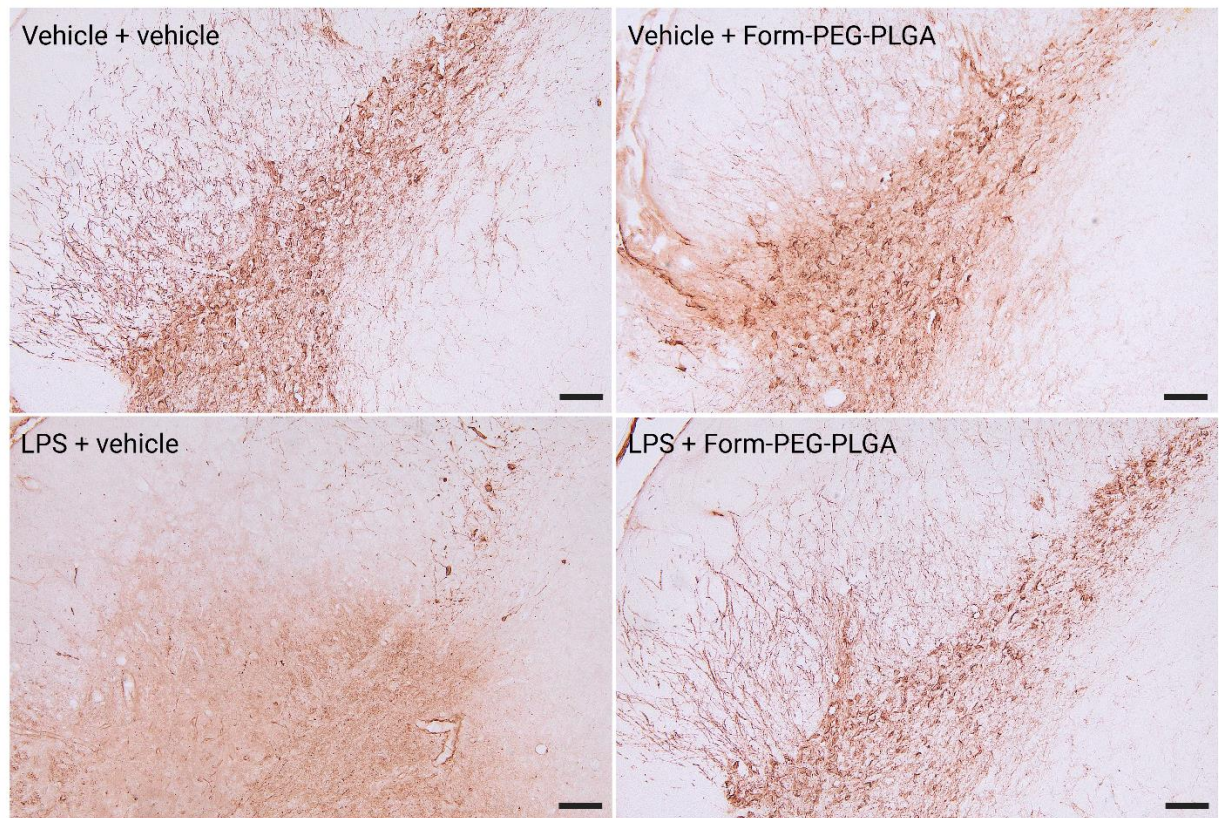
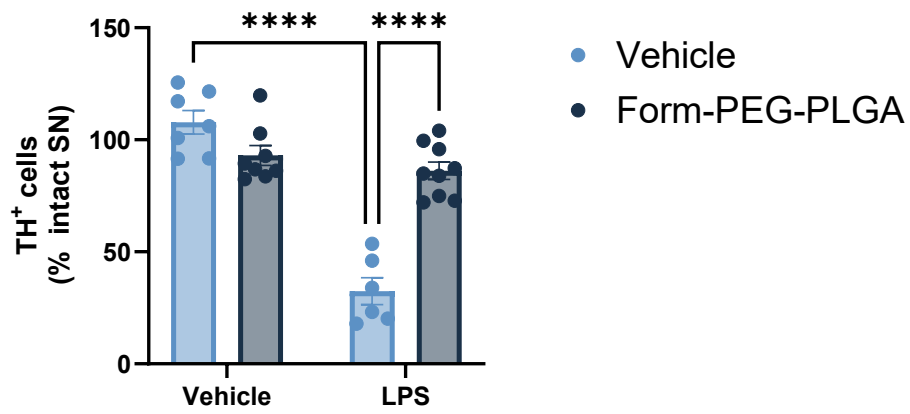


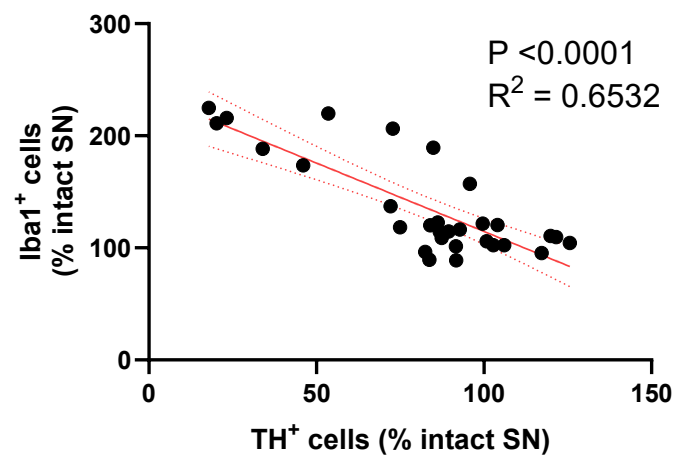
Figure 7.14. Unilateral intranigral LPS mediated TH⁺ cell loss is rescued by intranasal Form-PEG-PLGA.

Representative photomicrographs of TH DAB-stained substantia nigra tissue displaying LPS (5 µg/µl) induced loss of TH⁺ cell bodies is rescued by intranasal Form-PEG-PLGA (0.1 mg/kg). Magnification 10X where scale bar = 100 µm.

a



b



c

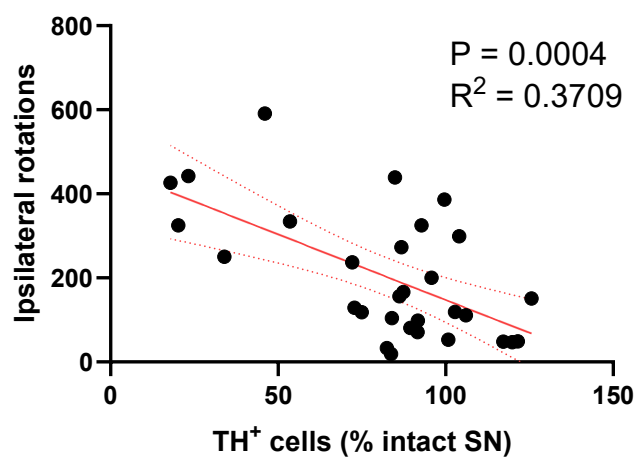


Figure 7.15. Unilateral intranigral LPS mediated TH⁺ cell loss is rescued by intranasal Form-PEG-PLGA.

(a) TH⁺ cell counts following intranigral LPS lesion (5 µg/ µl) and intranasal Form-PEG-PLGA (0.1 mg/kg). TH⁺ cell counts negatively correlate with (b) IBA1⁺ cell counts and (c) ipsilateral rotations in the D-amphetamine challenge. Data were analysed by Two-way ANOVA followed by Tukey's multiple comparisons and are expressed as mean percentage of TH⁺ cells in the intact substantia nigra (SN) ± SEM, where N=6-9. Significant differences between groups are denoted by **** where P<0.0001.

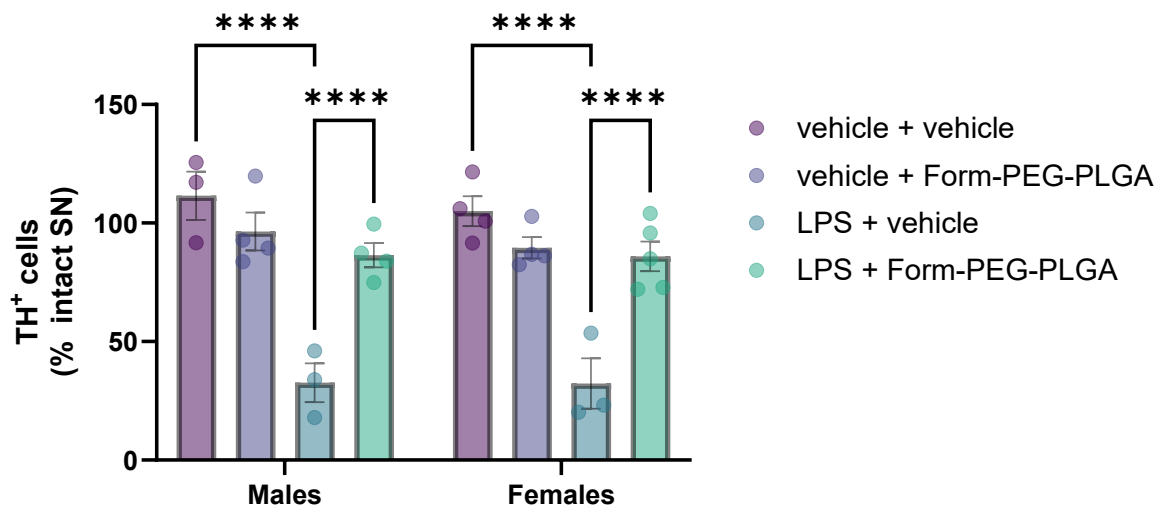


Figure 7.16. Sex did not affect TH⁺ cell loss following unilateral intranigral LPS.

TH⁺ cell counts following intranigral LPS lesion (5 µg/ µl) and intranasal Form-PEG-PLGA (0.1 mg/kg) did not differ between males and females. Data were analysed by Three-way ANOVA followed by Tukey's multiple comparisons and are expressed as mean percentage of TH⁺ cells in the intact substantia nigra (SN) ± SEM, where N=3-5. Significant differences between groups are denoted by **** where P<0.0001.

7.5. Discussion

The pathophysiology of PD is characterised by extensive degeneration of midbrain dopaminergic neurons accompanied by chronic neuroinflammation. This study simulated these disease features by local delivery of LPS to induce reactive microgliosis in the substantia nigra. On average, there was a two-fold increase in microglial density accompanied by a 68% loss of TH⁺ dopaminergic cells two weeks post-lesion when compared to vehicle control. The average area of individual IBA1⁺ cells in the substantia nigra was also analysed. Reactive microglia have an amoeboid or circular cell morphology corresponding to a larger cell area when compared to resting ramified cells. In line with this, LPS lesion caused a ~1.8-fold increase in the cell area of microglia in the substantia nigra compared to vehicle control brains. These findings agree with previous studies conducted by the Harkin lab and others, where intranigral LPS triggered significant microglial reactivity, upregulation of pro-inflammatory IL-1 β production and comparable reductions in TH immunoreactivity (Deng et al., 2020; Machado et al., 2011; O'Neill et al., 2020; Yssel et al., 2018). Pre-treatment with intranasal Form-PEG-PLGA (0.1 mg/kg; intranasal in hydrogel) attenuated LPS mediated increases in microgliosis by at least 50% and significantly reduced individual microglia cell area in the substantia nigra towards a quiescent morphology. In LPS-lesioned animals that were pre-dosed with Form-PEG-PLGA, there was on average a 14% loss in TH⁺ cell bodies compared to 68% in vehicle treated animals. This corroborates previous findings where intraperitoneal administration of formoterol attenuated intranigral and intra-locus coeruleus LPS-mediated microglial reactivity and catecholaminergic neuronal cell loss (McAuslan, PhD thesis; O'Neill et al., 2020).

7.5.1. Motor dysfunction in the intranigral LPS model

Motor deficits are a hallmark feature of PD. In this study we used a battery of motor function tests to examine the effects of targeting the β_2 AR with a PLGA drug nanocarrier delivered intranasally, focusing on asymmetric limb use, forelimb akinesia, and overall motor co-ordination and balance. In the cylinder test, contralateral forelimb use for weight shifting movements during spontaneous vertical apparatus exploration was reduced in animals that were lesioned with LPS in line with previous reports (Cankara et al., 2022; O'Neill et al., 2020; Schallert et al., 2000; Yssel et al., 2018). Intranasal administration of Form-PEG-PLGA prior to intranigral lesion significantly attenuated the LPS-mediated preference for the ipsilateral limb in the cylinder test.

Intranigral LPS lesion did not cause akinesia in the contralateral forelimb in the stepping test. Several 'pitfalls' of the stepping test have been described including a learning aspect which can result in animals dragging their paw instead of taking steps. This may be the case in the present study, where animals were trained for several days prior to baseline testing to habituate them to the required restraint. The results do not align with previous data

generated by the Harkin lab and others (Hoban et al., 2013; O'Neill et al., 2020; Yssel et al., 2018), where intranigral LPS reduced the number of contralateral adjusting steps taken in the forehand and backhand direction post-lesion. Owing to a possible habituation effect, analysis of backhand steps in either forelimb was not feasible. This measure of the stepping test may be more sensitive to intranigral lesions than the parameters reported here.

Similarly, we did not report an LPS effect on rotarod performance, in contrast with literature reports of significant reductions in the time spent on the rotarod in animals that received a unilateral intranigral LPS injection (Cankara et al., 2022; He et al., 2016; Lee et al., 2022; Sharma et al., 2017; Tai et al., 2013; Tu et al., 2019). Again, habituation to the apparatus may explain these discrepancies as details of training and baseline testing are often absent in publications and where reported, training and testing regimes vary from study to study making direct comparisons difficult. At the time of behavioural testing LPS-lesioned animals had notably worse grip strength on the rotarod with the contralateral hindlimb, but this did not prevent animals from remaining on the rotarod for the full test duration. In future studies, it may be worth recording rotarod sessions on video so that these nuances can be scored and quantified.

While the cylinder, stepping and rotarod tests are widely used as indicators of a midbrain lesion, rotational behaviour in the D-amphetamine-induced rotations test is directly related to midbrain dopamine cell integrity, where the magnitude of ipsilateral rotations is correlated to lesion severity (Björklund & Dunnett, 2019). The present study aligns with several others that describe increased rotational behaviour in the ipsilateral direction to the LPS-lesioned nigra (Cankara et al., 2022; Cui et al., 2012; Lee et al., 2022; Lee et al., 2021; Tai et al., 2013). Furthermore, a negative correlation between TH⁺ cell numbers and ipsilateral rotational behaviours was found. Intranasal delivery of Form-PEG-PLGA rescued these behaviours, attesting to protection of dopaminergic neurons in the nigrostriatal tract against LPS insult.

Cylinder and stepping test scoring are subjective and differ between individuals. While scoring in each behavioural paradigm was performed by the same individual, human error and unconscious bias may still be at play. Various free software tools have been developed to objectively analyse videos of animals performing behavioural tasks. These include DeepLabCut, SimBA and BORIS. However, these software packages require training, knowledge of coding and high-quality videos for automated workflow. Any-maze software was used to automate scoring of rotational behaviour following D-amphetamine challenge, eliminating the risk of observational biases to provide objective data. However, licenses for such software are expensive with brief free trial periods and therefore may be inaccessible to many research groups.

Interesting data trends were evident in both the stepping and rotarod tests post-LPS lesion that may hint at LPS effects, especially in male rats. It is possible that these experiments are not sufficiently powered as data from related past experiments was initially used to

calculate the effect size. Historically, these types of experiments only included male rats and may not accurately capture the parameters of the current investigation. Using data from the stepping test collected here, a retrospective power analysis was performed using G*power. An effect size of at least 136 animals where N=18 (9 males and 9 females) per group would be required to detect differences between vehicle and LPS lesioned rats in the stepping test.

7.5.2. Sex differences in PD and in preclinical models

Subtle sex differences were observed in motor function. Typically males are more likely to be diagnosed with PD than women and preclinical research is heavily reliant on male rodents. Only very recently has there been a concerted effort to include females in scientific research and so, many behavioural paradigms were initially validated using male rodents (Schallert et al., 2000). To this day, there is limited information on female performance in many behavioural tasks. In the studies referred to above that reported significant LPS effects in the stepping and rotarod tests, male rodents were used exclusively (Cankara et al., 2022; He et al., 2016; Hoban et al., 2013; Lee et al., 2022; O'Neill et al., 2020; Sharma et al., 2017; Tai et al., 2013; Yssel et al., 2018) or the investigators did not specify (Tu et al., 2019). This may further explain the discrepancies between effects reported in the literature with those found in the present study, as group numbers are low when assessing at males and females in isolation.

Sex differences in symptomology are present in the clinic; generally female patients present with tremor whereas males more often display postural instability and abnormal gait (Reekes et al., 2020). Women tend to have a higher incidence of non-motor symptoms like anxiety and autonomic dysfunction while men are more likely to suffer from sleep disturbances and cognitive impairment (Maas et al., 2024). Future studies may include behavioural tasks that can capture the non-motor symptoms of PD such as the elevated plus maze or light/dark box for anxiety related behaviours and novel object preference or operant conditioning to assess cognition. Song et al. (2019b) described a 'two-hit' PD model involving pretreatment with DSP4 prior to systemic LPS challenge induced neurodegeneration, where motor symptoms and non-motor symptoms like hyposmia, anxiety and impaired learning in mice were assessed. Such a model may be more appropriate to recapitulate differences between the sexes relating to presenting symptoms.

Preclinically, female rats are reported to be less vulnerable to 6-OHDA lesion with a higher degree of behavioural recovery in the open field test than male rats (Tamás et al., 2005). However, 6-OHDA lesion in aged female rats resulted in pathology and motor deficits comparable to both young and aged males, although aged animals did not fare as well post-surgery raising animal welfare concerns (Diwakar et al., 2006). Field et al. (2006) report no sex differences in performance in the cylinder test in 6-OHDA lesioned males and females,

reflecting the results shown here. Several studies have used female rodents in the intranigral LPS model, however no motor function tests were performed and although pathology is evident, males were not used so sex differences cannot be elucidated (Castano et al., 2002; Castaño et al., 1998; Herrera et al., 2000; Tomás-Camardiel et al., 2004). A literature search using key phrases 'intranigral LPS' and 'female' did not yield a single research article that compared motor function or pathology in wild-type male and female rodents following a unilateral intranigral LPS lesion.

Oestrogen is widely recognised as neuroprotective, making both the age of the animals and the stage in the oestrus cycle at the time of lesion important considerations in experimental design. During oestrous, females may exhibit identifiable behaviours such as a twitching or shaking in response to touch. In this study we observed that these behaviours were associated with poorer rotarod performance, both at baseline and post-lesion, regardless of whether animals received LPS or sham treatment. However, such effects were not apparent during other tasks, during drug administration, or on the day of surgery.

Behavioural testing in the stepping, cylinder and rotarod tests were carried out across several days, and average scores were obtained to minimise the impact of this variability. Nevertheless, in future studies it may be valuable to track the oestrous cycle of female animals to further investigate these.

In this study, the degree of LPS-mediated microgliosis was comparable in male and female rat brains. However, neurotoxins like rotenone produce dose-dependent effects on microglial reactivity in the substantia nigra of female rats and higher doses are required to reproduce the IBA1 and CD68 immunoreactivity observed in male counterparts (De Miranda et al., 2019). Evidence from primary cultures isolated from the adult rat brain support this. *Ex vivo* LPS stimulation of hippocampal microglia increased *Il1β*, and *Cd14* mRNA in males compared to females, but when female reproductive organs were removed, male and female microglia responses to LPS were comparable. Furthermore, these effects were rescued by estradiol therapy, pointing to a role for female hormones in influencing microglia responses to inflammatory stimuli like LPS (Loram et al., 2012). The presence of sexually dimorphic microglia is likely specific to brain region and age. In 3-month-old rats, females showed a higher percentage of 'activated' microglia at baseline compared with males in the periaqueductal grey area (PAG). Following systemic (i.p.) LPS injection, females also exhibited a more pronounced increase in non-ramified microglia in the PAG and amygdala, but not the substantia nigra (Doyle et al., 2017). Because these models rely on systemic administration of neurotoxins to induce neurodegeneration and neuroinflammation, sex differences in microglia may not appear, or may be less significant, when LPS is delivered directly to the substantia nigra.

Overall, the evidence suggests that sexually dimorphic microglial activity is likely region specific and changes across the lifespan, highlighting the need for further investigation in preclinical PD models.

Bekhat et al. (2016) reported that brief exposure to isoflurane anaesthesia, as used in this study for daily intranasal administration, increases plasma corticosterone concentrations in female rats but not males, indicating a stress response and activation of the HPA axis. Under physiological conditions, activation of the HPA axis acts to dampen neuroinflammatory signalling. However, repeated activation of the HPA can prime microglia and they become hyper-responsive to immune stimuli (Frank et al., 2016). There is no direct evidence linking repeated isoflurane exposure to microglia priming but it is a factor for consideration in future studies where animals are repeatedly subjected to isoflurane anaesthesia prior to LPS insult. It is critical that vehicle controls are exposed to anaesthesia for the same amount of time as drug-treated animals.

In this study, LPS induced increases in ipsilateral rotations across various measures in males compared to sham lesioned animals following the D-amphetamine challenge, but this difference was not significant in females. While insufficient power may be responsible, there is strong evidence to suggest that the male brain is more vulnerable to dopaminergic cell loss in 6-OHDA and MPTP models (Dluzen, 1997; Kenchappa et al., 2004; Tamás et al., 2005). This is supported by findings that oestrogen replacement therapy protected against an MPTP-induced reduction in dopamine concentrations in ovariectomised mice (Dluzen et al., 1996) and pre-treatment with an oestrogen receptor antagonist rendered female mice more susceptible to MPTP induced mitochondrial dysfunction (Diwakar et al., 2006; Dluzen et al., 2001). Female mice were also less susceptible to i.p. LPS-mediated dopaminergic cell loss and associated motor deficits than males (Liu et al., 2008).

While there is no evidence of gross anatomical sex differences in the structure of the nasal cavity, several physiological processes may introduce variability in intranasal drug targeting to the brain in females compared to males. Mucociliary clearance is slower in females than males, particularly during the dioestrus phase of the oestrous cycle and epithelial expression of acidic glycoproteins are lower in females (Saldiva et al., 1995). Together, these findings would suggest that nose to brain drug delivery is superior in females. However, the authors also found that mucus from the nasal cavity of female rats in oestrus is more rigid than males. This may trap drug molecules or particles in the nasal cavity and decrease particle and/or drug mobility. Sexual dimorphisms in olfactory circuitry are a conserved phenomenon allowing for pheromone detection in rats (Segovia & Guillemin, 1993). Since the olfactory pathway is a major route for drug delivery to the CNS from the nasal cavity, these sex differences may result in variations in drug uptake between males and females.

7.5.3. Form-PEG-PLGA attenuated dopaminergic cell loss in the substantia nigra

The Harkin lab has previously demonstrated that intranigral LPS lesion reduced TH immunoreactivity in the striatum and that i.p. formoterol can attenuate this cell loss (O'Neill et al., 2020). Future work should examine whether nigral lesion in the present study

also impacted striatal TH⁺ immunoreactivity and whether intranasal Form-PEG-PLGA protects against this. Additional immunohistochemical analysis of brain tissue from this study may provide further insight into the LPS-mediated microgliosis and subsequent dopaminergic cell loss in the substantia nigra, the ability of intranasal Form-PEG-PLGA to attenuate these effects and any tenuous sex differences that may exist. An upregulation in pro-inflammatory cytokine IL-1 β , phagocytic markers like CD68 and apoptosis marker caspase 3 have been demonstrated in the 6-OHDA and MPTP models of PD through immunohistochemical staining in addition to TH cell loss (Lee et al., 2019; Marinova-Mutafchieva et al., 2009). Previous members of the Harkin lab have investigated the role of astrogliosis in intranigral LPS-mediated neuroinflammation and degeneration through immunohistochemical GFAP and S100 β assays (O'Neill et al., 2019). However, a role for astrocytes in the protective effects conferred through pharmacological targeting of the noradrenergic system have not been explored. The substantia nigra is innervated by D β H⁺ LC noradrenergic projection neurons and the NET is trafficked to these axon terminals (Mandela & Ordway, 2006; Mejías-Aponte et al., 2009). D β H and NET expression in the intranigral LPS model has not yet been characterised, but these markers may unveil further details of the interplay between the noradrenergic system and dopaminergic cell loss in neuroinflammation, as well as how targeting this system has potential therapeutic effects. Both LPS activation of TLR4 and formoterol binding to the β_2 AR modulate *SNCA* transcription. LPS increased α -synuclein expression in the olfactory bulb and striatum of wildtype mice following intranasal and intrastriatal delivery, respectively (Deng et al., 2021; Niu et al., 2020). Lomeli-Lepe and colleagues recently described this phenomenon in the intranigral LPS model, where after 7 days, the emergence of α -synuclein oligomers coincided with severity of TH⁺ cell loss (Lomeli-Lepe et al., 2024). Syn-1 or pSer129 staining for wild-type or misfolded α -synuclein proteins, respectively, in the intranigral LPS model would give more insight into the translatability of this model and how pharmacotherapies like formoterol can affect PD pathology.

This study adds to a growing body of research that demonstrates the therapeutic potential of targeting the β_2 AR in neurodegenerative disease models. Low doses of salmeterol (0.001 or 0.01 mg/kg/day delivered via an infusion pump) were sufficient to reverse motor deficits in rotarod and rescue dopaminergic cell death in the substantia nigra of mice three months after a systemic LPS challenge (5 mg/kg; i.p.). This effect was shown to be β_2 AR-dependent in knockout mice (Qian et al., 2011). Clenbuterol treatment (10 mg/kg; i.p. daily for 4 weeks) was associated with a reduction in pro-inflammatory signalling and rescued TH⁺ cell loss in mice overexpressing human wildtype α -synuclein (Torrente et al., 2023). Clenbuterol administration (0.5 mg/kg; i.p.) prior to intrastriatal injection of pro-inflammatory cytokine IL-1 β prevented increases in the expression of *Tnfa* and TUNEL⁺ staining used to measure apoptosis (Griffin et al., 2018). These studies are supported by findings in human cohorts that long-term use of β_2 AR agonists like salbutamol is associated with a decreased risk of developing PD (Mittal et al., 2017). β_2 AR agonists have also been investigated in preclinical

models of AD, stroke, Down syndrome and excitotoxicity, where anti-inflammatory effects, reduced pathology and improved behavioural performance were reported (Chai et al., 2016; Chai et al., 2022; Dang et al., 2014; Gleeson et al., 2010; Lechtenberg et al., 2019; Rasheed et al., 2018).

To the best of our knowledge, this is the first time that formoterol-loaded PLGA nanoparticles have been delivered intranasally or in a preclinical model of neurodegenerative disease (see chapter 5 where studies that loaded nanoparticles with formoterol or other β_2 AR agonists were discussed). Several other studies have administered drug loaded PLGA nanoparticles intranasally to preclinical models of PD, highlighting the benefits of this route of administration and use of nanocarrier systems in the efficacy of dopamine, L-Dopa, rotigotine and bromocriptine over oral and unencapsulated intranasal formulations in the unilateral 6-OHDA rat and MPTP mouse models of PD (Arisoy et al., 2020; Cong et al., 2025; Gambaryan et al., 2014; Li et al., 2025b; Tang et al., 2019; Yan et al., 2018). The efficacy of intranasal nanocarriers for delivery of anti-inflammatory agents like ibuprofen in the MPTP mouse model of PD has been demonstrated (Mandal et al., 2016). However, the formulation used was a lipid-based nanoemulsion which has distinct physicochemical properties from PLGA nanoparticles. According to our current analysis, no other group to date has reported the intranasal administration of PLGA nanoparticles loaded with an anti-inflammatory drug in a rodent model of PD.

7.5.4. Pre-dosing with Form-PEG-PLGA is necessary to confer immunomodulatory and neuroprotective effects against intranigral LPS

In this study, dosing animals prior to stereotactic injection of LPS was critical in providing robust protection against neuroinflammatory driven neurodegeneration. These results prompt further investigation as to whether the four doses post-lesion are necessary or whether a single Form-PEG-PLGA administration prior to LPS could produce the same effects. In a subsequent study, we tested the efficacy of intranasal Form-PEG-PLGA administration immediately post-LPS lesion and daily for 7 days (see appendix I). However, this dosing regime was unable to confer robust anti-inflammatory and neuroprotective actions observed when animals received Form-PEG-PLGA prior to the lesion.

We hypothesise that intranasal Form-PEG-PLGA therapy may be more effective when administered in a slower progressing PD model such as α -synuclein models involving stereotactic injection of pre-formed fibrils or adeno-associated virus vectors. Although the intranigral LPS model displays key pathological features and motor defects associated with PD, it may be inappropriate for the testing of slow-release formulations due to the fast and robust induction of microglial reactivity and reports of substantial loss of TH⁺ cells just one day after injection (Iravani et al., 2005).

Both a single high dose and chronic low doses of LPS administered via the intraperitoneal route are reported to induce microglial reactivity and dopaminergic cell loss over many weeks to months that leads to motor deficits (Beier et al., 2017; Bodea et al., 2014; Qin et al., 2007). However, sex differences have been described in this model with a single high dose LPS challenge failing to produce neurodegeneration in the substantia nigra of female rodents (Liu et al., 2008). The resulting degeneration is bilateral which has implications for the experimental design. Chronic intranasal administration of LPS causes substantial reductions in the number of TH⁺ dopaminergic neurons in the substantia nigra and striatum unilaterally when administered repeatedly to the same nostril (Zhao et al., 2018) and may be performed immediately before or alongside intranasal drug administration. A major caveat in the search for new PD therapies is that when patients first present with symptoms there is already extensive dopaminergic cell loss. Therefore, to identify disease-modifying therapies that can help patients in the near future, it is important to utilise models and study design that reflect this.

Other studies that administered anti-Parkinson's therapies intranasally post-lesion used longer dosing schedules consisting of daily administration for up to two weeks (Gambaryan et al., 2014). Therefore, seven days of Form-PEG-PLGA therapy may have been insufficient to show protective effects of this therapy against intranigral LPS insult. Nevertheless, standard practices for intranasal drug delivery in rodents are non-existent. Many publications exclude key information about administration techniques, applicators and whether brief anaesthesia was used. Furthermore, there is extensive variation in dose volumes used in rats, with some studies describing volumes of up to 200 µl (Yan et al., 2018).

7.5.5. Future directions

The experimental Form-PEG-PLGA therapy proposed here may have clinical relevance as a prophylactic medication, even though administration prior to intranigral LPS lesion was a requisite for the observed anti-inflammatory and neuroprotective effects. In the PD brain, microglia become primed by pathology and chronic neuroinflammation, and on subsequent encounter with inflammatory stimuli the pro-inflammatory response elicited is exaggerated with detrimental effects to healthy tissue (Perry & Holmes, 2014). This becomes clinically relevant in the context of delirium, where a secondary immune challenge (often arising in the periphery) can exacerbate neurodegeneration and worsen symptom severity (Green et al., 2021; Pott Godoy et al., 2008). To investigate this, a future experiment may involve lesioning the nigra and beginning daily intranasal Form-PEG-PLGA administration prior to a secondary peripheral immune challenge to examine whether the associated advancement of disease severity can be prevented.

Chapter 8: Concluding remarks

8.1. Summary of main findings

This thesis details the experimental pharmaceutical, cell culture and *in vivo* methods used to develop formoterol-loaded PEG-PLGA nanoparticles for intranasal delivery in the LPS-induced rat model of PD-associated neuroinflammation and degeneration.

When introducing a foreign particle to the body, there is a risk that immune cells may recognise particles as an invading pathogen and launch an immune response. In CNS diseases where a chronic neuroinflammatory environment already exists, the introduction of a further insult may accelerate neuroinflammation and neurodegeneration. In chapter 4, the compatibility of PEG-PLGA nanoparticles with primary rat glial and neuronal cultures was demonstrated. Direct application of PEG-PLGA did not induce the expression of pro-inflammatory *Il1b*, *Tnfα*, *Nos2* or *Cd40* mRNA in glial cells or compromise neuronal complexity. Furthermore, the transfer of CM from glia treated with PEG-PLGA to neurons did not alter neuronal morphology. Together, these results indicate the biocompatibility of PEG-PLGA and their safety for potential use as *in vivo* drug delivery vehicles.

Drug loading with formoterol did not dramatically change the physicochemical properties of PEG-PLGA nanoparticles and, in agreement with the literature, Form-PEG-PLGA exhibited an initial burst release of drug followed by a secondary release phase over 24 hours as the polymer degraded. As the intended use of these nanoparticles was for intranasal delivery, Form-PEG-PLGA were incorporated into a mucoadhesive, thermosensitive hydrogel, which further decelerated drug release over a 72-hour period. Nanoparticles released drug in an *in vitro* model of neuroinflammation to produce anti-inflammatory effects. When applied to glial cells pre-stimulated with IFN γ or LPS, Form-PEG-PLGA reduced the expression of pro-inflammatory *Il1b*, *Tnfα*, *Nos2* or *Cd40* mRNA. Furthermore, this reduction of microglial reactivity was neuroprotective. Neurons treated with CM from IFN γ or LPS treated glia had a reduced complexity and this was rescued by exposure to CM from Form-PEG-PLGA treated glia.

To verify that the hydrogel system was an appropriate vehicle for intranasal drug delivery, contrast agent Mn²⁺ was embedded in the gel and delivered to rats via the intranasal route. Contrast enhanced MRI confirmed brain uptake. Following this, the anti-inflammatory effects of formoterol released from Form-PEG-PLGA (0.1 mg/kg; intranasal; ~30 μ l gel) were described in an acute LPS (0.25 mg/kg; i.p) model of neuroinflammation, where treatment attenuated the expression of *Il1b*, *Tnfα*, *Nos2* or *Cd40* mRNA in the rat cortex and striatum. However, intranasal delivery of Form-PEG-PLGA also reduced inflammatory signalling in the spleen, suggesting that drug may have also entered the systemic circulation. Quantification of drug concentrations in the brain and peripheral tissues is necessary to further assess the

distribution profile of formoterol when administered intranasally via a nanoparticle-hydrogel system.

The anti-inflammatory and neuroprotective effects of Form-PEG-PLGA treatment (0.1 mg/kg; intranasal; ~30 μ l gel) was then tested in the intranigral LPS model of PD-related neuroinflammation and neurodegeneration. This unilateral LPS model exhibits several cardinal features of PD pathology including microgliosis in the substantia nigra, degeneration of dopaminergic neurons along the nigro-striatal tract and motor deficits contralateral to the lesion. Formoterol released from Form-PEG-PLGA after intranasal instillation of the gel, rescued asymmetry in forelimb use in the cylinder test, reduced the number of ipsilateral rotations following D-amphetamine challenge, suppressed microglial activation and protected against dopaminergic cell loss in the substantia nigra of rats that had received a unilateral injection of LPS into the substantia nigra.

Taken together, these findings highlight the potential of PLGA nanocarriers for drug delivery to the brain for the treatment of PD and other neurological diseases and disorders; they are compatible with brain cells, display a sustained release profile, exhibit anti-inflammatory effects *in vitro* and *in vivo* and can be administered intranasally to protect against dopaminergic cell death in the substantia nigra which translates to preservation of motor function.

8.2. Considerations for PLGA nanoparticle design

PLGA nanoparticle optimisation is complicated by the fact that minor changes in the technique or synthesis materials used can have major effects on particle size, charge and drug encapsulation. Therefore, experimentally, minor adjustments must be made in each batch of nanoparticles until the desired characteristics are achieved. This is both time consuming and costly.

While the nanoparticles used in this study displayed favourable size and charge characteristics for CNS drug delivery, drug loading and release mechanisms may be improved with adjustments to the nanoparticle design. Initial studies were performed to optimise drug loading found that 65% drug (w/w) achieved higher encapsulation than lower and higher amounts of formoterol. However, fluctuations in drug encapsulation were observed throughout this project, which were attributed to external factors such as air temperature and humidity that could not be easily controlled, particularly in colder months. A warmer aqueous phase (25 to 30°C) is reported to improve drug encapsulation in polymeric nanoparticles as higher temperatures lead to faster precipitation (Sun et al., 2024). However, some drugs become more water soluble at higher temperatures and dissociate from polymers which would reduce encapsulation (Hamdallah et al., 2024). Furthermore, the temperature during nanoparticle formation needs to be maintained below the glass transition temperature (approximately 40°C for PLGA) to preserve particle

morphology as polymers are more rigid below this temperature and are more flexible above this temperature (Kamaly et al., 2016; Makadia & Siegel, 2011). Raising the polymer concentration, adjusting the phase ratio and increasing the rate of phase mixing are common strategies for increasing drug loading in PLGA nanoparticles (Hamdallah et al., 2024). However, these changes will likely impact the physicochemical properties of the resulting nanoparticles which may impede CNS targeting, for example, increasing the concentration of polymer increases particle size (Chatterjee & Chanda, 2022).

Hamdallah et al. (2024) reported that drug loading in PLGA nanoparticles is primarily governed by drug solubility in the organic phase. In the present study, drug was dissolved in DMSO and then added to the organic phase as formoterol is not soluble in tetrahydrofuran. As DMSO is water-miscible the formation of nanoparticles may be slower and drug loading reduced. The use of methanol for drug preparation may be preferable for PLGA nanoparticle loading as methanol is less water-miscible than DMSO and may facilitate faster nanoprecipitation to increase encapsulation efficiency.

Adjusting the polymer concentration used in nanoparticle synthesis would increase the batch size produced. PLGA nanoparticles are typically stable at 4°C for weeks to months but for large batches, lyophilisation of particles is recommended. Cryoprotectants are necessary during the freeze-drying process to prevent particle aggregation and structural collapse. However, cryoprotectants can affect nanoparticle size, uniformity, re-dispersibility, porosity, surface chemistry, stability, drug encapsulation and release profile (Fonte et al., 2016). Therefore, optimisation of these conditions is also required.

In this study, a simple nanoprecipitation method was used as it is a scalable process that can achieve nanoparticles that are less than 100 nm in size. Single emulsion methods may enable higher drug loading but tend to yield larger particles than nanoprecipitation (Ghaly et al., 2025). Flash nanoprecipitation using microfluidics involves fast and controlled mixing of phases, leading to rapid formation of small, uniform nanoparticles and more efficient encapsulation of hydrophobic drugs (Kamaly et al., 2016; Tao et al., 2019).

To improve the drug release profile and decelerate polymer degradation, the ratio of PLA: PGA in the PLGA copolymer can be increased. Increasing the PLA content in the polymer affects the resulting nanoparticles in several ways; the glass transition temperature increases, the particles become more hydrophobic and there are stronger interactions with hydrophobic drugs in the particle core (Makadia & Siegel, 2011). The use of high molecular weight polymers also slows drug release as they have low elasticity and erode more slowly than low molecular weight polymers which are highly elastic and easily swell as osmotic pressure increases, allowing water molecules to enter and hydrolyse the polymers (Makadia & Siegel, 2011). However, the use of high molecular weight polymers typically results in larger nanoparticles and slows the rate of nanoparticle biodegradation which could have negative effects in biological systems (Lim et al., 2022). The PLGA copolymer used in this study was carboxylic acid terminated. Ester terminated PEG-PLGA is more

hydrophobic and repels water molecules, therefore slowing nanoparticle breakdown and drug release (Kamaly et al., 2016).

Surface modifications can improve drug delivery to the brain. Lactoferrin, a ligand for the lactoferrin receptor, is frequently conjugated to the surface of nanoparticles for brain targeting as it facilitates receptor mediated transcytosis across endothelial BBB cells (Akdaşçi et al., 2024; Fillebeen et al., 1999). Studies have shown that lactoferrin coated PLGA nanoparticles achieve higher brain concentrations of drug than uncoated particles following intranasal delivery (Cong et al., 2025; Tang et al., 2019; Yan et al., 2018). Lactoferrin could easily be conjugated to the PLGA copolymer used to synthesise nanoparticles in this study; the presence of the carboxylic acid terminal facilitates amide bonding with primary amines on lactoferrin. Modifications to the surface of the particle affects the charge, size and interactions in biological tissues. However, these particles are generally still less than 200 nm with a slight negative charge (Tang et al., 2019; Yan et al., 2018).

While PLGA nanoparticles display favourable characteristics for CNS targeting and have been extensively studied for this purpose, other classes of nanoparticles may also be suitable for this application. Lipid nanoparticles and liposomes are similar in that they can cross the BBB, are biocompatible, biodegradable and can be loaded with a range of therapeutics. However, release mechanisms from these particles are much more rapid and they offer fast drug release when needed (Zheng et al., 2024). A new generation of nanoparticles are emerging that are hybrids between several classes of inorganic and organic particles, harnessing the distinct benefits of these materials for drug delivery systems. For example, silica nanoparticles have huge potential as drug delivery systems due to their high surface area and stability, however, their toxicity remains an issue (Ding et al., 2023). Encapsulation of silica nanoparticles within a lipid structure may improve the biocompatibility of these particles and their transport across the BBB (Fahmy et al., 2022). Overall, the choice of nanoparticle is dependent on the application. In the context of this study, PLGA nanoparticles were advantageous because of their small size and controlled drug release properties.

Nanoparticle optimisation is a juggling act; smaller nanoparticles have lower drug encapsulation and faster release, but larger nanoparticles with higher drug loading may not enter the brain. *In silico* models for nanoparticle design can be used to predict how changes to variables in the synthesis method will affect the physicochemical properties, drug loading capabilities and release profile, saving time on experiments and cutting the associated costs (Dalbanjan et al., 2025). Furthermore, molecular modelling can be used to examine to what degree small-molecule drugs interact with variations of PLGA polymers, which may maximise drug loading (Lim et al., 2022). However, this is dependent on sufficient *in vitro* and *in vivo* data to improve predictions. As the field continues to grow and

more evidence emerges, *in silico* approaches are likely to become a valuable tool in nanoparticle design.

8.3. Intranasal Form-PEG-PLGA dosing schedules

In this study, pre-treatment with Form-PEG-PLGA prior to intranigral LPS delivery was necessary to protect against LPS-mediated microglial activation, dopaminergic neurodegeneration in the substantia nigra and loss of motor function. Administration of Form-PEG-PLGA after intranigral LPS injection did not attenuate microgliosis, loss of dopaminergic neurons in the substantia nigra or rescue motor deficits (see appendix I).

Several studies do not disclose the timeline of intranasal therapies so direct comparisons are difficult to make. However, where listed as taking place after neurotoxin treatment, high doses and long treatment periods are reported, typically ranging from 3-13 weeks (Cong et al., 2025; Gambaryan et al., 2014; Li et al., 2025b; Yan et al., 2018). We hypothesised that the slow release of formoterol from the nanoparticles and nasal hydrogel did not accumulate in the brain fast enough to combat the effects of LPS when treatment was delivered immediately after LPS lesion for 7 days (see appendix I). It may be possible that when pathology was induced and Form-PEG-PLGA was subsequently administered intranasally, the duration of drug exposure was insufficient to produce a therapeutic effect.

Disdier et al. (2025) administered a nasal emulsion containing dodecyl creatine one day prior to stereotactic injection of 6-OHDA in rats and daily for a further 5 weeks. The authors reported that this dosing schedule reduced amphetamine induced rotations, preserved nigrostriatal dopamine content and promoted the expression of neurofilament and BDNF in the striatum (Disdier et al., 2025). Notably, liposomes loaded with bambuterol, a prodrug of β_2 AR agonist terbutaline, administered intranasally via a thermosensitive gel, improved long-term memory and protected hippocampal neurons against degeneration in a mouse model of AD when treatment commenced one day before the stereotactic injection of A β_{1-42} and continued for 18 days post-surgery at a dose three times higher than what was used in this study (Groo et al., 2025). In the present work, animals were administered Form-PEG-PLGA intranasally for 3 days prior to LPS lesion, however, according to these studies, a single dose one day before surgery may have been sufficient for the anti-inflammatory and neuroprotective effects of formoterol to be conferred.

8.4. Clinical relevance of these findings

Current PD therapies are associated with systemic side effects including gastrointestinal issues, low blood pressure and dry mouth. A dual drug delivery system comprised of a nanoparticle and a hydrogel for intranasal drug delivery can facilitate brain targeting, thus reducing systemic drug exposure and drug side effects, and introducing the potential for

lowering dosages. Sustained release intranasal formulations reduce the frequency of dosing and allow for self-administration, giving patients independence and relieving pressure on health care systems.

When hospitalised, PD patients are at a greater chance of acquiring infections and subsequent delirium, a neuropsychiatric condition caused by neuroinflammation. Moreover, delirium is often under diagnosed due to shared clinical features but is associated with an increased risk of developing PD dementia and mortality (Green et al., 2021). A recent study reported that over 67% of PD patients developed delirium following hospitalisation compared to 39% of non-PD participants (Gerakios et al., 2024). In chapter 6 and 7, pretreatment with Form-PEG-PLGA attenuated inflammatory responses in the brain following LPS challenge. Therefore, this study suggests that anti-inflammatory therapies such as formoterol, may be beneficial in preventing delirium. If such a therapy was incorporated into the standard of care for PD patients, the worsening of disease during secondary infection may be prevented to reduce mortality and increase the time of less severe symptoms, improving quality of life.

Additionally, therapies like this may be beneficial for individuals who are at risk of developing PD. As pathology is already extensive at the time of diagnosis, current research aims to identify early markers of PD. Several blood and urine biomarkers are emerging including neurofilament light chain, immature reticulocyte fraction, C-reactive protein and insulin-like growth factor 1 (Gao et al., 2025). A recent systematic review assessed the use of AI in PD diagnosis and data from wearable sensors may provide further insight into physiological changes that predate clinical symptoms (Topol, 2019). As AI technology continues to develop in the coming years, it is likely that novel PD biomarkers will be identified, and early interventions, including anti-inflammatory therapies, may slow disease progression and prolong the time that patients experience mild to moderate symptoms.

8.5. Translating rodent studies to the human condition

While the LPS model of PD associated neuroinflammation and degeneration is a valuable model for investigating the role of inflammation in PD pathogenesis, it does not accurately reflect human pathology. While a perfect animal model does not exist, the direct injection of α -synuclein fibrils to the brain or adeno-associated viral vector (AAV)-mediated delivery of mutant α -synuclein may be a more appropriate model for testing novel therapeutic strategies for PD. Advances in recombinant AAV development have led to the generation of serotypes that produce increased transduction and lead to greater propagation of α -synuclein in the brain (McFarland et al., 2009). More recently, simultaneous injection of AAV-human α -synuclein and preformed fibrils resulted in a greater accumulation of α -synuclein aggregates and neuroinflammatory responses compared to injection of AAV or fibrils alone (Negrini et al., 2022). Furthermore, systemic delivery of a novel AAV9-PHP.e capsid carrier resulted in α -synuclein overexpression, dopaminergic cell loss and

neuroinflammation in the substantia nigra as well as motor deficits after a single dose compared to PBS control, avoiding invasive stereotactic surgery (Bérard et al., 2023).

Emerging therapies for PD are unlikely to be tested clinically as a monotherapy but as adjuncts to standard of care treatment plans. Therefore, drugs may proceed to clinical testing more rapidly if *in vivo* studies reflect this. Therefore, a future study may investigate whether Form-PEG-PLGA can boost or mimic the activity of drugs like L-DOPA. It is possible to incorporate multiple drugs into nanoparticle delivery systems, depending on the drug properties. While L-DOPA has previously been encapsulated in PLGA nanoparticles (Ahmad et al., 2022a; Arisoy et al., 2020; Gambaryan et al., 2014), it is hydrophilic drug so loading efficiency may be low and release faster than formoterol. However, adjusting the polymer molecular weight, using an ester capped polymer or ion-pairing L-DOPA prior to nanoprecipitation may improve loading and release profiles (Lozoya-Agullo et al., 2019).

As the use of nanoparticle delivery systems for intranasal drug delivery in PD is an emerging research field, information in the literature surrounding dosing volumes, technique and delivery device is inconsistent or not reported. This type of research is often carried out as a collaborative effort between pharmaceutical scientists and neuroscientists, so often key pieces of information from either domain are missing in research articles. Therefore, standardised procedures and reporting are required to further advance the field.

Typically, nasal formulations are marketed as nasal sprays. Nasal sprays are limited in the volume that can be administered and only a small 5-8% initial dose reaches the olfactory epithelium. This is due to the large surface area reached by the plume created, with the remainder of the drug swallowed, inhaled or cleared from the nasal cavity (Keller et al., 2022; Morin et al., 2024), thus highlighting the need for thermosensitive mucoadhesive hydrogels for intranasal drug delivery.

Several factors need to be considered in the design of devices and formulations for intranasal drug delivery to rodents and humans including formula viscosity, sheer force, spray velocity, droplet size and plume geometry (Gao et al., 2020), which all determine if the formulation can reach the targeted region in the nasal cavity to facilitate absorption into the brain. In some reports, high shear force can increase nanoparticle aggregation and cause drug leakage (Ho et al., 2025). However, this seems to be dependent on the nanoparticle and device used. External factors can also interfere with drug delivery deep in the nasal cavity. These include the depth and angle of the delivery device in the nose, head position and breathing patterns of the recipient (Gao et al., 2020). Furthermore, if patients have a tremor or are experiencing dyskinesia, self-administration may be difficult. Patients and therapists should be consulted in the design process for further optimisation of intranasal drug delivery devices.

In the present body of work, a catheter and syringe were used for administration. This is quite a rudimentary applicator, and small volumes are difficult to control. However, this

application method allows for deeper deposition of drug formulations in the nasal cavity compared to the use of a rigid micropipette tip (Morin et al., 2025). Morin et al. (2024) developed a canula-based laminar fluid ejection (LFE) method for precise delivery of nasal formulations to the olfactory epithelium and demonstrated its superiority over conventional nasal sprays when methylene blue was delivered to human participants using this technique. Notably, LFE devices were found to preserve nanoparticle structure and did not cause drug leakage compared to commercial nebulisers and sprays (Ho et al., 2025).

In this study, a sweet scent was introduced to the recovery cage to stimulate olfactory neurons and encourage brain uptake. Similarly, a pleasant scent may be included in the formulation for delivery to patients for the same reason, but neurons may become desensitised following repeated exposure. The scent was also used to encourage animals to sniff while waking instead of trying to expel the formulation. This would likely not be a problem in humans as clear administration instructions can be provided, assuming the formulation does not irritate the nasal cavity.

Nasal anatomy in rats and humans differs greatly and so formulations tested in rodent models may need to be adapted for delivery to humans. The ratio of surface area to luminal volume in the nose is four times greater in rats than humans (Veronesi et al., 2020). As rodents have poor vision, they are more reliant on their sense of smell and therefore have a proportionally larger olfactory mucosa than humans (Tian et al., 2019). As the transport along olfactory neurons to the olfactory bulb is a major pathway for nose-to-brain drug delivery, results from rodent studies may overestimate trafficking to the brain via this route in humans (Veronesi et al., 2020).

Anosmia is a prodromal feature of PD and neurodegeneration within the olfactory bulb occurs early in PD, so transport via the olfactory pathway likely plays a minor role in drug delivery to the brain in PD patients. Trigeminal nerve innervation and branching in the nasal passage is comparable in rodents and primates (Bon, 2022; McCulloch et al., 2018). However, deeper deposition in the nasal cavity is required to target trigeminal nerves, but this is achieved more easily in humans as rats have a complicated nasal anatomy consisting of turbinates made of cartilage that must be navigated (Alvites et al., 2018).

Rat models are less accurate for predicting drug pharmacokinetics in humans due to differences in CNS expression of cytochrome p450, sulfotransferase and UGT between species. Formoterol is predominantly metabolised via O-glucuronidation by UGT enzymes in the brain, however, UGT activity in the human brain is significantly lower than in rat (Gherzi-Egea et al., 1993; Ouzzine et al., 2014). Generally, drug metabolism in rats is faster; formoterol was more rapidly metabolised in rats than dogs when administered systemically (Sasaki et al., 1982). Therefore, slower metabolism and prolonged drug effects would be expected if Form-PEG-PLGA was delivered to patients. In the human brain, vascular endothelial cells exhibit higher UGT expression than other CNS cells (Ouzzine et al., 2014),

and therefore bypassing the BBB by intranasal drug delivery may prevent premature drug metabolism and increase CNS bioavailability.

8.6. Scale-up of biomaterial formulations

Efficient and uniform drug encapsulation is a major barrier to industrial scale manufacturing of PLGA nanoparticle-based medicines, where large batches of drug-loaded nanoparticles are synthesised, separated and packaged to be sold separately. Equal loading of nanoparticles is necessary to avoid inconsistent dosing, where too much or too little drug could be administered. The use of microfluidics in flash nanoprecipitation is a valuable technique for producing uniformly sized PLGA nanoparticles with high drug encapsulation and may enable high-throughput manufacturing (Marecki et al., 2024; Valencia et al., 2012). Similarly, achieving heterogenous distribution of nanoparticles within a bulk hydrogel is a challenge and may result in uneven dosages and drug release (Yoon et al., 2025) so specific instructions, such as 'shake before use' may need to be included on packaging to redisperse nanoparticles prior to administration.

Regulatory and quality frameworks for drug products that contain nanomaterials have been introduced by the FDA to ensure that extra checks are in place to monitor nanoparticle size, PDI, surface charge, drug loading and release, removal of residual solvent and sterility of each batch produced. It also needs to be demonstrated that these parameters remain stable when batches are scaled up from milligram to kilogram quantities and when a delivery device is used.

In the coming years, it is likely that nanoparticle drug delivery formulations will feature prominently in pipelines of pharmaceutical companies, not only for the treatment of CNS disorders but also various types of cancer, cardiovascular disease, inflammatory conditions and others. Indeed, several pharmaceutical companies have nanomedicines in the pipeline for CNS disorders. Liposomal formulations of radiotherapeutic agents developed by Plus Nanotherapeutics® in the US were recently reported to be safe and increase the survival rate of patients with recurring glioma in phase I trials (Brenner et al., 2025). Evox therapeutics Ltd. in the UK has partnered with Eli Lilly to develop exosome nanoparticles to deliver gene therapies to the brain for neurodegenerative disease, but these studies are still in the preclinical phase. Furthermore, BIORCHESTRA in Korea are conducting preclinical investigations into polymeric nanoparticles for CNS delivery of gene therapies for PD. Serina therapeutics in the US developed polymer-rotigotine drug conjugates and showed that once weekly injections were sufficient to maintain stable plasma concentrations well tolerated in PD patients in a Phase I trial (Olanow et al., 2020).

8.7. Future directions

8.7.1. Efficiency of intranasal PLGA-mediated CNS transport

While this study showed that formoterol delivered intranasally via PLGA nanoparticles has anti-inflammatory effects in the brain, the route from the nasal cavity to the brain is unclear. Several studies have loaded polymeric nanoparticles with radiolabelled drug to confirm nose-to-brain delivery, however, this does not tell us whether nanoparticles can also reach the brain as the nanoparticle may simply release the drug load which can then travel to brain. Incorporation of an imaging agent into the polymer matrix of the nanoparticle may be necessary for future work as previously described (Li et al., 2019). However, changes to nanoparticle structure may have knock on effects on physicochemical properties that may alter brain uptake.

A major gap in the current study is that we failed to develop a quantification method for formoterol in brain and peripheral tissues of rodents in chapter 6. A HPLC-UV method was developed based on the protocol described by Nadarassan et al. (2007), however, this was not sensitive enough to detect the drug quantities in the brain following intranasal delivery of a relatively conservative dose of formoterol. Currently, a higher sensitivity liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method is being optimised for the detection of formoterol based on the conditions previously described (Yan et al., 2023). It is expected that intranasal administration of Form-PEG-PLGA-hydrogel causes a more gradual increase in brain concentrations of formoterol as the biomaterials are broken down by intrinsic mechanisms, when compared to intranasal delivery of unencapsulated drug. This study could be further expanded to include comparisons with systemic routes of administration and standard of care PD therapies like L-DOPA.

In this study we also noted that intranasal delivery of Form-PEG-PLGA attenuated the expression of pro-inflammatory cytokines in the spleen in response to systemic LPS administration. Future investigations may explore this further by injecting a peripherally acting β_2 AR antagonist such as nadolol prior to intranasal administration of Form-PEG-PLGA. If the anti-inflammatory response in the spleen is blocked by nadolol treatment, then it is likely that a portion of Form-PEG-PLGA entered blood capillaries in the nose to gain access to the general circulation and reduce LPS-induced inflammation in the spleen as described in chapter 6. However, if these effects remain in the presence of nadolol, then it is likely that the peripheral anti-inflammatory response are mediated via activation of central β_2 ARs following intranasal Form-PEG-PLGA delivery. Spleen tissue and serum were collected from animals as described in chapter 6 to be used to determine whether lower circulating and peripheral tissue drug concentrations are achievable following intranasal delivery, compared to alternative parenteral routes of administration.

8.7.2. A role for astrocytes in β_2 AR-mediated neuroprotection

Astrocytes are the most functionally diverse cells in the CNS and their role in neuroinflammation is often overlooked. While microglia are the dominant immune cell in the CNS, astrocytes play a critical role in pro- and anti-inflammatory signalling. Moreover, formoterol activation of astrocytic β_2 ARs promotes the secretion of neurotrophic factors that are vital for neuronal survival and synapse formation (Day et al., 2014). In the healthy brain, the density of astrocytes in the substantia nigra is lower than other brain regions. As astrocytes play a vital support role to neurons, fewer astrocytes in the midbrain may contribute to the selective vulnerability of dopaminergic neurons in the substantia nigra to PD pathology and degeneration (Damier et al., 1993). Therefore, astrocyte dysfunction in neurological disease is equally as detrimental as microglia overactivation.

The ability of PLGA nanoparticles to release formoterol and promote anti-inflammatory and neurotrophic signalling can be studied *in vitro* in cultures where astrocytes are the predominant cell type (also known as astroglial cultures). Typically, reactive astrocytes are triggered by the release of cytokines like TNF α from microglia. Under neuroinflammatory conditions, astrocytes release pro-inflammatory cytokines IL-1 β and IL-6, as well as chemokines including CXCL10, CCL2 and CCL5. (Zhao et al., 2024). Based on this, a future experiment may repeat the primary cell culture experiments conducted in chapter 5 using astroglial cultures stimulated by TNF α to test the efficacy of Form-PEG-PLGA and measuring mRNA levels of pro-inflammatory markers like *Il6*, *Il1b*, *CCL5* and neurotrophic factor *Bdnf* and *Ngf*.

8.7.3. Formoterol and protection of mitochondrial function

Along with dopaminergic neurodegeneration, protein misfolding and neuroinflammation, mitochondrial dysfunction is also a salient feature of PD. As midbrain dopaminergic neurons have a high energy demand, mitochondrial dysfunction is thought to be linked to the selective degeneration of this neuronal population in PD. Formoterol has recently been shown to induce mitochondrial biogenesis and improve mitochondrial function *in vitro* and *in vivo* (Chang et al., 2024; Khidr et al., 2023; Vekaria et al., 2020). Activation of the β_2 AR by formoterol is thought to improve trafficking of mitochondria to synapses and the capacity of mitochondria to buffer intracellular calcium levels in neurons (Chang et al., 2024; Vekaria et al., 2020). In chronic neuroinflammatory conditions, prolonged ROS production leads to oxidative stress and mitochondrial damage. Therefore, the anti-inflammatory and neuroprotective effects of formoterol likely also protect against mitochondrial dysfunction. As LPS can induce mitochondrial damage, immunohistochemical analysis of mitochondrial proteins such as complex I, II and IV in brain tissue from animals used in chapter 7, may further elucidate these processes.

8.7.4. Efficacy of Form-PEG-PLGA against subsequent immune challenges

The Harkin lab have previously reported that formoterol (0.1 mg/kg; i.p.) protects against an exacerbation of motor symptoms and pathology when rats treated with LPS, delivered unilaterally to the substantia nigra, received a subsequent systemic LPS challenge (O'Neill et al., 2020). In the context of delirium in PD patients, such treatments may attenuate the effects of a systemic infection on disease progression. Future experiments may involve repeating the study conducted by O'Neill et al. (2020) to determine whether intranasal Form-PEG-PLGA can produce the same effects with reduced systemic drug exposure. This would involve lesioning the substantia nigra with LPS and daily intranasal Form-PEG-PLGA administration for up to one week followed by an i.p. LPS challenge and assessment of motor function, microglial reactivity and TH⁺ cell loss. Given the anti-inflammatory and neuroprotective effects of Form-PEG-PLGA, as demonstrated in the present study when administered prior to intranigral LPS injection, it is expected that administration of Form-PEG-PLGA would prevent a worsening of central pathology subsequent to a secondary inflammatory challenge.

8.7.5. Progressing to human iPSC models

Advanced modelling and testing of new therapeutic strategies can be achieved using patient-derived iPSCs differentiated into CNS cells. Demonstrating the efficacy of Form-PEG-PLGA in human stem cell models would greatly enhance the translatability of the nanomedicine to the clinic.

Drug free PLGA nanoparticles (0.1 mg/ml; 24 hours) are compatible with neurons derived from healthy control and AD patient iPSCs (Wu et al., 2022). Several studies have used BBB models consisting of human iPSC-derived brain microvascular endothelial cells to investigate the transport of PLGA nanoparticles to the brain and reported no changes in cell viability upon exposure to unloaded particles (Danz et al., 2024; Onyema et al., 2021). However, there is no evidence in the literature that PLGA nanoparticles have been tested in microglia or astrocytes derived from human iPSCs, which is a gap in the existing knowledge that may hinder clinical translation of these treatments. To further progress the current study, unloaded PEG-PLGA could be tested in neuronal and glial cells derived from healthy and PD patient iPSCs, mimicking the study carried out in chapter 4 of this thesis to confirm that these nanoparticles are compatible with CNS cells of human origin.

Furthermore, β_2 AR agonist clenbuterol (20 μ M) reduced *SNCA* transcription, attenuated ROS production and increased cell viability in neurons derived from iPSCs from a patient carrying an *SNCA* mutation (Mittal et al., 2017). Similarly, another β_2 AR agonist isoproterenol (30 μ M) reduced the expression of endogenous tau in human iPSC derived neurons from healthy controls (Wang et al., 2017a). Collectively, this evidence supports the testing of Form-PEG-PLGA in an iPSC-based PD model, where, for example, experiments

conducted using primary cultures in chapter 5 may be replicated using patient derived stem cells differentiated into neurons and glia.

iPSC-derived neurons and glia that have either been isolated from PD patients or that have been modified to harbour disease specific mutations are now commercially available through companies like FUJIFIL Cellular Dynamics (iCell), STEMCELL technologies, Axol Bioscience and BrainXell. The availability of differentiated cells are facilitating scientists to utilise human stem cell technologies as a platform for fundamental neuroscience and drug screening in clinically relevant models. Users require cell culture experience, but expertise in stem cell maturation and differentiation, and the lengthy process that this involves, is eliminated. Accessibility to such tools is likely to revolutionise drug screening in the coming years and play a major role in personalised medicine to predict what cohorts will respond best to certain therapies or develop adverse effects and for the testing of customised cell or gene therapy for an individual.

Appendix I: Intranasal administration of Form-PEG-PLGA post-lesion does not rescue pathology or motor deficits in the intranigral LPS rat model of PD

A1.1. Aims and objectives

The aim of this study was to determine if intranasal delivery of the β_2 AR agonist formoterol-loaded PEG-PLGA nanoparticles encapsulated in a mucoadhesive, thermosensitive hydrogel can attenuate the microglial reactivity, dopaminergic cell loss and motor impairments induced by stereotactic delivery of LPS into the rat substantia nigra. From herein, all Form-PEG-PLGA formulations were incorporated into hydrogel to facilitate intranasal delivery but are referred to as Form-PEG-PLGA for simplicity.

A1.2. Experimental design

32 adult Wistar rats (15 male, 17 female) underwent baseline behavioural testing in the stepping, cylinder and rotarod tests before being randomly assigned to treatment groups (below). All animals underwent stereotactic surgery and received a unilateral 2 μ l injection of vehicle (0.89% sterile saline) or LPS (5 μ g/ μ l) to the substantia nigra (female co-ordinates from bregma: AP -5.3, ML +2.0, DV -7.5; male co-ordinates from bregma: AP -5.3, ML +2.0, DV -8.5). Rats were administered vehicle (unloaded nanoparticles in hydrogel, intranasal) or Form-PEG-PLGA (100 μ g/kg in hydrogel, intranasal) immediately after surgery and daily for 7 days (**Fig A1.1**). Two weeks post-surgery the stepping, cylinder and rotarod tests were repeated along with the amphetamine-induced rotations test. Four weeks post-surgery rats were euthanised by sodium pentobarbital overdose and transcardial perfusion/fixation. Immunohistochemical analysis of IBA1+ microglia and TH+ neuronal cell bodies in the substantia nigra was carried out to investigate microglial reactivity and neuronal integrity.

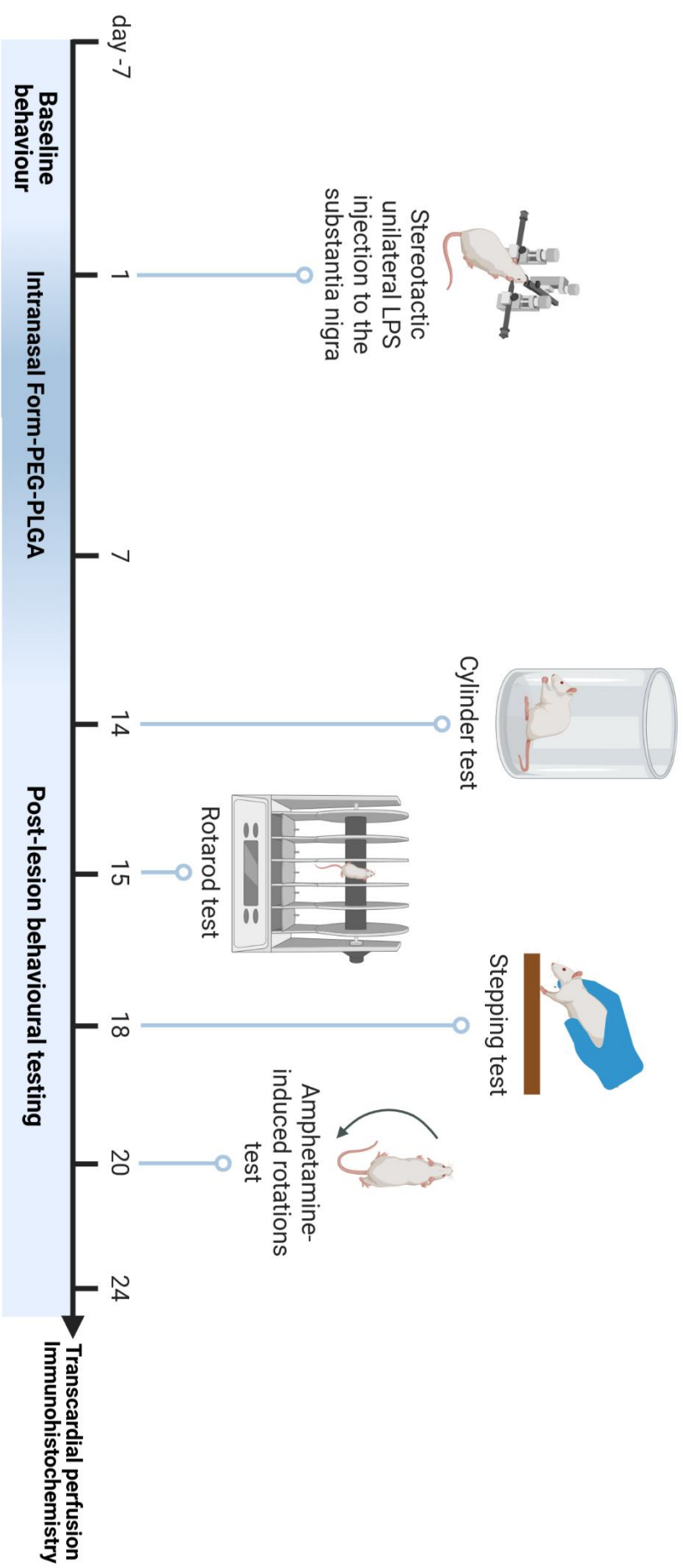


Figure A1.1 Experimental timeline.

A1.3. Results

Two-way ANOVA of the total number of rotations in the D-amphetamine-induced rotations test showed an effect of LPS [$F_{(1, 28)} = 10.44$, $P < 0.01$] (**Fig. A1.2a**). Fisher's LSD did not reveal any relevant differences between groups.

Two-way ANOVA of the number of contralateral rotations showed no effect of LPS or treatment (**Fig. A1.2b**).

Two-way ANOVA of the number of ipsilateral rotations showed an effect of LPS [$F_{(1, 28)} = 13.98$, $P < 0.001$] and treatment [$F_{(1, 28)} = 5.411$, $P < 0.05$] (**Fig. A1.2c**). Fisher's LSD revealed that the number of ipsilateral rotations made by animals that were lesioned with LPS was higher compared to vehicle control ($P < 0.05$) and this was not reduced as expected but increased in animals treated with Form-PEG-PLGA ($P < 0.05$).

Two-way ANOVA of the ratio of ipsilateral to contralateral rotations showed an effect of LPS [$F_{(1, 28)} = 7.064$, $P < 0.05$] (**Fig. A1.2d**). Fisher's LSD did not reveal any relevant differences between groups.

Two-way ANOVA of the net ipsilateral rotations showed an effect of LPS [$F_{(1, 28)} = 12.61$, $P < 0.01$] and treatment [$F_{(1, 28)} = 4.937$, $P < 0.05$] (**Fig. A1.2e**). Fisher's LSD revealed higher net ipsilateral rotations following intranigral LPS compared to vehicle control ($P < 0.05$).

Two-way ANOVA of the number of ipsilateral rotations per minute showed an effect of LPS [$F_{(1, 28)} = 12.61$, $P < 0.01$] and treatment [$F_{(1, 28)} = 4.937$, $P < 0.05$] (**Fig. A1.2f**). Fisher's LSD revealed that the number of ipsilateral rotations per minute made by animals lesioned with LPS was higher compared to vehicle control ($P < 0.05$).

Representative images of IBA1⁺ cells in the substantia nigra depicts LPS induced microgliosis that is not attenuated by Form-PEG-PLGA (**fig. A1.3**). Representative images of TH⁺ cell bodies in the substantia nigra shows cell loss in LPS treated animals that is not rescued by Form-PEG-PLGA treatment (**fig. A1.4**).

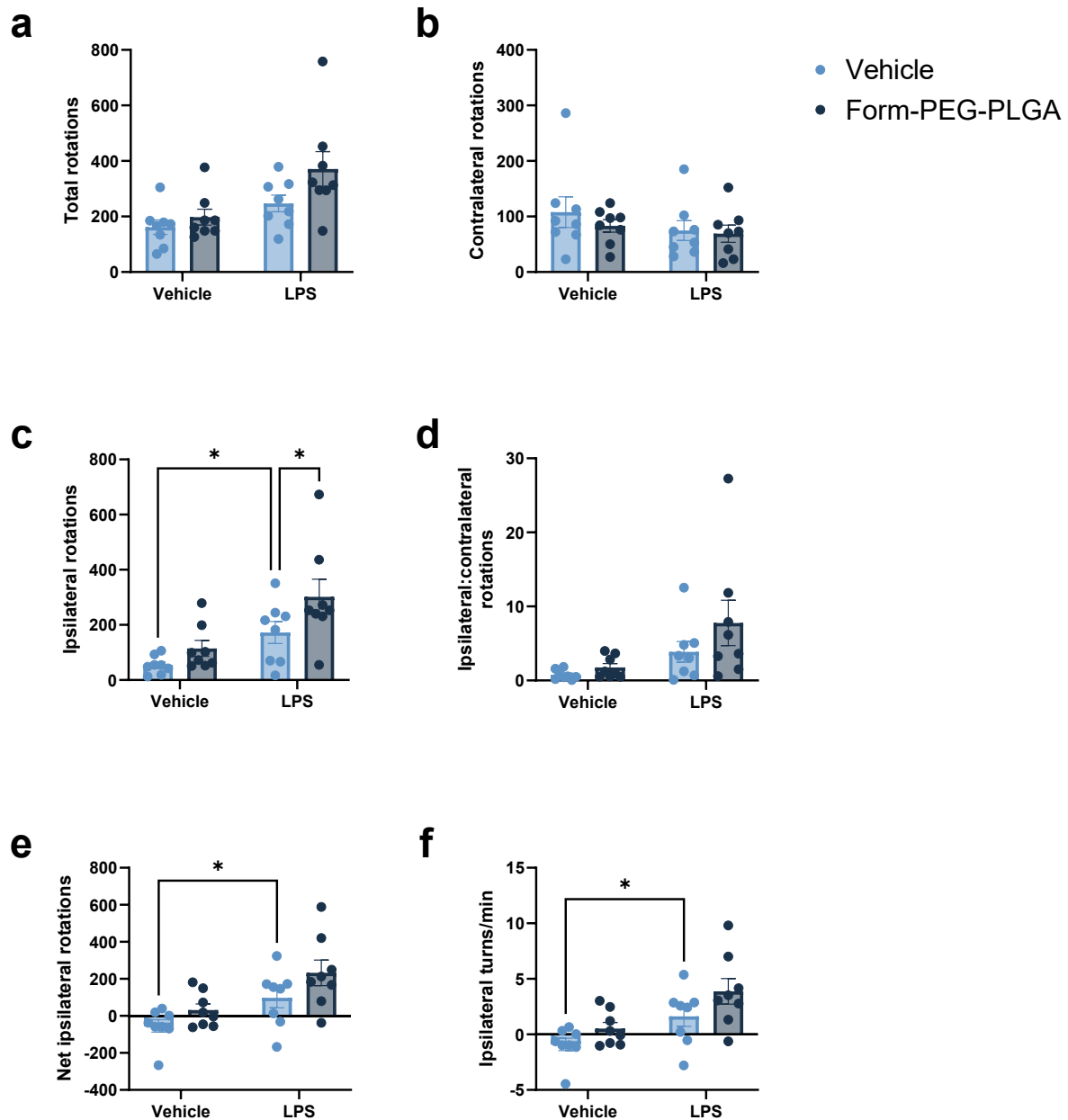


Figure A1.2. Increases in ipsilateral rotations induced by unilateral lesion of the substantia nigra in the D-amphetamine rotations test is not attenuated by intranasal Form-PEG-PLGA.

Total rotations, (b) rotations contralateral to intranigral LPS lesion, (c) rotations ipsilateral to intranigral LPS lesion, (d) ratio of ipsilateral to contralateral rotations, (e) net ipsilateral rotations and (f) ipsilateral rotations per minute in the D-amphetamine induced rotations test following intranigral LPS injection (5 $\mu\text{g}/\mu\text{l}$) and intranasal Form-PEG-PLGA (0.1 mg/kg). Data were analysed by two-way ANOVA followed by Fisher's LSD and are expressed as the mean $\pm$ SEM, where N=8.

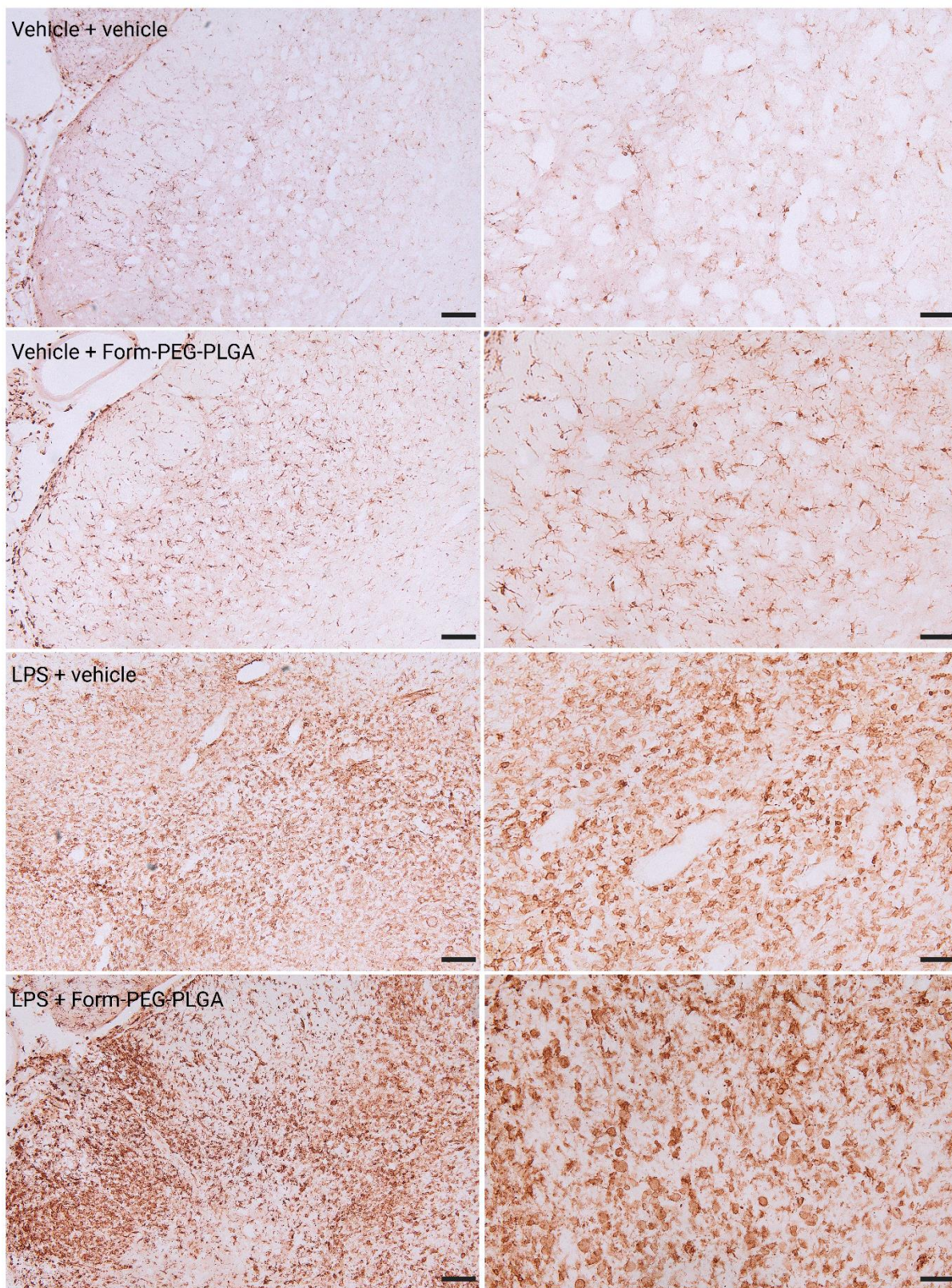


Figure A1.3. Unilateral intranigral LPS induced increases in microglial cell density is not attenuated by intranasal Form-PEG-PLGA.

Representative photomicrographs of IBA1 DAB-stained substantia nigra tissue displaying LPS (5 $\mu\text{g}/\mu\text{l}$) induced increases in microglial cell density and morphological changes are not rescued by intranasal Form-PEG-PLGA (0.1 mg/kg). Left panel: magnification 20X, scale bar = 100 μm , right panel: magnification 40X, scale bar = 50 μm .

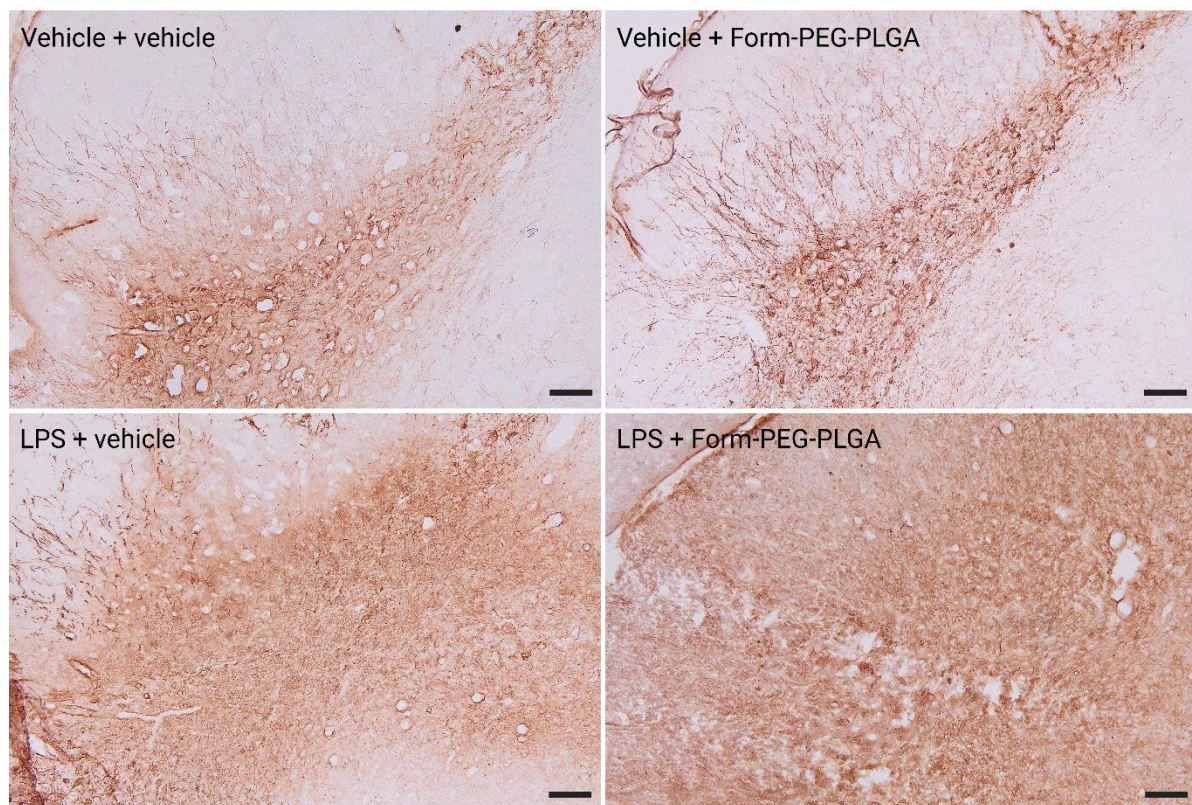


Figure A1.4. Unilateral intranigral LPS mediated TH⁺ cell loss is not rescued by intranasal Form-PEG-PLGA.

Representative photomicrographs of TH DAB-stained substantia nigra tissue displaying LPS (5 $\mu\text{g}/\mu\text{l}$) induced loss of TH⁺ cell bodies is not rescued by intranasal Form-PEG-PLGA (0.1 mg/kg). Magnification 20X where scale bar = 100 μm .

A1.4. Brief conclusion

In this study, stereotactic injection of LPS to the substantia nigra induced ipsilateral rotation behaviour following injection of D-amphetamine, which is indicative of the presence of a unilateral lesion. However, intranasal Form-PEG-PLGA administration for seven days post-lesion did not rescue ipsilateral rotational behaviour, suggesting that the formoterol released from PLGA nanoparticles was unable to alleviate LPS-mediated dopaminergic cell loss in the substantia nigra.

As discussed in chapter 7, intranigral LPS lesion did not affect motor function in the stepping or rotarod tests (data not shown). Additionally, Form-PEG-PLGA treatment had no effect on forelimb asymmetry in the cylinder test, in line with the results from the amphetamine induced rotations test (data not shown).

Due to time constraints, images from randomly selected animals from each treatment group are shown here. Extensive microgliosis is obvious in animals that received an intranigral LPS injection compared to vehicle control, however, intranasal delivery of Form-PEG-PLGA beginning immediately after surgery did not reduce microglial activation. Similarly, TH⁺ cell bodies in the substantia nigra have been obliterated by LPS injection when compared to the brains of vehicle injected animals but this cell loss was not rescued by Form-PEG-PLGA treatment. These results compliment findings from the amphetamine induced rotations test and highlight that post-lesion intranasal dosing with Form-PEG-PLGA is ineffective against LPS. However, brain tissue from the remaining animals needs to be stained so that cell counts can be performed and compared between groups.

The lack of effect observed in this study is likely attributed to slow drug release from the hydrogel-PLGA nanoparticle system and insufficient brain concentrations to immediately combat the effects of intranigral LPS. Therefore, these findings indicate that pre-dosing of intranasal Form-PEG-PLGA is necessary to attenuate motor deficits, microglial reactivity and the dopaminergic cell in the LPS rat model of PD.

Appendix II: Dissemination

List of publications

1. **Maher, R.**; Moreno-Borralló, A.; Jindal, D.; Mai, B.T.; Ruiz-Hernandez, E.; Harkin, A. Intranasal Polymeric and Lipid-Based Nanocarriers for CNS Drug Delivery. *Pharmaceutics* 2023, 15, 746. <https://doi.org/10.3390/pharmaceutics15030746>
2. Moreno-Borralló, A.; **Maher, R.**; Gómez-González, E.; Jain, A.; Santosh Alex, T.; Harkin, A.; Mai, B.T.; Ruiz-Hernandez, E. Stimuli-Responsive Polymer Biomaterials: Shaping the Future of Glioblastoma Therapy (in preparation)

Dissemination plan

Title for manuscript: Development of Formoterol-loaded PLGA nanoparticles for CNS targeting; *in vitro* validation and *in vivo* pharmacokinetics

Authors: Rebecca Maher, Devin Seward, Almudena Moreno-Borralló, Thomson Santosh Alex, Elisabet Gomez Gonzalez, Ayushi Jain, Christopher Sheridan, Eduardo Ruiz-Hernandez, Andrew Harkin.

Title for manuscript: Anti-inflammatory and neuroprotective effects of Formoterol-loaded PLGA nanoparticles in the unilateral intranigral LPS model of PD associated neuroinflammation

Authors: Rebecca Maher, Myles Corrigan, Almudena Moreno-Borralló, Thomson Santosh Alex, Eduardo Ruiz-Hernandez, Andrew Harkin.

Poster presentations

‘An *in vitro* assessment of the biocompatibility of polymeric nanoparticles in primary neuronal and glial cell cultures.’ Rebecca Maher, Almudena Moreno-Borralló, Eduardo Ruiz-Hernandez & Andrew Harkin. *All Ireland School of Pharmacy Conference, Belfast 2023*

‘PLGA nanoparticles as drug delivery tools for brain degenerative disorders; *in vitro* assessment and release properties.’ Rebecca Maher, Almudena Moreno-Borralló, Thomson Santosh Alex, Elisabet Gomez Gonzalez, Ayushi Jain, Eduardo Ruiz-Hernandez & Andrew Harkin. *Federation of European Neuroscience (FENS), Vienna 2024, All Ireland School of Pharmacy Conference, Dublin 2024*

‘PLGA nanoparticles as drug delivery tools for brain degenerative disorders; *in vitro* assessment and release properties.’ Rebecca Maher, Almudena Moreno-Borralló, Thomson Santosh Alex, Elisabet Gomez Gonzalez, Ayushi Jain, Eduardo Ruiz-Hernandez & Andrew Harkin. *All Ireland School of Pharmacy Conference, Dublin 2024*

‘Formoterol-PEG-PLGA nanoparticles produce anti-inflammatory effects in primary glial cultures and in the rat brain following intranasal delivery.’ Rebecca Maher, Almudena Moreno-Borrillo, Thomson Santosh Alex, Eduardo Ruiz-Hernandez & Andrew Harkin. *British Neuroscience Association, Liverpool 2025*

‘Formoterol-PEG-PLGA nanoparticles produce anti-inflammatory effects in primary glial cultures and in the rat brain following intranasal delivery.’ Rebecca Maher, Almudena Moreno-Borrillo, Thomson Santosh Alex, Oliviero L. Gobbo, Eduardo Ruiz-Hernandez & Andrew Harkin. *European Society for Biomaterials (ESB), Turin 2025*

Oral presentations

‘Development of a Drug Nanocarrier Targeting Neuroinflammation and Degeneration.’ *School of pharmacy seminar series, 2024*

‘Development of a Drug Nanocarrier Targeting Neuroinflammation and Degeneration.’ *TCIN Neuroscience research day, 2025*

Public and Patient Involvement (PPI)/outreach

Lewy Body Ireland (LBI) 2021-2023

LBI is an Irish charity that aims to raise awareness about Lewy Body dementia (LBD) and support both patients and caregivers affected by this disease. This work involved attending monthly online support groups ‘Tea-time with Lewy’ to share recent scientific advances to attendees and listen to how LBD affects patients and their families daily to best inform research. During this time, I also collated a research directory of relevant scientific articles including a lay summary of each article and a list of clinicians, researchers, nurses and advocates who specialise in LBD for the LBI website.

Culture night 2022, 2023

The Trinity College Pratchett project is an interdisciplinary endeavour to bring researchers together from diverse fields of study inspired by the life and work of Sir Terry Pratchett. Terry Pratchett was diagnosed with a rare form of Alzheimer’s disease called posterior cortical atrophy (PCA). Following this diagnosis, he became an advocate for patients living with the disease and generously donated to fund research. Since his death, the Pratchett project in Trinity College has continued to honour his legacy. Over the course of my project a few events were organised on culture night in Dublin in 2022 and 2023, in collaboration with scientists, clinicians, poets, actors, patients and advocates, to raise awareness of PCA, highlight the importance of brain health and the role of the arts in promoting it. This work included supporting event promotion, coordinating speaker invitations and logistics, and presenting on PPI activities with LBI.

Bibliography

- Abdulla, D., & Renton, K. W. (2005). β -Adrenergic receptor modulation of the LPS-mediated depression in CYP1A activity in astrocytes. *Biochemical Pharmacology*, 69(5), 741-750. <https://doi.org/https://doi.org/10.1016/j.bcp.2004.11.020>
- Abou-Taleb, B. A., & El-Ganainy, S. O. (2023). Thermoresponsive Gel-loaded Oxcarbazepine Nanosystems for Nose- To-Brain Delivery: Enhanced Antiepileptic Activity in Rats. *Pharm Res*, 40(7), 1835-1852. <https://doi.org/10.1007/s11095-023-03552-7>
- af Bjerkén, S., Stenmark Persson, R., Barkander, A., Karalija, N., Pelegrina-Hidalgo, N., Gerhardt, G. A., Virel, A., & Strömberg, I. (2019). Noradrenaline is crucial for the substantia nigra dopaminergic cell maintenance. *Neurochemistry international*, 131, 104551. <https://doi.org/https://doi.org/10.1016/j.neuint.2019.104551>
- Agrawal, M., Saraf, S., Saraf, S., Dubey, S. K., Puri, A., Gupta, U., Kesharwani, P., Ravichandiran, V., Kumar, P., Naidu, V. G. M., Murty, U. S., Ajazuddin, & Alexander, A. (2020). Stimuli-responsive In situ gelling system for nose-to-brain drug delivery. *Journal of Controlled Release*, 327, 235-265. <https://doi.org/https://doi.org/10.1016/j.jconrel.2020.07.044>
- Ahlström Emanuelsson, C., Andersson, M., Persson, C. G. A., Thorsson, L., & Greiff, L. (2007). Effects of topical formoterol alone and in combination with budesonide in a pollen season model of allergic rhinitis. *Respiratory Medicine*, 101(6), 1106-1112. <https://doi.org/https://doi.org/10.1016/j.rmed.2006.11.017>
- Ahmad, M. Z., Sabri, A. H. B., Anjani, Q. K., Domínguez-Robles, J., Abdul Latip, N., & Hamid, K. A. (2022a). Design and Development of Levodopa Loaded Polymeric Nanoparticles for Intranasal Delivery. *Pharmaceuticals (Basel)*, 15(3). <https://doi.org/10.3390/ph15030370>
- Ahmad, N., Ahmad, R., Alrasheed, R., Almatar, H., Al-Ramadan, A., Amir, M., & Sarafroz, M. (2020). Quantification and Evaluations of Catechin Hydrate Polymeric Nanoparticles Used in Brain Targeting for the Treatment of Epilepsy. *Pharmaceutics*, 12(3), 203. <https://doi.org/10.3390/pharmaceutics12030203>
- Ahmad, S., Khan, I., Pandit, J., Emad, N. A., Bano, S., Dar, K. I., Rizvi, M. M. A., Ansari, M. D., Aqil, M., & Sultana, Y. (2022b). Brain targeted delivery of carmustine using chitosan coated nanoparticles via nasal route for glioblastoma treatment. *Int J Biol Macromol*, 221, 435-445. <https://doi.org/10.1016/j.ijbiomac.2022.08.210>
- Akdaşçı, E., Eker, F., Duman, H., Singh, P., Bechelany, M., & Karav, S. (2024). Lactoferrin as a Versatile Agent in Nanoparticle Applications: From Therapeutics to Agriculture. *Nanomaterials*, 14(24), 2018. <https://www.mdpi.com/2079-4991/14/24/2018>
- Akiyama, H., & McGeer, P. L. (1989). Microglial response to 6-hydroxydopamine-induced substantia nigra lesions. *Brain Res*, 489(2), 247-253. [https://doi.org/10.1016/0006-8993\(89\)90857-3](https://doi.org/10.1016/0006-8993(89)90857-3)
- Aksungur, P., Demirbilek, M., Denkbaş, E. B., Vandervoort, J., Ludwig, A., & Ünlü, N. (2011). Development and characterization of Cyclosporine A loaded

- nanoparticles for ocular drug delivery: Cellular toxicity, uptake, and kinetic studies. *Journal of Controlled Release*, 151(3), 286-294. <https://doi.org/https://doi.org/10.1016/j.jconrel.2011.01.010>
- Al-Bachari, S., Naish, J. H., Parker, G. J., Emsley, H. C., & Parkes, L. M. (2020). Blood–brain barrier leakage is increased in Parkinson’s disease. *Frontiers in physiology*, 11, 593026.
- Aldred, J., & Nutt, J. G. (2010). Levodopa. In K. Kompoliti & L. V. Metman (Eds.), *Encyclopedia of Movement Disorders* (pp. 132-137). Academic Press. <https://doi.org/https://doi.org/10.1016/B978-0-12-374105-9.00340-3>
- Alghareeb, S., Asare-Addo, K., Conway, B. R., & Adebisi, A. O. (2024). PLGA nanoparticles for nasal drug delivery. *Journal of Drug Delivery Science and Technology*, 95, 105564. <https://doi.org/https://doi.org/10.1016/j.jddst.2024.105564>
- Almahmoud, A., Parekh, H. S., Paterson, B. M., Tupally, K. R., & Vegh, V. (2024). Intranasal delivery of imaging agents to the brain. *Theranostics*, 14(13), 5022.
- Alqahtani, M. S., Kazi, M., Alsenaidy, M. A., & Ahmad, M. Z. (2021). Advances in oral drug delivery. *Frontiers in pharmacology*, 12, 618411.
- Alsaab, H. O., Alharbi, F. D., Alhibs, A. S., Alanazi, N. B., Alshehri, B. Y., Saleh, M. A., Alshehri, F. S., Algarni, M. A., Almugaiteeb, T., Uddin, M. N., & Alzhrani, R. M. (2022). PLGA-Based Nanomedicine: History of Advancement and Development in Clinical Applications of Multiple Diseases. *Pharmaceutics*, 14(12). <https://doi.org/10.3390/pharmaceutics14122728>
- Alvites, R. D., Caseiro, A. R., Pedrosa, S. S., Branquinho, M. E., Varejão, A. S. P., & Maurício, A. C. (2018). The Nasal Cavity of the Rat and Mouse-Source of Mesenchymal Stem Cells for Treatment of Peripheral Nerve Injury. *Anat Rec (Hoboken)*, 301(10), 1678-1689. <https://doi.org/10.1002/ar.23844>
- Annu, Sartaj, A., Qamar, Z., Md, S., Alhakamy, N. A., Baboota, S., & Ali, J. (2022). An Insight to Brain Targeting Utilizing Polymeric Nanoparticles: Effective Treatment Modalities for Neurological Disorders and Brain Tumor. *Front Bioeng Biotechnol*, 10, 788128. <https://doi.org/10.3389/fbioe.2022.788128>
- Anselmo, A. C., & Mitragotri, S. (2021). Nanoparticles in the clinic: An update post <scp>COVID</scp> -19 vaccines. *Bioengineering & Translational Medicine*, 6(3). <https://doi.org/10.1002/btm2.10246>
- Aoki, I., Wu, Y.-J. L., Silva, A. C., Lynch, R. M., & Koretsky, A. P. (2004). In vivo detection of neuroarchitecture in the rodent brain using manganese-enhanced MRI. *Neuroimage*, 22(3), 1046-1059.
- Arafa, M. G., Mousa, H. A., Kataia, M. M., M, S., & Afifi, N. N. (2023). Functionalized surface of PLGA nanoparticles in thermosensitive gel to enhance the efficacy of antibiotics against antibiotic resistant infections in endodontics: A randomized clinical trial. *Int J Pharm X*, 6, 100219. <https://doi.org/10.1016/j.ijpx.2023.100219>
- Arai, H., Furuya, T., Yasuda, T., Miura, M., Mizuno, Y., & Mochizuki, H. (2004). Neurotoxic effects of lipopolysaccharide on nigral dopaminergic neurons are mediated by microglial activation, interleukin-1beta, and expression of caspase-11 in mice. *J Biol Chem*, 279(49), 51647-51653. <https://doi.org/10.1074/jbc.M407328200>

- Archer, T., & Fredriksson, A. (2006). Influence of noradrenaline denervation on MPTP-induced deficits in mice. *J Neural Transm (Vienna)*, 113(9), 1119-1129. <https://doi.org/10.1007/s00702-005-0402-5>
- Arisoy, S., Sayiner, O., Comoglu, T., Onal, D., Atalay, O., & Pehlivanoglu, B. (2020). *In vitro* and *in vivo* evaluation of levodopa-loaded nanoparticles for nose to brain delivery. *Pharmaceutical Development and Technology*, 25(6), 735-747. <https://doi.org/10.1080/10837450.2020.1740257>
- Aryal, M., Arvanitis, C. D., Alexander, P. M., & McDannold, N. (2014). Ultrasound-mediated blood-brain barrier disruption for targeted drug delivery in the central nervous system. *Adv Drug Deliv Rev*, 72, 94-109. <https://doi.org/10.1016/j.addr.2014.01.008>
- Bakhaidar, R., Green, J., Alfahad, K., Samanani, S., Moollan, N., O'Neill, S., & Ramtoola, Z. (2019). Effect of Size and Concentration of PLGA-PEG Nanoparticles on Activation and Aggregation of Washed Human Platelets. *Pharmaceutics*, 11(10). <https://doi.org/10.3390/pharmaceutics11100514>
- Bakhaidar, R., O'Neill, S., & Ramtoola, Z. (2020). PLGA-PEG Nanoparticles Show Minimal Risks of Interference with Platelet Function of Human Platelet-Rich Plasma. *Int J Mol Sci*, 21(24). <https://doi.org/10.3390/ijms21249716>
- Baloyannis, S. J., Costa, V., & Baloyannis, I. S. (2006). Morphological alterations of the synapses in the locus coeruleus in Parkinson's disease. *J Neurol Sci*, 248(1-2), 35-41. <https://doi.org/10.1016/j.jns.2006.05.006>
- Barrett, M. J., Sargent, L., Nawaz, H., Weintraub, D., Price, E. T., & Willis, A. W. (2021). Antimuscarinic Anticholinergic Medications in Parkinson Disease: To Prescribe or Deprescribe? *Mov Disord Clin Pract*, 8(8), 1181-1188. <https://doi.org/10.1002/mdc3.13347>
- Batta, A., Kalra, B. S., & Khirasaria, R. (2020). Trends in FDA drug approvals over last 2 decades: An observational study. *J Family Med Prim Care*, 9(1), 105-114. https://doi.org/10.4103/jfmprc.jfmprc_578_19
- Begines, B., Ortiz, T., Pérez-Aranda, M., Martínez, G., Merinero, M., Argüelles-Arias, F., & Alcudia, A. (2020). Polymeric nanoparticles for drug delivery: Recent developments and future prospects. *Nanomaterials*, 10(7), 1403.
- Beier, E. E., Neal, M., Alam, G., Edler, M., Wu, L.-J., & Richardson, J. R. (2017). Alternative microglial activation is associated with cessation of progressive dopamine neuron loss in mice systemically administered lipopolysaccharide. *Neurobiology of disease*, 108, 115-127. <https://doi.org/10.1016/j.nbd.2017.08.009>
- Bekhat, M., Merrill, L., Kelly, S. D., Lee, V. K., & Neigh, G. N. (2016). Brief anesthesia by isoflurane alters plasma corticosterone levels distinctly in male and female rats: implications for tissue collection methods. *Behavioural brain research*, 305, 122-125.
- Benveniste, E. N., Nguyen, V. T., & Wesemann, D. R. (2004). Molecular regulation of CD40 gene expression in macrophages and microglia. *Brain, behavior, and immunity*, 18(1), 7-12. <https://doi.org/10.1016/j.bbi.2003.09.001>
- Bérard, M., Martínez-Drudis, L., Sheta, R., El-Agnaf, O. M. A., & Oueslati, A. (2023). Non-invasive systemic viral delivery of human alpha-synuclein mimics selective and progressive neuropathology of Parkinson's disease in rodent

- brains. *Mol Neurodegener*, 18(1), 91. <https://doi.org/10.1186/s13024-023-00683-8>
- Bertrand, E., Lechowicz, W., Szpak, G. M., & Dymecki, J. (1997). Qualitative and quantitative analysis of locus coeruleus neurons in Parkinson's disease. *Folia Neuropathol*, 35(2), 80-86.
- Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., & Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci*, 3(12), 1301-1306. <https://doi.org/10.1038/81834>
- Bhattacharjee, S. (2016). DLS and zeta potential—what they are and what they are not? *Journal of Controlled Release*, 235, 337-351.
- Bi, C., Wang, A., Chu, Y., Liu, S., Mu, H., Liu, W., Wu, Z., Sun, K., & Li, Y. (2016). Intranasal delivery of rotigotine to the brain with lactoferrin-modified PEG-PLGA nanoparticles for Parkinson's disease treatment. *Int J Nanomedicine*, 11, 6547-6559. <https://doi.org/10.2147/ijn.S120939>
- Bianchetti, M. E., Ferreira, A. F. F., & Britto, L. R. G. (2024). Inhibition of neuroinflammation by GIBH-130 (AD-16) reduces neurodegeneration, motor deficits, and proinflammatory cytokines in a hemiparkinsonian model. *Front Neuroanat*, 18, 1511951. <https://doi.org/10.3389/fnana.2024.1511951>
- Bicker, J., Fortuna, A., Alves, G., Soares-da-Silva, P., & Falcão, A. (2017). Elucidation of the Impact of P-glycoprotein and Breast Cancer Resistance Protein on the Brain Distribution of Catechol-O-Methyltransferase Inhibitors. *Drug Metab Dispos*, 45(12), 1282-1291. <https://doi.org/10.1124/dmd.117.077883>
- Björklund, A., & Dunnett, S. B. (2019). The amphetamine induced rotation test: a re-assessment of its use as a tool to monitor motor impairment and functional recovery in rodent models of Parkinson's disease. *Journal of Parkinson's disease*, 9(1), 17-29.
- Bloem, B. R., Okun, M. S., & Klein, C. (2021). Parkinson's disease. *Lancet*, 397(10291), 2284-2303. [https://doi.org/10.1016/s0140-6736\(21\)00218-x](https://doi.org/10.1016/s0140-6736(21)00218-x)
- Bodea, L. G., Wang, Y., Linnartz-Gerlach, B., Kopatz, J., Sinkkonen, L., Musgrove, R., Kaoma, T., Muller, A., Vallar, L., Di Monte, D. A., Balling, R., & Neumann, H. (2014). Neurodegeneration by activation of the microglial complement-phagosome pathway. *J Neurosci*, 34(25), 8546-8556. <https://doi.org/10.1523/jneurosci.5002-13.2014>
- Bon, I. E. K., T.V.; Kokhan, N.V. (2022). "Trigeminal Sensor System of the Rat, Development and Morphofunctional Features". *Biomedical Journal of Scientific & Technical Research*, 44(4). <https://doi.org/10.26717/bjstr.2022.44.007091>
- Bondarenko, O., & Saarma, M. (2021). Neurotrophic Factors in Parkinson's Disease: Clinical Trials, Open Challenges and Nanoparticle-Mediated Delivery to the Brain. *Frontiers in cellular neuroscience*, 15. <https://doi.org/10.3389/fncel.2021.682597>
- Bovone, G., Cousin, L., Steiner, F., & Tibbitt, M. W. (2022). Solvent Controls Nanoparticle Size during Nanoprecipitation by Limiting Block Copolymer Assembly. *Macromolecules*, 55(18), 8040-8048. <https://doi.org/10.1021/acs.macromol.2c00907>

- Braak, H., & Del Tredici, K. (2016). Potential Pathways of Abnormal Tau and α -Synuclein Dissemination in Sporadic Alzheimer's and Parkinson's Diseases. *Cold Spring Harb Perspect Biol*, 8(11). <https://doi.org/10.1101/cshperspect.a023630>
- Braak, H., Del Tredici, K., Rüb, U., de Vos, R. A., Jansen Steur, E. N., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging*, 24(2), 197-211. [https://doi.org/10.1016/s0197-4580\(02\)00065-9](https://doi.org/10.1016/s0197-4580(02)00065-9)
- Braak, H., Ghebremedhin, E., Rüb, U., Bratzke, H., & Del Tredici, K. (2004). Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res*, 318(1), 121-134. <https://doi.org/10.1007/s00441-004-0956-9>
- Brenner, A. J., Patel, T., Bao, A., Phillips, W. T., Michalek, J. E., Youssef, M., Weinberg, J. S., Kamiya Matsuoka, C., Hedrick, M. H., LaFrance, N., Moore, M., & Floyd, J. R. (2025). Convection enhanced delivery of Rhenium (186Re) Obisbameda (186RNL) in recurrent glioma: a multicenter, single arm, phase 1 clinical trial. *Nature communications*, 16(1), 2079. <https://doi.org/10.1038/s41467-025-57263-1>
- Caestecker, S., Lescrauwaet, E., Boon, P., Carrette, E., Raedt, R., & Vonck, K. (2025). The Locus Coeruleus-Noradrenergic System in the Healthy and Diseased Brain: A Narrative Review. *Eur J Neurol*, 32(9), e70337. <https://doi.org/10.1111/ene.70337>
- Calabresi, P., Mechelli, A., Natale, G., Volpicelli-Daley, L., Di Lazzaro, G., & Ghiglieri, V. (2023). Alpha-synuclein in Parkinson's disease and other synucleinopathies: from overt neurodegeneration back to early synaptic dysfunction. *Cell Death & Disease*, 14(3), 176. <https://doi.org/10.1038/s41419-023-05672-9>
- Caldera-Villalobos, M., Ramos-Montañez, D. G., Cabrera-Munguía, D. A., Becerra-Rodríguez, J. J., Rodríguez-Fuentes, N., & Claudio-Rizo, J. A. (2025). Biocompatible hydrogels comprised of collagen, chitosan and polyurethane with potential applications for wound healing and controlled drug release. *Polymer International*, 74(1), 9-19. <https://doi.org/https://doi.org/10.1002/pi.6590>
- Cankara, F. N., Kuş, M. S., Günaydin, C., Şafak, S., Bilge, S. S., Ozmen, O., Tural, E., & Kortholt, A. (2022). The beneficial effect of salubrinal on neuroinflammation and neuronal loss in intranigral LPS-induced hemi-Parkinson disease model in rats. *Immunopharmacology and Immunotoxicology*, 44(2), 168-177.
- Cano, A., Ettcheto, M., Espina, M., Auladell, C., Folch, J., Kühne, B. A., Barenys, M., Sánchez-López, E., Souto, E. B., & García, M. L. (2020). Epigallocatechin-3-gallate PEGylated poly (lactic-co-glycolic) acid nanoparticles mitigate striatal pathology and motor deficits in 3-nitropropionic acid intoxicated mice. *Nanomedicine*, 16(1), 19-35.
- Casanova, Y., Negro, S., Slowing, K., García-García, L., Fernández-Carballido, A., Rahmani, M., & Barcia, E. (2022). Micro- and Nano-Systems Developed for Tolcapone in Parkinson's Disease. *Pharmaceutics*, 14(5). <https://doi.org/10.3390/pharmaceutics14051080>
- Casas, A. I., Hassan, A. A., Larsen, S. J., Gomez-Rangel, V., Elbatreek, M., Kleikers, P. W. M., Guney, E., Egea, J., López, M. G., Baumbach, J., & Schmidt, H. H. H. W. (2019). From single drug targets to synergistic network pharmacology in ischemic stroke. *Proceedings of the National Academy of Sciences*, 116(14), 7129-7136. <https://doi.org/doi:10.1073/pnas.1820799116>

- Casey, L. M., Kakade, S., Decker, J. T., Rose, J. A., Deans, K., Shea, L. D., & Pearson, R. M. (2019). Cargo-less nanoparticles program innate immune cell responses to toll-like receptor activation. *Biomaterials*, 218, 119333. <https://doi.org/10.1016/j.biomaterials.2019.119333>
- Castano, A., Herrera, A., Cano, J., & Machado, A. (2002). The degenerative effect of a single intranigral injection of LPS on the dopaminergic system is prevented by dexamethasone, and not mimicked by rh-TNF- α , IL-1 β and IFN- γ . *Journal of neurochemistry*, 81(1), 150-157.
- Castaño, A., Herrera, A. J., Cano, J., & Machado, A. (1998). Lipopolysaccharide intranigral injection induces inflammatory reaction and damage in nigrostriatal dopaminergic system. *J Neurochem*, 70(4), 1584-1592. <https://doi.org/10.1046/j.1471-4159.1998.70041584.x>
- Castellanos, G., Fernández-Seara, M. A., Lorenzo-Betancor, O., Ortega-Cubero, S., Puigvert, M., Uranga, J., Vidorreta, M., Irigoyen, J., Lorenzo, E., Muñoz-Barrutia, A., Ortiz-de-Solorzano, C., Pastor, P., & Pastor, M. A. (2015). Automated neuromelanin imaging as a diagnostic biomarker for Parkinson's disease. *Mov Disord*, 30(7), 945-952. <https://doi.org/10.1002/mds.26201>
- Cayero-Otero, M. D., Gomes, M. J., Martins, C., Álvarez-Fuentes, J., Fernández-Arévalo, M., Sarmento, B., & Martín-Banderas, L. (2019). In vivo biodistribution of venlafaxine-PLGA nanoparticles for brain delivery: plain vs. functionalized nanoparticles. *Expert Opin Drug Deliv*, 16(12), 1413-1427. <https://doi.org/10.1080/17425247.2019.1690452>
- Chai, G.-s., Wang, Y.-y., Yasheng, A., & Zhao, P. (2016). Beta 2-adrenergic receptor activation enhances neurogenesis in Alzheimer's disease mice. *Neural regeneration research*, 11(10), 1617-1624.
- Chai, G.-s., Wu, J.-j., Gong, J., Zhou, J.-l., Jiang, Z.-q., Yi, H.-y., Gu, Y., Huang, H.-h., Yao, Z.-y., & Zhang, Y.-q. (2022). Activation of β 2-adrenergic receptor ameliorates amyloid- β -induced mitophagy defects and tau pathology in mice. *Neuroscience*, 505, 34-50.
- Chan-Palay, V., & Asan, E. (1989). Alterations in catecholamine neurons of the locus coeruleus in senile dementia of the Alzheimer type and in Parkinson's disease with and without dementia and depression. *J Comp Neurol*, 287(3), 373-392. <https://doi.org/10.1002/cne.902870308>
- Chan, J. M., Valencia, P. M., Zhang, L., Langer, R., & Farokhzad, O. C. (2010). Polymeric nanoparticles for drug delivery. In *Cancer Nanotechnology* (pp. 163-175). Springer.
- Chang, J. C., Chang, H. S., Chao, Y. C., Huang, C. S., Lin, C. H., Wu, Z. S., Chang, H. J., Liu, C. S., & Chuang, C. S. (2024). Formoterol Acting via β 2-Adrenoreceptor Restores Mitochondrial Dysfunction Caused by Parkinson's Disease-Related UQCRC1 Mutation and Improves Mitochondrial Homeostasis Including Dynamic and Transport. *Biology (Basel)*, 13(4). <https://doi.org/10.3390/biology13040231>
- Chaplin, A., Gao, H., Asase, C., Rengasamy, P., Park, B., Skander, D., Bebek, G., Rajagopalan, S., & Maisseyeu, A. (2020). Systemically-delivered biodegradable PLGA alters gut microbiota and induces transcriptomic reprogramming in the liver in an obesity mouse model. *Sci Rep*, 10(1), 13786. <https://doi.org/10.1038/s41598-020-69745-x>

- Chatterjee, M., & Chanda, N. (2022). Formulation of PLGA nano-carriers: specialized modification for cancer therapeutic applications. *Materials Advances*, 3(2), 837-858. <https://doi.org/10.1039/d1ma00600b>
- Chatzitaki, A. T., Jesus, S., Karavasili, C., Andreadis, D., Fatouros, D. G., & Borges, O. (2020). Chitosan-coated PLGA nanoparticles for the nasal delivery of ropinirole hydrochloride: In vitro and ex vivo evaluation of efficacy and safety. *Int J Pharm*, 589, 119776. <https://doi.org/10.1016/j.ijpharm.2020.119776>
- Chen, H., Jacobs, E., Schwarzschild, M. A., McCullough, M. L., Calle, E. E., Thun, M. J., & Ascherio, A. (2005). Nonsteroidal antiinflammatory drug use and the risk for Parkinson's disease. *Ann Neurol*, 58(6), 963-967. <https://doi.org/10.1002/ana.20682>
- Chen, X., Hu, Y., Cao, Z., Liu, Q., & Cheng, Y. (2018). Cerebrospinal Fluid Inflammatory Cytokine Aberrations in Alzheimer's Disease, Parkinson's Disease and Amyotrophic Lateral Sclerosis: A Systematic Review and Meta-Analysis. *Front Immunol*, 9, 2122. <https://doi.org/10.3389/fimmu.2018.02122>
- Chen, Y.-B., Qiao, T., Wang, Y.-Q., Cui, Y.-L., & Wang, Q.-S. (2022). Hydrogen bond-enhanced nanogel delivery system for potential intranasal therapy of Parkinson's disease. *Materials & Design*, 219, 110741. <https://doi.org/10.1016/j.matdes.2022.110741>
- Cheng, Z., Huang, H., Yin, M., & Liu, H. (2025). Applications of liposomes and lipid nanoparticles in cancer therapy: current advances and prospects. *Experimental Hematology & Oncology*, 14(1), 11. <https://doi.org/10.1186/s40164-025-00602-1>
- Cho, B. P., Song, D. Y., Sugama, S., Shin, D. H., Shimizu, Y., Kim, S. S., Kim, Y. S., & Joh, T. H. (2006). Pathological dynamics of activated microglia following medial forebrain bundle transection. *Glia*, 53(1), 92-102. <https://doi.org/10.1002/glia.20265>
- Chruscinski, A., Brede, M. E., Meinel, L., Lohse, M. J., Kobilka, B. K., & Hein, L. (2001). Differential distribution of beta-adrenergic receptor subtypes in blood vessels of knockout mice lacking beta(1)- or beta(2)-adrenergic receptors. *Mol Pharmacol*, 60(5), 955-962. <https://doi.org/10.1124/mol.60.5.955>
- Chung, S., Peters, J. M., Detyniecki, K., Tatum, W., Rabinowicz, A. L., & Carrazana, E. (2023). The nose has it: Opportunities and challenges for intranasal drug administration for neurologic conditions including seizure clusters. *Epilepsy Behav Rep*, 21, 100581. <https://doi.org/10.1016/j.ebr.2022.100581>
- Cicchetti, F., Drouin-Ouellet, J., & Gross, R. E. (2009). Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? *Trends Pharmacol Sci*, 30(9), 475-483. <https://doi.org/10.1016/j.tips.2009.06.005>
- Çomoğlu, T. (2022). Evaluation of Drug Release Kinetics of Temozolomide Loaded Plga Nanoparticles in Pluronic® F-127 Hydrogel. *Bezmialem Science*, 10(6), 735-741. <https://doi.org/10.14235/bas.galenos.2022.40427>
- Cong, H., Hu, J., Wang, J., Chang, B., Li, R., Cui, X., Zhang, C., Ji, H., Lin, C., Tang, J., & Liu, J. (2025). Bromocriptine mesylate-loaded nanoparticles co-modified with low molecular weight protamine and lactoferrin for enhanced nose-to-brain delivery in Parkinson's disease treatment. *Int J Pharm*, 669, 125054. <https://doi.org/10.1016/j.ijpharm.2024.125054>

- Cooper, W., Ray, S., Aurora, S. K., Shrewsbury, S. B., Fuller, C., Davies, G., & Hoekman, J. (2022). Delivery of Dihydroergotamine Mesylate to the Upper Nasal Space for the Acute Treatment of Migraine: Technology in Action. *Journal of Aerosol Medicine and Pulmonary Drug Delivery*, 35(6), 321-332. <https://doi.org/10.1089/jamp.2022.0005>
- Correale, J., & Villa, A. (2009). Cellular Elements of the Blood-Brain Barrier. *Neurochemical Research*, 34(12), 2067-2077. <https://doi.org/10.1007/s11064-009-0081-y>
- Creemers, J. H. A., Pawlitzky, I., Grosios, K., Gileadi, U., Middleton, M. R., Gerritsen, W. R., Mehra, N., Rivoltini, L., Walters, I., Figdor, C. G., Ottevanger, P. B., & de Vries, I. J. M. (2021). Assessing the safety, tolerability and efficacy of PLGA-based immunomodulatory nanoparticles in patients with advanced NY-ESO-1-positive cancers: a first-in-human phase I open-label dose-escalation study protocol. *BMJ Open*, 11(11), e050725. <https://doi.org/10.1136/bmjopen-2021-050725>
- Crowley, T., Fitzpatrick, J.-M., Kuijper, T., Cryan, J. F., O'Toole, O., O'Leary, O. F., & Downer, E. J. (2015). Modulation of TLR3/TLR4 inflammatory signaling by the GABAB receptor agonist baclofen in glia and immune cells: relevance to therapeutic effects in multiple sclerosis [Original Research]. *Frontiers in cellular neuroscience*, Volume 9 - 2015. <https://doi.org/10.3389/fncel.2015.00284>
- Cruz, L. J., Stammes, M. A., Que, I., van Beek, E. R., Knol-Blankevoort, V. T., Snoeks, T. J. A., Chan, A., Kaijzel, E. L., & Löwik, C. (2016). Effect of PLGA NP size on efficiency to target traumatic brain injury. *J Control Release*, 223, 31-41. <https://doi.org/10.1016/j.jconrel.2015.12.029>
- Cui, Y. Q., Jia, Y. J., Zhang, T., Zhang, Q. B., & Wang, X. M. (2012). Fucoidan protects against lipopolysaccharide-induced rat neuronal damage and inhibits the production of proinflammatory mediators in primary microglia. *CNS neuroscience & therapeutics*, 18(10), 827-833.
- Cullen, K. P., Grant, L. M., Kelm-Nelson, C. A., Brauer, A. F. L., Bickelhaupt, L. B., Russell, J. A., & Ciucci, M. R. (2018). Pink1 ^{-/-} Rats Show Early-Onset Swallowing Deficits and Correlative Brainstem Pathology. *Dysphagia*, 33(6), 749-758. <https://doi.org/10.1007/s00455-018-9896-5>
- Dai, X. J., Li, N., Yu, L., Chen, Z. Y., Hua, R., Qin, X., & Zhang, Y. M. (2015). Activation of BV2 microglia by lipopolysaccharide triggers an inflammatory reaction in PC12 cell apoptosis through a toll-like receptor 4-dependent pathway. *Cell Stress Chaperones*, 20(2), 321-331. <https://doi.org/10.1007/s12192-014-0552-1>
- Dalbanjan, N. P., Korgaonkar, K., Eelager, M. P., Gonal, B. N., Kadapure, A. J., Arakera, S. B., & Kumar S.K, P. (2025). In-silico strategies in nano-drug design: Bridging nanomaterials and pharmacological applications. *Nano TransMed*, 4, 100091. <https://doi.org/https://doi.org/10.1016/j.ntm.2025.100091>
- Dallapiazza, R. F., De Vloo, P., Fomenko, A., Lee, D. J., Hamani, C., Munhoz, R. P., Hodaie, M., Lozano, A. M., Fasano, A., & Kalia, S. K. (2018). Considerations for Patient and Target Selection in Deep Brain Stimulation Surgery for Parkinson's Disease. In (pp. 145-160). Codon Publications. <https://doi.org/10.15586/codonpublications.parkinsonsdisease.2018.ch8>

- Damier, P., Hirsch, E. C., Zhang, P., Agid, Y., & Javoy-Agid, F. (1993). Glutathione peroxidase, glial cells and Parkinson's disease. *Neuroscience*, 52(1), 1-6. [https://doi.org/10.1016/0306-4522\(93\)90175-f](https://doi.org/10.1016/0306-4522(93)90175-f)
- Dang, V., Medina, B., Das, D., Moghadam, S., Martin, K. J., Lin, B., Naik, P., Patel, D., Nosheny, R., & Ashford, J. W. (2014). Formoterol, a long-acting β_2 adrenergic agonist, improves cognitive function and promotes dendritic complexity in a mouse model of Down syndrome. *Biological psychiatry*, 75(3), 179-188.
- Danz, K., Fleddermann, J., Koch, M., Fecioru, E., Maahs, L., Kinsinger, N., Krämer, J., Kraegeloh, A., & Wagner, S. (2024). Evaluation of the Transport and Binding of Dopamine-Loaded PLGA Nanoparticles for the Treatment of Parkinson's Disease Using In Vitro Model Systems. *Pharmaceutics*, 16(5), 571. <https://www.mdpi.com/1999-4923/16/5/571>
- Das, A., Kim, S. H., Arifuzzaman, S., Yoon, T., Chai, J. C., Lee, Y. S., Park, K. S., Jung, K. H., & Chai, Y. G. (2016). Transcriptome sequencing reveals that LPS-triggered transcriptional responses in established microglia BV2 cell lines are poorly representative of primary microglia. *J Neuroinflammation*, 13(1), 182. <https://doi.org/10.1186/s12974-016-0644-1>
- Dashputre, N. L., Laddha, U. D., Pagare, T. D., Kadam, J. D., Patil, S. B., Tajanpure, A. B., Udavant, P. B., Kakad, S. P., & Kshirsagar, S. J. (2023). Fabrication of nanoparticulate system for oral delivery of Naringenin against paraquat-induced Parkinson's disorder in Wistar rats. *European Journal of Medicinal Chemistry Reports*, 8, 100105. <https://doi.org/https://doi.org/10.1016/j.ejmcr.2023.100105>
- Dawson, T. M., Ko, H. S., & Dawson, V. L. (2010). Genetic animal models of Parkinson's disease. *Neuron*, 66(5), 646-661. <https://doi.org/10.1016/j.neuron.2010.04.034>
- Day, J. S., O'Neill, E., Cawley, C., Aretz, N. K., Kilroy, D., Gibney, S. M., Harkin, A., & Connor, T. J. (2014). Noradrenaline acting on astrocytic β_2 -adrenoceptors induces neurite outgrowth in primary cortical neurons. *Neuropharmacology*, 77, 234-248. <https://doi.org/https://doi.org/10.1016/j.neuropharm.2013.09.027>
- De Miranda, B. R., Fazzari, M., Rocha, E. M., Castro, S., & Greenamyre, J. T. (2019). Sex differences in rotenone sensitivity reflect the male-to-female ratio in human Parkinson's disease incidence. *Toxicological Sciences*, 170(1), 133-143.
- de Oliveira, A. M., Jäger, E., Jäger, A., Stepánek, P., & Giacomelli, F. C. (2013). Physicochemical aspects behind the size of biodegradable polymeric nanoparticles: A step forward. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 436, 1092-1102.
- De, R., Mahata, M. K., & Kim, K. T. (2022). Structure-Based Varieties of Polymeric Nanocarriers and Influences of Their Physicochemical Properties on Drug Delivery Profiles. *Advanced Science*, 9(10), 2105373. <https://doi.org/10.1002/advs.202105373>
- de S. L. Oliveira, A. L. C., dos Santos-Silva, A. M., da Silva-Júnior, A. A., Garcia, V. B., de Araújo, A. A., de Geus-Oei, L.-F., Chan, A. B., Cruz, L. J., & de Araújo Júnior, R. F. (2020). Cholesterol-functionalized carvedilol-loaded PLGA nanoparticles: anti-inflammatory, antioxidant, and antitumor effects. *Journal of Nanoparticle Research*, 22(5), 115. <https://doi.org/10.1007/s11051-020-04832-8>

- De Waal, E. J., De Jong, W. H., Van Der Vliet, H., Verlaan, B., & Van Loveren, H. (1996). An immunotoxicity screening study on salmeterol in rats. *International Journal of Immunopharmacology*, 18(8), 523-528. [https://doi.org/https://doi.org/10.1016/S0192-0561\(96\)00029-X](https://doi.org/https://doi.org/10.1016/S0192-0561(96)00029-X)
- Dello Russo, C., Boullerne, A. I., Gavrilyuk, V., & Feinstein, D. L. (2004). Inhibition of microglial inflammatory responses by norepinephrine: effects on nitric oxide and interleukin-1 β production. *Journal of neuroinflammation*, 1(1), 9. <https://doi.org/10.1186/1742-2094-1-9>
- Deng, I., Corrigan, F., Garg, S., Zhou, X.-F., & Bobrovskaya, L. (2021). Further characterization of intrastriatal lipopolysaccharide model of Parkinson's disease in C57BL/6 mice. *International Journal of Molecular Sciences*, 22(14), 7380.
- Deng, I., Corrigan, F., Zhai, G., Zhou, X.-F., & Bobrovskaya, L. (2020). Lipopolysaccharide animal models of Parkinson's disease: Recent progress and relevance to clinical disease. *Brain, behavior, & immunity-health*, 4, 100060.
- Deng, Q., Parker, E., Wu, C., Zhu, L., Liu, T. C., Duan, R., & Yang, L. (2024). Repurposing Ketamine in the Therapy of Depression and Depression-Related Disorders: Recent Advances and Future Potential. *Aging Dis*, 16(2), 804-840. <https://doi.org/10.14336/ad.2024.0239>
- Depino, A. M., Earl, C., Kaczmarczyk, E., Ferrari, C., Besedovsky, H., del Rey, A., Pitossi, F. J., & Oertel, W. H. (2003). Microglial activation with atypical proinflammatory cytokine expression in a rat model of Parkinson's disease. *Eur J Neurosci*, 18(10), 2731-2742. <https://doi.org/10.1111/j.1460-9568.2003.03014.x>
- Devi, S., Alexandre, Y. O., Loi, J. K., Gillis, R., Ghazanfari, N., Creed, S. J., Holz, L. E., Shackelford, D., Mackay, L. K., & Heath, W. R. (2021). Adrenergic regulation of the vasculature impairs leukocyte interstitial migration and suppresses immune responses. *Immunity*, 54(6), 1219-1230. e1217.
- Di, J., Gao, X., Du, Y., Zhang, H., Gao, J., & Zheng, A. (2021). Size, shape, charge and “stealthy” surface: Carrier properties affect the drug circulation time in vivo. *Asian journal of pharmaceutical sciences*, 16(4), 444-458.
- Ding, R., Li, Y., Yu, Y., Sun, Z., & Duan, J. (2023). Prospects and hazards of silica nanoparticles: Biological impacts and implicated mechanisms. *Biotechnology Advances*, 69, 108277. <https://doi.org/https://doi.org/10.1016/j.biotechadv.2023.108277>
- Disdier, C., Lhotellier, C., Wagner, S., Andriambeloson, E., Théodoro, F., Pruvost, A., Joudinaud, T., Bénech, H., & Mabondzo, A. (2025). Intranasal delivery of dodecyl creatine ester alleviates motor deficits and increases dopamine levels in a 6-OHDA rat model of parkinsonism. *Front Aging Neurosci*, 17, 1597263. <https://doi.org/10.3389/fnagi.2025.1597263>
- Diwakar, L., Kenchappa, R. S., Annepu, J., Saeed, U., Sujanitha, R., & Ravindranath, V. (2006). Down-regulation of glutaredoxin by estrogen receptor antagonist renders female mice susceptible to excitatory amino acid mediated complex I inhibition in CNS. *Brain research*, 1125(1), 176-184.
- Dluzen, D. (1997). Estrogen decreases corpus striatal neurotoxicity in response to 6-hydroxydopamine. *Brain research*, 767(2), 340-344.

- Dluzen, D. E., McDermott, J. L., & Anderson, L. I. (2001). Tamoxifen eliminates estrogen's neuroprotective effect upon MPTP-induced neurotoxicity of the nigrostriatal dopaminergic system. *Neurotoxicity research*, 3(3), 291-300.
- Dluzen, D. E., McDermott, J. L., & Liu, B. (1996). Estrogen alters MPTP-induced neurotoxicity in female mice: effects on striatal dopamine concentrations and release. *Journal of neurochemistry*, 66(2), 658-666.
- Dorsey, E. R., Sherer, T., Okun, M. S., & Bloem, B. R. (2018). The emerging evidence of the Parkinson pandemic. *Journal of Parkinson's disease*, 8(s1), S3-S8.
- Dougherty, K. D., Dreyfus, C. F., & Black, I. B. (2000). Brain-Derived Neurotrophic Factor in Astrocytes, Oligodendrocytes, and Microglia/Macrophages after Spinal Cord Injury. *Neurobiology of disease*, 7(6), 574-585. <https://doi.org/https://doi.org/10.1006/nbdi.2000.0318>
- Dovonou, A., Bolduc, C., Soto Linan, V., Gora, C., Peralta Iii, M. R., & Lévesque, M. (2023). Animal models of Parkinson's disease: bridging the gap between disease hallmarks and research questions. *Translational neurodegeneration*, 12(1), 36. <https://doi.org/10.1186/s40035-023-00368-8>
- Doyle, H. H., Eidson, L. N., Sinkiewicz, D. M., & Murphy, A. Z. (2017). Sex differences in microglia activity within the periaqueductal gray of the rat: a potential mechanism driving the dimorphic effects of morphine. *Journal of Neuroscience*, 37(12), 3202-3214.
- Dunham, N. W., & Miya, T. S. (1957). A note on a simple apparatus for detecting neurological deficit in rats and mice. *J Am Pharm Assoc Am Pharm Assoc*, 46(3), 208-209. <https://doi.org/10.1002/jps.3030460322>
- Eijsvogel, P., Borghans, L., Prins, S., Moss, L., van Kraaij, S. J. W., van Brummelen, E., Klaassen, E., Martin, R. S., Bautista, E., Ford, A. P., Kremer, P. H. C., Groeneveld, G. J., & Vargas, G. A. (2024). Cognitive Effects of Three β -Adrenoceptor Acting Drugs in Healthy Volunteers and Patients with Parkinson's Disease. *J Parkinsons Dis*, 14(6), 1149-1161. <https://doi.org/10.3233/jpd-240039>
- El-Bachá, R. S., Leclerc, S., Netter, P., Magdalou, J., & Minn, A. (2000). Glucuronidation of apomorphine. *Life Sci*, 67(14), 1735-1745. [https://doi.org/10.1016/s0024-3205\(00\)00764-5](https://doi.org/10.1016/s0024-3205(00)00764-5)
- El-Hammadi, M. M., & Arias, J. L. (2022). Recent Advances in the Surface Functionalization of PLGA-Based Nanomedicines. *Nanomaterials*, 12(3), 354. <https://www.mdpi.com/2079-4991/12/3/354>
- Erdem, M., Erdem, Ş., Alver, A., Kiran, T. R., & Karahan, S. C. (2024). β 2-adrenoceptor agonist formoterol attenuates NLRP3 inflammasome activation and GSDMD-mediated pyroptosis in microglia through enhancing I κ B α /NF- κ B inhibition, SQSTM1/p62-dependent selective autophagy and ESCRT-III-mediated plasma membrane repair. *Molecular and Cellular Neuroscience*, 130, 103956. <https://doi.org/https://doi.org/10.1016/j.mcn.2024.103956>
- Evans, A. K., Ardestani, P. M., Yi, B., Park, H. H., Lam, R. K., & Shamloo, M. (2020). Beta-adrenergic receptor antagonism is proinflammatory and exacerbates neuroinflammation in a mouse model of Alzheimer's Disease. *Neurobiology of disease*, 146, 105089.
- Evans, S. S., Repasky, E. A., & Fisher, D. T. (2015). Fever and the thermal regulation of immunity: the immune system feels the heat. *Nature Reviews Immunology*, 15(6), 335-349.

- Ewii, U. E., Attama, A. A., Olorunsola, E. O., Onugwu, A. L., Nwakpa, F. U., Anyiam, C., Chijioke, C., & Ogbulie, T. (2025). Nanoparticles for drug delivery: Insight into in vitro and in vivo drug release from nanomedicines. *Nano TransMed*, 4, 100083. <https://doi.org/https://doi.org/10.1016/j.ntm.2025.100083>
- Fahmy, H. M., Ahmed, M. M., Mohamed, A. S., Shams-Eldin, E., Abd El-Daim, T. M., El-Feky, A. S., Mustafa, A. B., Abd Alrahman, M. W., Mohammed, F. F., & Fathy, M. M. (2022). Novel lipid-coated mesoporous silica nanoparticles loaded with thymoquinone formulation to increase its bioavailability in the brain and organs of Wistar rats. *BMC Pharmacol Toxicol*, 23(1), 71. <https://doi.org/10.1186/s40360-022-00616-z>
- Färber, K., Pannasch, U., & Kettenmann, H. (2005). Dopamine and noradrenaline control distinct functions in rodent microglial cells. *Mol Cell Neurosci*, 29(1), 128-138. <https://doi.org/10.1016/j.mcn.2005.01.003>
- Feinstein, D. L., Heneka, M. T., Gavriluk, V., Dello Russo, C., Weinberg, G., & Galea, E. (2002). Noradrenergic regulation of inflammatory gene expression in brain. *Neurochem Int*, 41(5), 357-365. [https://doi.org/10.1016/s0197-0186\(02\)00049-9](https://doi.org/10.1016/s0197-0186(02)00049-9)
- Ferreira, F. J. N., & Carneiro, A. S. (2025). AI-Driven Drug Discovery: A Comprehensive Review. *ACS Omega*, 10(23), 23889-23903. <https://doi.org/10.1021/acsomega.5c00549>
- Field, E. F., Metz, G. A., Pellis, S. M., & Whishaw, I. Q. (2006). Sexually dimorphic postural adjustments during vertical behaviour are altered in a unilateral 6-OHDA rat model of Parkinson's disease. *Behavioural brain research*, 174(1), 39-48.
- Filippov, S. K., Khusnutdinov, R., Murmiliuk, A., Inam, W., Zakharova, L. Y., Zhang, H., & Khutoryanskiy, V. V. (2023). Dynamic light scattering and transmission electron microscopy in drug delivery: a roadmap for correct characterization of nanoparticles and interpretation of results. *Mater Horiz*, 10(12), 5354-5370. <https://doi.org/10.1039/d3mh00717k>
- Fillebeen, C., Descamps, L., Dehouck, M.-P., Fenart, L., Benaïssa, M., Spik, G., Cecchelli, R., & Pierce, A. (1999). Receptor-mediated Transcytosis of Lactoferrin through the Blood-Brain Barrier*. *Journal of Biological Chemistry*, 274(11), 7011-7017. <https://doi.org/https://doi.org/10.1074/jbc.274.11.7011>
- Foehring, R. C., Zhang, X. F., Lee, J. C., & Callaway, J. C. (2009). Endogenous calcium buffering capacity of substantia nigral dopamine neurons. *J Neurophysiol*, 102(4), 2326-2333. <https://doi.org/10.1152/jn.00038.2009>
- Foltynie, T., Bruno, V., Fox, S., Kühn, A. A., Lindop, F., & Lees, A. J. (2024). Medical, surgical, and physical treatments for Parkinson's disease. *The Lancet*, 403(10423), 305-324. [https://doi.org/10.1016/S0140-6736\(23\)01429-0](https://doi.org/10.1016/S0140-6736(23)01429-0)
- Fonseca-Gomes, J., Loureiro, J. A., Tanqueiro, S. R., Mouro, F. M., Ruivo, P., Carvalho, T., Sebastião, A. M., Diógenes, M. J., & Pereira, M. C. (2020). <p>In vivo Bio-Distribution and Toxicity Evaluation of Polymeric and Lipid-Based Nanoparticles: A Potential Approach for Chronic Diseases Treatment</p>. *International Journal of Nanomedicine*, Volume 15, 8609-8621. <https://doi.org/10.2147/ijn.s267007>

- Fonte, P., Reis, S., & Sarmiento, B. (2016). Facts and evidences on the lyophilization of polymeric nanoparticles for drug delivery. *Journal of Controlled Release*, 225, 75-86. <https://doi.org/https://doi.org/10.1016/j.jconrel.2016.01.034>
- Frank, M. G., Weber, M. D., Watkins, L. R., & Maier, S. F. (2016). Stress-induced neuroinflammatory priming: A liability factor in the etiology of psychiatric disorders. *Neurobiol Stress*, 4, 62-70. <https://doi.org/10.1016/j.ynstr.2015.12.004>
- Frazer, A., Ordway, G., O'Donnell, J., Vos, P., & Wolfe, B. (2007). Effect of Repeated Administration of Clenbuterol on the Regulation of β -Adrenoceptors in the Central Nervous System of the Rat. Ciba Foundation Symposium 123- Antidepressants and Receptor Function: Antidepressants and Receptor Function: Ciba Foundation Symposium 123,
- Fujita, H., Tanaka, J., Maeda, N., & Sakanaka, M. (1998). Adrenergic agonists suppress the proliferation of microglia through beta 2-adrenergic receptor. *Neurosci Lett*, 242(1), 37-40. [https://doi.org/10.1016/s0304-3940\(98\)00003-2](https://doi.org/10.1016/s0304-3940(98)00003-2)
- Gambaryan, P. Y., Kondrasheva, I. G., Severin, E. S., Guseva, A. A., & Kamensky, A. A. (2014). Increasing the Efficiency of Parkinson's Disease Treatment Using a poly(lactic-co-glycolic acid) (PLGA) Based L-DOPA Delivery System. *Exp Neurobiol*, 23(3), 246-252. <https://doi.org/10.5607/en.2014.23.3.246>
- Gänger, S., & Schindowski, K. (2018). Tailoring Formulations for Intranasal Nose-to-Brain Delivery: A Review on Architecture, Physico-Chemical Characteristics and Mucociliary Clearance of the Nasal Olfactory Mucosa. *Pharmaceutics*, 10(3), 116. <https://doi.org/10.3390/pharmaceutics10030116>
- Gao, C., Jiang, J., Tan, Y., & Chen, S. (2023). Microglia in neurodegenerative diseases: mechanism and potential therapeutic targets. *Signal Transduction and Targeted Therapy*, 8(1), 359. <https://doi.org/10.1038/s41392-023-01588-0>
- Gao, M., Shen, X., & Mao, S. (2020). Factors influencing drug deposition in the nasal cavity upon delivery via nasal sprays. *Journal of Pharmaceutical Investigation*, 50(3), 251-259. <https://doi.org/10.1007/s40005-020-00482-z>
- Gao, S., Wang, Z., Huang, Y., Yang, G., Wang, Y., Yi, Y., Zhou, Q., Jian, X., Zhao, G., Li, B., Xu, L., Xia, K., Tang, B., & Li, J. (2025). Early detection of Parkinson's disease through multiplex blood and urine biomarkers prior to clinical diagnosis. *npj Parkinson's Disease*, 11(1), 35. <https://doi.org/10.1038/s41531-025-00888-2>
- Gao, Y., Joshi, M., Zhao, Z., & Mitragotri, S. (2024). PEGylated therapeutics in the clinic. *Bioeng Transl Med*, 9(1), e10600. <https://doi.org/10.1002/btm2.10600>
- García-Llano, M., Pedroso-Ibáñez, I., Morales-Chacón, L., Rodríguez-Obaya, T., Pérez-Ruiz, L., Sosa-Testé, I., Amaro-González, D., & Bringas-Vega, M. L. (2021). Short-term Tolerance of Nasally-administered NeuroEPO in Patients with Parkinson Disease. *MEDICC Rev*, 23(1), 49-54. <https://doi.org/10.37757/mr2021.V23.N1.10>
- Garcia, L., Palma-Florez, S., Espinosa, V., Soleimani Rokni, F., Lagunas, A., Mir, M., García-Celma, M. J., Samitier, J., Rodríguez-Abreu, C., & Grijalvo, S. (2023). Ferulic acid-loaded polymeric nanoparticles prepared from nano-emulsion templates facilitate internalisation across the blood-brain barrier in model membranes. *Nanoscale*, 15(17), 7929-7944. <https://doi.org/10.1039/d2nr07256d>

- Gerakios, F., Yarnall, A. J., Bate, G., Wright, L., Davis, D., Stephan, B. C. M., Robinson, L., Brayne, C., Stebbins, G., Taylor, J. P., Burn, D. J., Allan, L. M., Richardson, S. J., & Lawson, R. A. (2024). Delirium is more common and associated with worse outcomes in Parkinson's disease compared to older adult controls: results of two prospective longitudinal cohort studies. *Age Ageing*, 53(3). <https://doi.org/10.1093/ageing/afae046>
- German, D. C., Manaye, K. F., White, C. L., 3rd, Woodward, D. J., McIntire, D. D., Smith, W. K., Kalaria, R. N., & Mann, D. M. (1992). Disease-specific patterns of locus coeruleus cell loss. *Ann Neurol*, 32(5), 667-676. <https://doi.org/10.1002/ana.410320510>
- Gesi, M., Soldani, P., Giorgi, F. S., Santinami, A., Bonaccorsi, I., & Fornai, F. (2000). The role of the locus coeruleus in the development of Parkinson's disease. *Neurosci Biobehav Rev*, 24(6), 655-668. [https://doi.org/10.1016/s0149-7634\(00\)00028-2](https://doi.org/10.1016/s0149-7634(00)00028-2)
- Ghaly, H. S. A., Seyedasli, N., & Varamini, P. (2025). Enhanced Nanoprecipitation Method for the Production of PLGA Nanoparticles for Oncology Applications. *The AAPS Journal*, 27(5), 113. <https://doi.org/10.1208/s12248-025-01096-9>
- Gherzi-Egea, J. F., Perrin, R., Leininger-Muller, B., Grassiot, M. C., Jeandel, C., Floquet, J., Cuny, G., Siest, G., & Minn, A. (1993). Subcellular localization of cytochrome P450, and activities of several enzymes responsible for drug metabolism in the human brain. *Biochem Pharmacol*, 45(3), 647-658. [https://doi.org/10.1016/0006-2952\(93\)90139-n](https://doi.org/10.1016/0006-2952(93)90139-n)
- Giaime, E., Tong, Y., Wagner, L. K., Yuan, Y., Huang, G., & Shen, J. (2017). Age-Dependent Dopaminergic Neurodegeneration and Impairment of the Autophagy-Lysosomal Pathway in LRRK-Deficient Mice. *Neuron*, 96(4), 796-807.e796. <https://doi.org/10.1016/j.neuron.2017.09.036>
- Gleeson, L. C., Ryan, K. J., Griffin, É. W., Connor, T. J., & Harkin, A. (2010). The β 2-adrenoceptor agonist clenbuterol elicits neuroprotective, anti-inflammatory and neurotrophic actions in the kainic acid model of excitotoxicity. *Brain, behavior, and immunity*, 24(8), 1354-1361.
- Gobbo, O. L., Petit, F., Gurden, H., & Dhenain, M. (2012). In vivo detection of excitotoxicity by manganese-enhanced MRI: Comparison with physiological stimulation. *Magnetic resonance in medicine*, 68(1), 234-240.
- Goldberg, N. R., Haack, A. K., Lim, N. S., Janson, O. K., & Meshul, C. K. (2011). Dopaminergic and behavioral correlates of progressive lesioning of the nigrostriatal pathway with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Neuroscience*, 180, 256-271. <https://doi.org/10.1016/j.neuroscience.2011.02.027>
- Gordon, J., Amini, S., & White, M. K. (2013). General overview of neuronal cell culture. *Methods Mol Biol*, 1078, 1-8. https://doi.org/10.1007/978-1-62703-640-5_1
- Gordon, P. H., Moore, D. H., Miller, R. G., Florence, J. M., Verheijde, J. L., Doorish, C., Hilton, J. F., Spitalny, G. M., MacArthur, R. B., Mitsumoto, H., Neville, H. E., Boylan, K., Mozaffar, T., Belsh, J. M., Ravits, J., Bedlack, R. S., Graves, M. C., McCluskey, L. F., Barohn, R. J., & Tandan, R. (2007). Efficacy of minocycline in patients with amyotrophic lateral sclerosis: a phase III randomised trial. *The Lancet Neurology*, 6(12), 1045-1053. [https://doi.org/https://doi.org/10.1016/S1474-4422\(07\)70270-3](https://doi.org/https://doi.org/10.1016/S1474-4422(07)70270-3)

- Gordon, R., Albornoz, E. A., Christie, D. C., Langley, M. R., Kumar, V., Mantovani, S., Robertson, A. A. B., Butler, M. S., Rowe, D. B., O'Neill, L. A., Kanthasamy, A. G., Schroder, K., Cooper, M. A., & Woodruff, T. M. (2018). Inflammasome inhibition prevents α -synuclein pathology and dopaminergic neurodegeneration in mice. *Sci Transl Med*, 10(465). <https://doi.org/10.1126/scitranslmed.aah4066>
- Gorecki, A. M., Anyaegbu, C. C., & Anderton, R. S. (2021). TLR2 and TLR4 in Parkinson's disease pathogenesis: the environment takes a toll on the gut. *Transl Neurodegener*, 10(1), 47. <https://doi.org/10.1186/s40035-021-00271-0>
- Goren, A., Bao, Z., Martinez Lozano, J. P., & Allen, C. (2025). A formulation dataset of poly(lactide-co-glycolide) nanoparticles for small molecule delivery. *Scientific Data*, 12(1), 1182. <https://doi.org/10.1038/s41597-025-05520-9>
- Gosselin, D., Skola, D., Coufal, N. G., Holtman, I. R., Schlachetzki, J. C. M., Sajti, E., Jaeger, B. N., O'Connor, C., Fitzpatrick, C., Pasillas, M. P., Pena, M., Adair, A., Gonda, D. D., Levy, M. L., Ransohoff, R. M., Gage, F. H., & Glass, C. K. (2017). An environment-dependent transcriptional network specifies human microglia identity. *Science*, 356(6344). <https://doi.org/10.1126/science.aal3222>
- Gouda, N. A., Elkamhawy, A., & Cho, J. (2022). Emerging Therapeutic Strategies for Parkinson's Disease and Future Prospects: A 2021 Update. *Biomedicines*, 10(2). <https://doi.org/10.3390/biomedicines10020371>
- Grabrucker, A. M., Garner, C. C., Boeckers, T. M., Bondioli, L., Ruozi, B., Forni, F., Vandelli, M. A., & Tosi, G. (2011). Development of novel Zn²⁺ loaded nanoparticles designed for cell-type targeted drug release in CNS neurons: in vitro evidences. *PLoS One*, 6(3), e17851. <https://doi.org/10.1371/journal.pone.0017851>
- Gray, M. T., & Woulfe, J. M. (2015). Striatal blood–brain barrier permeability in Parkinson's disease. *Journal of Cerebral Blood Flow & Metabolism*, 35(5), 747–750.
- Green, S., Perrott, S. L., McCleary, A., Sleeman, I., Maple-Grødem, J., Counsell, C. E., & Macleod, A. D. (2021). First delirium episode in Parkinson's disease and parkinsonism: incidence, predictors, and outcomes. *NPJ Parkinsons Dis*, 7(1), 92. <https://doi.org/10.1038/s41531-021-00234-2>
- Greffard, S., Verny, M., Bonnet, A. M., Beinis, J. Y., Gallinari, C., Meaume, S., Piette, F., Hauw, J. J., & Duyckaerts, C. (2006). Motor score of the Unified Parkinson Disease Rating Scale as a good predictor of Lewy body-associated neuronal loss in the substantia nigra. *Arch Neurol*, 63(4), 584–588. <https://doi.org/10.1001/archneur.63.4.584>
- Griffin, É. W., Yssel, J. D., O'Neill, E., Ryan, K. J., Boyle, N., Harper, P., Harkin, A., & Connor, T. (2018). The β 2-adrenoceptor agonist clenbuterol reduces the neuroinflammatory response, neutrophil infiltration and apoptosis following intra-striatal IL-1 β administration to rats. *Immunopharmacology and Immunotoxicology*, 40(2), 99–106.
- Groo, A. C., Cudel, T., Malzert-Fréon, A., Ségué, L., Bento, O., Corvaisier, S., Culerier, T., Legrand, R., Callizot, N., Henriques, A., Culley, G., Claeys, S., Rochais, C., & Dallemagne, P. (2025). Evidence from a mouse model supports repurposing an anti-asthmatic drug, bambuterol, against Alzheimer's disease by administration through an intranasal route. *Commun Biol*, 8(1), 155. <https://doi.org/10.1038/s42003-025-07599-7>

- Gross, G. (2015). Strategies for enhancing oral bioavailability and brain penetration. In *The Practice of Medicinal Chemistry* (pp. 631-655). Elsevier.
- Guo, X., Zuo, X., Zhou, Z., Gu, Y., Zheng, H., Wang, X., Wang, G., Xu, C., & Wang, F. (2023). PLGA-Based Micro/Nanoparticles: An Overview of Their Applications in Respiratory Diseases. *Int J Mol Sci*, 24(5). <https://doi.org/10.3390/ijms24054333>
- Gupta, R., Chen, Y., Sarkar, M., & Xie, H. (2022). Surfactant Mediated Accelerated and Discriminatory In Vitro Drug Release Method for PLGA Nanoparticles of Poorly Water-Soluble Drug. *Pharmaceuticals (Basel)*, 15(12). <https://doi.org/10.3390/ph15121489>
- Gutierrez, H., & Davies, A. M. (2007). A fast and accurate procedure for deriving the Sholl profile in quantitative studies of neuronal morphology. *Journal of neuroscience methods*, 163(1), 24-30.
- Halder, J., Pradhan, D., Kar, B., Ghosh, G., & Rath, G. (2022). Nanotherapeutics approaches to overcome P-glycoprotein-mediated multi-drug resistance in cancer. *Nanomedicine*, 40, 102494. <https://doi.org/10.1016/j.nano.2021.102494>
- Hamdallah, S. I., Zoqlam, R., Yang, B., Campbell, A., Booth, R., Booth, J., Belton, P., & Qi, S. (2024). Using a systematic and quantitative approach to generate new insights into drug loading of PLGA nanoparticles using nanoprecipitation. *Nanoscale Advances*, 6(12), 3188-3198. <https://doi.org/10.1039/d4na00087k>
- Han, G., Ghosh, P., & Rotello, V. M. (2007). Functionalized gold nanoparticles for drug delivery. *Nanomedicine (Lond)*, 2(1), 113-123. <https://doi.org/10.2217/17435889.2.1.113>
- Hanisch, U. K., & Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci*, 10(11), 1387-1394. <https://doi.org/10.1038/nn1997>
- Hard, S., Shivakumar, H. N., Bafail, D. A., & Moqbel Redhwan, M. A. (2025). Development of in vitro and in vivo evaluation of mucoadhesive in-situ gel for intranasal delivery of vinpocetine. *J Drug Target*, 33(4), 528-545. <https://doi.org/10.1080/1061186x.2024.2433557>
- Harilal, S., Jose, J., Parambi, D. G. T., Kumar, R., Unnikrishnan, M. K., Uddin, M. S., Mathew, G. E., Pratap, R., Marathakam, A., & Mathew, B. (2020). Revisiting the blood-brain barrier: A hard nut to crack in the transportation of drug molecules. *Brain Res Bull*, 160, 121-140. <https://doi.org/10.1016/j.brainresbull.2020.03.018>
- He, H., Duo, H., Hao, Y., Zhang, X., Zhou, X., Zeng, Y., Li, Y., & Li, B. (2023). Computational drug repurposing by exploiting large-scale gene expression data: Strategy, methods and applications. *Computers in Biology and Medicine*, 155, 106671. <https://doi.org/10.1016/j.compbiomed.2023.106671>
- He, H., Guo, W.-W., Xu, R.-R., Chen, X.-Q., Zhang, N., Wu, X., & Wang, X.-M. (2016). Alkaloids from piper longum protect dopaminergic neurons against inflammation-mediated damage induced by intranigral injection of lipopolysaccharide. *BMC Complementary and Alternative Medicine*, 16(1), 412.

- He, H., Zhang, X., He, H., Xu, G., Li, L., Yang, C., Liu, Y. E., You, Z., & Zhang, J. (2024). Microglial priming by IFN- γ involves STAT1-mediated activation of the NLRP3 inflammasome. *CNS Neurosci Ther*, 30(10), e70061. <https://doi.org/10.1111/cns.70061>
- He, Q., Yu, W., Wu, J., Chen, C., Lou, Z., Zhang, Q., Zhao, J., Wang, J., & Xiao, B. (2013). Intranasal LPS-mediated Parkinson's model challenges the pathogenesis of nasal cavity and environmental toxins. *PLoS One*, 8(11), e78418. <https://doi.org/10.1371/journal.pone.0078418>
- He, Y., Taylor, N., Yao, X., & Bhattacharya, A. (2021). Mouse primary microglia respond differently to LPS and poly(I:C) in vitro. *Scientific Reports*, 11(1), 10447. <https://doi.org/10.1038/s41598-021-89777-1>
- Heneka, M. T., Gavrilyuk, V., Landreth, G. E., O'Banion, M. K., Weinberg, G., & Feinstein, D. L. (2003). Noradrenergic depletion increases inflammatory responses in brain: effects on I κ B and HSP70 expression. *J Neurochem*, 85(2), 387-398. <https://doi.org/10.1046/j.1471-4159.2003.01694.x>
- Henn, A., Lund, S., Hedtj rn, M., Schratzenholz, A., P rzig, P., & Leist, M. (2009). The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. *Altx*, 26(2), 83-94. <https://doi.org/10.14573/altex.2009.2.83>
- Heredia, N. S., Vizuite, K., Flores-Calero, M., Pazmi o, V. K., Pilaquinga, F., Kumar, B., & Debut, A. (2022). Comparative statistical analysis of the release kinetics models for nanoprecipitated drug delivery systems based on poly(lactic-co-glycolic acid). *PLoS One*, 17(3), e0264825. <https://doi.org/10.1371/journal.pone.0264825>
- Hernandez-Baltazar, D., Zavala-Flores, L. M., & Villanueva-Olivo, A. (2017). The 6-hydroxydopamine model and parkinsonian pathophysiology: Novel findings in an older model. *Neurologia*, 32(8), 533-539. <https://doi.org/10.1016/j.nrl.2015.06.011> (El modelo de 6-hidroxidopamina y la fisiopatolog a parkinsoniana: nuevos hallazgos en un viejo modelo.)
- Hern ndez-Parra, H., Cort s, H., Avalos-Fuentes, J. A., Del Prado-Audelo, M., Flor n, B., Leyva-G mez, G., Sharifi-Rad, J., & Cho, W. C. (2022). Repositioning of drugs for Parkinson's disease and pharmaceutical nanotechnology tools for their optimization. *Journal of Nanobiotechnology*, 20(1). <https://doi.org/10.1186/s12951-022-01612-5>
- Hernandez, J. J., Pryszlak, M., Smith, L., Yanchus, C., Kurji, N., Shahani, V. M., & Molinski, S. V. (2017). Giving Drugs a Second Chance: Overcoming Regulatory and Financial Hurdles in Repurposing Approved Drugs As Cancer Therapeutics. *Front Oncol*, 7, 273. <https://doi.org/10.3389/fonc.2017.00273>
- Herrera, A., Castano, A., Venero, J., Cano, J., & Machado, A. (2000). The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. *Neurobiology of disease*, 7(4), 429-447.
- Heydel, J.-M., Leclerc, S., Bernard, P., Pelczar, H., Gradinaru, D., Magdalou, J., Minn, A., Artur, Y., & Goudonnet, H. (2001). Rat olfactory bulb and epithelium UDP-glucuronosyltransferase 2A1 (UGT2A1) expression: in situ mRNA localization and quantitative analysis. Also published on the World Wide Web on 2 April

2001. *Molecular Brain Research*, 90(1), 83-92. [https://doi.org/https://doi.org/10.1016/S0169-328X\(01\)00080-8](https://doi.org/https://doi.org/10.1016/S0169-328X(01)00080-8)
- Hirsch, E. C., & Hunot, S. (2009). Neuroinflammation in Parkinson's disease: a target for neuroprotection? *Lancet Neurol*, 8(4), 382-397. [https://doi.org/10.1016/s1474-4422\(09\)70062-6](https://doi.org/10.1016/s1474-4422(09)70062-6)
- Ho, C. H., Casmil, I. C., Sharma, M., Rees, T., Enright, K., Allan, N., & Blakney, A. K. (2025). Laminar fluid ejection device enables high yield and preservation of mRNA and SaRNA LNP formulations. *Scientific Reports*, 15(1), 18507. <https://doi.org/10.1038/s41598-025-03309-9>
- Hoban, D. B., Connaughton, E., Connaughton, C., Hogan, G., Thornton, C., Mulcahy, P., Moloney, T. C., & Dowd, E. (2013). Further characterisation of the LPS model of Parkinson's disease: a comparison of intra-nigral and intra-striatal lipopolysaccharide administration on motor function, microgliosis and nigrostriatal neurodegeneration in the rat. *Brain, behavior, and immunity*, 27, 91-100.
- Hohlbaum, K., Bert, B., Dietze, S., Palme, R., Fink, H., & Thöne-Reineke, C. (2017). Severity classification of repeated isoflurane anesthesia in C57BL/6J mice—Assessing the degree of distress. *PLoS One*, 12(6), e0179588.
- Hoogendijk, W. J., Pool, C. W., Troost, D., van Zwieten, E., & Swaab, D. F. (1995). Image analyser-assisted morphometry of the locus coeruleus in Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. *Brain*, 118 (Pt 1), 131-143. <https://doi.org/10.1093/brain/118.1.131>
- Hoogland, I. C., Houbolt, C., van Westerloo, D. J., van Gool, W. A., & van de Beek, D. (2015). Systemic inflammation and microglial activation: systematic review of animal experiments. *J Neuroinflammation*, 12, 114. <https://doi.org/10.1186/s12974-015-0332-6>
- Hsu, E., & Bajaj, T. (2025). Beta2-Agonists. In *StatPearls*. StatPearls Publishing
- Copyright © 2025, StatPearls Publishing LLC.
- Huntington, T. E., & Srinivasan, R. (2021). Adeno-Associated Virus Expression of α -Synuclein as a Tool to Model Parkinson's Disease: Current Understanding and Knowledge Gaps. *Aging Dis*, 12(4), 1120-1137. <https://doi.org/10.14336/ad.2021.0517>
- Iranzo, A., Tolosa, E., Gelpi, E., Molinuevo, J. L., Valldeoriola, F., Serradell, M., Sanchez-Valle, R., Vilaseca, I., Lomeña, F., Vilas, D., Lladó, A., Gaig, C., & Santamaria, J. (2013). Neurodegenerative disease status and post-mortem pathology in idiopathic rapid-eye-movement sleep behaviour disorder: an observational cohort study. *Lancet Neurol*, 12(5), 443-453. [https://doi.org/10.1016/s1474-4422\(13\)70056-5](https://doi.org/10.1016/s1474-4422(13)70056-5)
- Iravani, M. M., Leung, C. C., Sadeghian, M., Haddon, C. O., Rose, S., & Jenner, P. (2005). The acute and the long-term effects of nigral lipopolysaccharide administration on dopaminergic dysfunction and glial cell activation. *Eur J Neurosci*, 22(2), 317-330. <https://doi.org/10.1111/j.1460-9568.2005.04220.x>
- Ishida, Y., Matsushita, M., Yoneshiro, T., Saito, M., Fuse, S., Hamaoka, T., Kuroiwa, M., Tanaka, R., Kurosawa, Y., & Nishimura, T. (2024). Genetic evidence for involvement of β 2-adrenergic receptor in brown adipose tissue thermogenesis in humans. *International Journal of Obesity*, 48(8), 1110-1117.

- Jeon, S. G., Cha, M. Y., Kim, J. I., Hwang, T. W., Kim, K. A., Kim, T. H., Song, K. C., Kim, J. J., & Moon, M. (2019). Vitamin D-binding protein-loaded PLGA nanoparticles suppress Alzheimer's disease-related pathology in 5XFAD mice. *Nanomedicine*, 17, 297-307. <https://doi.org/10.1016/j.nano.2019.02.004>
- Jeong, S. H., Jang, J. H., & Lee, Y. B. (2023). Drug delivery to the brain via the nasal route of administration: exploration of key targets and major consideration factors. *J Pharm Investig*, 53(1), 119-152. <https://doi.org/10.1007/s40005-022-00589-5>
- Jiang, L., Chen, S. H., Chu, C. H., Wang, S. J., Oyarzabal, E., Wilson, B., Sanders, V., Xie, K., Wang, Q., & Hong, J. S. (2015). A novel role of microglial NADPH oxidase in mediating extra-synaptic function of norepinephrine in regulating brain immune homeostasis. *Glia*, 63(6), 1057-1072. <https://doi.org/10.1002/glia.22801>
- Jiang, S., Wang, H., Yang, C., Feng, F., Xu, D., Zhang, M., Xie, M., Cui, R., Zhu, Z., Jia, C., Liu, L., Wang, L., Yang, X., Yang, Y., Hao, H., Liu, Z., Wu, Z., Leng, L., Li, X.,...Wang, R. (2024). Phase 1 study of safety and preliminary efficacy of intranasal transplantation of human neural stem cells (ANGE-S003) in Parkinson's disease. *J Neurol Neurosurg Psychiatry*, 95(12), 1102-1111. <https://doi.org/10.1136/jnnp-2023-332921>
- Jiang, X., He, H., Mo, L., Liu, Q., Yang, F., Zhou, Y., Li, L., Su, D., Yi, S., & Zhang, J. (2022). Mapping the Plasticity of Morphology, Molecular Properties and Function in Mouse Primary Microglia [Original Research]. *Frontiers in cellular neuroscience*, Volume 15 - 2021. <https://www.frontiersin.org/journals/cellular-neuroscience/articles/10.3389/fncel.2021.811061>
- Jiang, X., Xin, H., Ren, Q., Gu, J., Zhu, L., Du, F., Feng, C., Xie, Y., Sha, X., & Fang, X. (2014). Nanoparticles of 2-deoxy-D-glucose functionalized poly(ethylene glycol)-co-poly(trimethylene carbonate) for dual-targeted drug delivery in glioma treatment. *Biomaterials*, 35(1), 518-529. <https://doi.org/10.1016/j.biomaterials.2013.09.094>
- Jo, D. H., Kim, J. H., Lee, T. G., & Kim, J. H. (2015). Size, surface charge, and shape determine therapeutic effects of nanoparticles on brain and retinal diseases. *Nanomedicine*, 11(7), 1603-1611. <https://doi.org/10.1016/j.nano.2015.04.015>
- Johnson, J., Kashyap, B., Gustafson, S., Wyman-Chick, K., O'Brien, M., Frey, W., Svitak, A., & Hanson, L. (2025). Safety and Tolerability of Intranasal Insulin in Parkinson's Disease (P11-5.018). *Neurology*, 104(7_Supplement_1), 5504. <https://doi.org/doi:10.1212/WNL.0000000000212405>
- Junker, V., Becker, A., Hühne, R., Zembatov, M., Ravati, A., Culmsee, C., & Kriegelstein, J. (2002). Stimulation of β -adrenoceptors activates astrocytes and provides neuroprotection. *European Journal of Pharmacology*, 446(1), 25-36. [https://doi.org/https://doi.org/10.1016/S0014-2999\(02\)01814-9](https://doi.org/https://doi.org/10.1016/S0014-2999(02)01814-9)
- Kakubari, I., Dejima, H., Miura, K., Koga, Y., Mizu, H., Takayasu, T., Yamauchi, H., Takayama, S., & Takayama, K. (2007). Determination of formoterol in rat plasma by liquid chromatography-electrospray ionisation mass spectrometry. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, 62(2), 94-95.
- Kalia, L. V., & Lang, A. E. (2015). Parkinson's disease. *Lancet*, 386(9996), 896-912. [https://doi.org/10.1016/s0140-6736\(14\)61393-3](https://doi.org/10.1016/s0140-6736(14)61393-3)
- Kamaly, N., Yameen, B., Wu, J., & Farokhzad, O. C. (2016). Degradable Controlled-Release Polymers and Polymeric Nanoparticles: Mechanisms of Controlling

- Drug Release. *Chem Rev*, 116(4), 2602-2663. <https://doi.org/10.1021/acs.chemrev.5b00346>
- Katila, N., Duwa, R., Bhurtel, S., Khanal, S., Maharjan, S., Jeong, J. H., Lee, S., Choi, D. Y., & Yook, S. (2022). Enhancement of blood-brain barrier penetration and the neuroprotective effect of resveratrol. *J Control Release*, 346, 1-19. <https://doi.org/10.1016/j.jconrel.2022.04.003>
- Kaur, A., Nigam, K., Tyagi, A., & Dang, S. (2022). A Preliminary Pharmacodynamic Study for the Management of Alzheimer's Disease Using Memantine-Loaded PLGA Nanoparticles. *AAPS PharmSciTech*, 23(8). <https://doi.org/10.1208/s12249-022-02449-9>
- Kaur, P., Garg, T., Rath, G., & Goyal, A. K. (2016). In situ nasal gel drug delivery: A novel approach for brain targeting through the mucosal membrane. *Artif Cells Nanomed Biotechnol*, 44(4), 1167-1176. <https://doi.org/10.3109/21691401.2015.1012260>
- Keller, L. A., Merkel, O., & Popp, A. (2022). Intranasal drug delivery: opportunities and toxicologic challenges during drug development. *Drug Deliv Transl Res*, 12(4), 735-757. <https://doi.org/10.1007/s13346-020-00891-5>
- Kenchappa, R. S., Diwakar, L., Annepu, J., & Ravindranath, V. (2004). Estrogen and neuroprotection: higher constitutive expression of glutaredoxin in female mice offers protection against MPTP-mediated neurodegeneration. *The FASEB journal*, 18(10), 1102-1104.
- Khan, S., Patil, K., Bobade, N., Yeole, P., & Gaikwad, R. (2010). Formulation of intranasal mucoadhesive temperature-mediated in situ gel containing ropinirole and evaluation of brain targeting efficiency in rats. *J Drug Target*, 18(3), 223-234. <https://doi.org/10.3109/10611860903386938>
- Khanal, S., Shin, E. J., Yoo, C. J., Kim, J., & Choi, D. Y. (2025). Inosine exerts dopaminergic neuroprotective effects via mitigation of NLRP3 inflammasome activation. *Neuropharmacology*, 266, 110278. <https://doi.org/10.1016/j.neuropharm.2024.110278>
- Khidr, H. Y., Hassan, N. F., Abdelrahman, S. S., El-Ansary, M. R., El-Yamany, M. F., & Rabie, M. A. (2023). Formoterol attenuated mitochondrial dysfunction in rotenone-induced Parkinson's disease in a rat model: Role of PINK-1/PARKIN and PI3K/Akt/CREB/BDNF/TrkB axis. *International Immunopharmacology*, 125, 111207. <https://doi.org/10.1016/j.intimp.2023.111207>
- Kim, D., You, B., Jo, E.-K., Han, S.-K., Simon, M. I., & Lee, S. J. (2010). NADPH oxidase 2-derived reactive oxygen species in spinal cord microglia contribute to peripheral nerve injury-induced neuropathic pain. *Proceedings of the National Academy of Sciences*, 107(33), 14851-14856. <https://doi.org/10.1073/pnas.1009926107>
- Kim, S. I., Shin, J., Tran, Q., Park, H., Kwon, H. H., Shin, N., Hwang, J. A., Shin, H. J., Lee, J., Lee, W. H., Lee, S. Y., & Kim, D. W. (2021). Application of PLGA nanoparticles to enhance the action of duloxetine on microglia in neuropathic pain. *Biomater Sci*, 9(18), 6295-6307. <https://doi.org/10.1039/d1bm00486g>
- Kim, S. I., Yang, J., Shin, J., Shin, N., Shin, H. J., Lee, J., Noh, C., Kim, D. W., & Lee, S. Y. (2024). Amitriptyline nanoparticle repositioning prolongs the anti-allodynic effect of enhanced microglia targeting. *Nanomedicine (Lond)*, 19(25), 2099-2112. <https://doi.org/10.1080/17435889.2024.2390349>

- Kinoshita, M., McDannold, N., Jolesz, F. A., & Hynynen, K. (2006). Noninvasive localized delivery of Herceptin to the mouse brain by MRI-guided focused ultrasound-induced blood-brain barrier disruption. *Proc Natl Acad Sci U S A*, 103(31), 11719-11723. <https://doi.org/10.1073/pnas.0604318103>
- Konnova, E. A., & Swanberg, M. (2018). Animal Models of Parkinson's Disease. In (pp. 83-106). Codon Publications. <https://doi.org/10.15586/codonpublications.parkinsonsdisease.2018.ch5>
- Kopin, I. J., & Markey, S. P. (1988). MPTP toxicity: implications for research in Parkinson's disease. *Annu Rev Neurosci*, 11, 81-96. <https://doi.org/10.1146/annurev.ne.11.030188.000501>
- Koretsky, A. P., & Silva, A. C. (2004). Manganese-enhanced magnetic resonance imaging (MEMRI). In (Vol. 17, pp. 527-531): Wiley Online Library.
- Kou, D., Xu, K., Yin, J., Wang, N., Zhang, R., Zeng, J., & Gao, M. (2025). Intranasal delivery of rotigotine to the brain for treating Parkinson's disease. *Fundamental Research*, 5(5), 1911-1919. <https://doi.org/https://doi.org/10.1016/j.fmre.2025.02.004>
- Kulkarni, S. A., & Feng, S. S. (2013). Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery. *Pharm Res*, 30(10), 2512-2522. <https://doi.org/10.1007/s11095-012-0958-3>
- Kumari, A., Yadav, S. K., & Yadav, S. C. (2010). Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids Surf B Biointerfaces*, 75(1), 1-18. <https://doi.org/10.1016/j.colsurfb.2009.09.001>
- Lababidi, J. M., & Azzazy, H. M. E.-S. (2025). Revamping Parkinson's disease therapy using PLGA-based drug delivery systems. *npj Parkinson's Disease*, 11(1), 248. <https://doi.org/10.1038/s41531-025-01081-1>
- Lai, S. K., Wang, Y. Y., & Hanes, J. (2009). Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev*, 61(2), 158-171. <https://doi.org/10.1016/j.addr.2008.11.002>
- Lamparelli, E. P., Marino, M., Scognamiglio, M. R., D'Auria, R., Santoro, A., & Della Porta, G. (2024). PLA/PLGA nanocarriers fabricated by microfluidics-assisted nanoprecipitation and loaded with Rhodamine or gold can be efficiently used to track their cellular uptake and distribution. *International Journal of Pharmaceutics*, 667, 124934. <https://doi.org/https://doi.org/10.1016/j.ijpharm.2024.124934>
- Lau, K., Kotzur, R., & Richter, F. (2024). Blood-brain barrier alterations and their impact on Parkinson's disease pathogenesis and therapy. *Translational neurodegeneration*, 13(1), 37.
- Laurencin, C., Lancelot, S., Brosse, S., Mérida, I., Redouté, J., Greusard, E., Lamberet, L., Liotier, V., Le Bars, D., Costes, N., Thobois, S., Boulinguez, P., & Ballanger, B. (2024). Noradrenergic alterations in Parkinson's disease: a combined 11C-yohimbine PET/neuromelanin MRI study. *Brain*, 147(4), 1377-1388. <https://doi.org/10.1093/brain/awad338>
- Le, L. H. D., Feidler, A. M., Rodriguez, L. C., Cealie, M., Plunk, E., Li, H., Kara-Pabani, K., Lamantia, C., O'Banion, M. K., & Majewska, A. K. (2025). Noradrenergic signaling controls Alzheimer's disease pathology via activation of microglial β_2 adrenergic receptors. *Brain, behavior, and immunity*, 128, 307-322. <https://doi.org/https://doi.org/10.1016/j.bbi.2025.04.022>

- Lechtenberg, K. J., Meyer, S. T., Doyle, J. B., Peterson, T. C., & Buckwalter, M. S. (2019). Augmented β 2-adrenergic signaling dampens the neuroinflammatory response following ischemic stroke and increases stroke size. *Journal of neuroinflammation*, 16(1), 112.
- Lee, E.-S. Y., Chen, H., King, J., & Charlton, C. (2008). The Role of 3-O-Methyldopa in the Side Effects of L-dopa. *Neurochemical Research*, 33(3), 401-411. <https://doi.org/10.1007/s11064-007-9442-6>
- Lee, E., Hwang, I., Park, S., Hong, S., Hwang, B., Cho, Y., Son, J., & Yu, J.-W. (2019). MPTP-driven NLRP3 inflammasome activation in microglia plays a central role in dopaminergic neurodegeneration. *Cell Death & Differentiation*, 26(2), 213-228.
- Lee, K. M., Kim, H. J., Son, J., Park, J.-H., Kwon, O.-S., & Lee, J. (2014). Simple quantitation of formoterol and 11-nor- Δ 9-tetrahydrocannabinol-9-carboxylic acid in human urine by liquid chromatography-tandem mass spectrometry in doping control. *Journal of Chromatography B*, 967, 8-12.
- Lee, S.-L., Hsu, J.-Y., Chen, T.-C., Huang, C.-C., Wu, T.-Y., & Chin, T.-Y. (2022). Erinacine A prevents lipopolysaccharide-mediated glial cell activation to protect dopaminergic neurons against inflammatory factor-induced cell death in vitro and in vivo. *International Journal of Molecular Sciences*, 23(2), 810.
- Lee, S.-L., Tu, S.-C., Hsu, M.-Y., & Chin, T.-Y. (2021). Diosgenin prevents microglial activation and protects dopaminergic neurons from lipopolysaccharide-induced neural damage in vitro and in vivo. *International Journal of Molecular Sciences*, 22(19), 10361.
- Lei, L., Tu, Q., Zhang, X., Xiang, S., Xiao, B., Zhai, S., Yu, H., Tang, L., Guo, B., Chen, X., & Zhang, C. (2023). Stimulus-responsive curcumin-based polydopamine nanoparticles for targeting Parkinson's disease by modulating α -synuclein aggregation and reactive oxygen species. *Chemical Engineering Journal*, 461, 141606. <https://doi.org/https://doi.org/10.1016/j.cej.2023.141606>
- Leyden, E., & Tadi, P. (2025). Carbidopa. In *StatPearls*. StatPearls Publishing
- Copyright © 2025, StatPearls Publishing LLC.
- Li, L., Gao, W., Ren, N., & Chen, L. (2025a). IL-2/anti-IL-2 complexes attenuates neuroinflammation and neurodegeneration in mice of experimental Parkinson's disease. *Brain Research Bulletin*, 223, 111273. <https://doi.org/https://doi.org/10.1016/j.brainresbull.2025.111273>
- Li, L., Sun, C., Cai, S., Hang, Z., Gao, X., Hou, L., Li, L., Wu, Y., Xing, C., & Du, H. (2025b). Biomimetic Nanoparticles Enhance Recovery of Movement Disorders in Parkinson's Disease by Improving Microglial Mitochondrial Homeostasis and Suppressing Neuroinflammation. *ACS Applied Materials & Interfaces*, 17(16), 23536-23552. <https://doi.org/10.1021/acsami.4c22181>
- Li, Y., Wang, C., Zong, S., Qi, J., Dong, X., Zhao, W., Wu, W., Fu, Q., Lu, Y., & Chen, Z. (2019). The Trigeminal Pathway Dominates the Nose-to-Brain Transportation of Intact Polymeric Nanoparticles: Evidence from Aggregation-Caused Quenching Probes. *J Biomed Nanotechnol*, 15(4), 686-702. <https://doi.org/10.1166/jbn.2019.2724>
- Lim, Y. W., Tan, W. S., Ho, K. L., Mariatulqabtiah, A. R., Abu Kasim, N. H., Abd. Rahman, N., Wong, T. W., & Chee, C. F. (2022). Challenges and Complications of

- Poly(lactic-co-glycolic acid)-Based Long-Acting Drug Product Development. *Pharmaceutics*, 14(3), 614. <https://www.mdpi.com/1999-4923/14/3/614>
- Lin, H., Xie, L., Lv, L., Chen, J., Feng, F., Liu, W., Han, L., & Liu, F. (2023). Intranasally administered thermosensitive gel for brain-targeted delivery of rhynchophylline to treat Parkinson's disease. *Colloids and Surfaces B: Biointerfaces*, 222, 113065. <https://doi.org/https://doi.org/10.1016/j.colsurfb.2022.113065>
- Lin, J. H., & Yamazaki, M. (2003). Role of P-Glycoprotein in Pharmacokinetics. *Clinical Pharmacokinetics*, 42(1), 59-98. <https://doi.org/10.2165/00003088-200342010-00003>
- Lipworth, B. J., & McDevitt, D. G. (1989). Beta-adrenoceptor responses to inhaled salbutamol in normal subjects. *Eur J Clin Pharmacol*, 36(3), 239-245. <https://doi.org/10.1007/bf00558154>
- Liu, Y., Li, P., Qiao, C., Wu, T., Sun, X., Wen, M., & Zhang, W. (2020). Chitosan Hydrogel Enhances the Therapeutic Efficacy of Bone Marrow-Derived Mesenchymal Stem Cells for Myocardial Infarction by Alleviating Vascular Endothelial Cell Pyroptosis. *J Cardiovasc Pharmacol*, 75(1), 75-83. <https://doi.org/10.1097/fjc.0000000000000760>
- Liu, Y., Qin, L., Wilson, B., Wu, X., Qian, L., Granholm, A. -C., Crews, F. T., & Hong, J. -S. (2008). Endotoxin induces a delayed loss of TH-IR neurons in substantia nigra and motor behavioral deficits. *Neurotoxicology*, 29(5), 864-870.
- Liu, Y. L., Huang, H. J., Sheu, S. Y., Liu, Y. C., Lee, I. J., Chiang, S. C., & Lin, A. M. (2023). Oral ellagic acid attenuated LPS-induced neuroinflammation in rat brain: MEK1 interaction and M2 microglial polarization. *Exp Biol Med (Maywood)*, 248(7), 656-664. <https://doi.org/10.1177/15353702231182230>
- Lockman, P. R., Koziara, J. M., Mumper, R. J., & Allen, D. D. (2004). Nanoparticle surface charges alter blood-brain barrier integrity and permeability. *J Drug Target*, 12(9-10), 635-641. <https://doi.org/10.1080/10611860400015936>
- Lodeweyckx, T., de Hoon, J., Van Laere, K., Bautista, E., Rizzo, G., Bishop, C., Rabiner, E., Martin, R. S., Ford, A., & Vargas, G. (2024). Effects on cerebral blood flow after single doses of the $\beta(2)$ agonist, clenbuterol, in healthy volunteers and patients with mild cognitive impairment or Parkinson's disease. *Br J Clin Pharmacol*, 90(10), 2638-2651. <https://doi.org/10.1111/bcp.16160>
- Lomeli-Lepe, A. K., López-Pérez, S. J., Castañeda-Cabral, J. L., & Ureña-Guerrero, M. E. (2024). Early expression of monomeric and oligomeric alpha-synuclein and reduction of tyrosine hydroxylase following intranigral injection of lipopolysaccharide. *Molecular Biology Reports*, 51(1), 996.
- Lopez-Mitjavila, J. J., Palma-Florez, S., Lagunas, A., Mir, M., Samitier, J., Rodriguez-Abreu, C., & Grijalvo, S. (2025). PEGylated PLGA nanoparticles prepared from nano-emulsion templates as versatile platforms to cross blood-brain barrier models. *Journal of Drug Delivery Science and Technology*, 110, 107057. <https://doi.org/https://doi.org/10.1016/j.jddst.2025.107057>
- Loram, L. C., Sholar, P. W., Taylor, F. R., Wiesler, J. L., Babb, J. A., Strand, K. A., Berkelhammer, D., Day, H. E., Maier, S. F., & Watkins, L. R. (2012). Sex and estradiol influence glial pro-inflammatory responses to lipopolysaccharide in rats. *Psychoneuroendocrinology*, 37(10), 1688-1699.
- Loureiro, J. A., Gomes, B., Fricker, G., Coelho, M. A. N., Rocha, S., & Pereira, M. C. (2016). Cellular uptake of PLGA nanoparticles targeted with anti-amyloid and

- anti-transferrin receptor antibodies for Alzheimer's disease treatment. *Colloids Surf B Biointerfaces*, 145, 8-13. <https://doi.org/10.1016/j.colsurfb.2016.04.041>
- Lozoya-Agullo, I., Planelles, M., Merino-Sanjuán, M., Bermejo, M., Sarmiento, B., González-Álvarez, I., & González-Álvarez, M. (2019). Ion-pair approach coupled with nanoparticle formation to increase bioavailability of a low permeability charged drug. *International Journal of Pharmaceutics*, 557, 36-42. <https://doi.org/10.1016/j.ijpharm.2018.12.038>
- Lu, Y., Wang, J. T.-W., Li, N., Zhu, X., Li, Y., Bansal, S., Wang, Y., & Al-Jamal, K. T. (2023). Intranasal administration of edaravone nanoparticles improves its stability and brain bioavailability. *Journal of Controlled Release*, 359, 257-267.
- Luk, K. C., Kehm, V., Carroll, J., Zhang, B., O'Brien, P., Trojanowski, J. Q., & Lee, V. M. (2012). Pathological α -synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. *Science*, 338(6109), 949-953. <https://doi.org/10.1126/science.1227157>
- Lull, M. E., & Block, M. L. (2010). Microglial activation and chronic neurodegeneration. *Neurotherapeutics*, 7(4), 354-365. <https://doi.org/10.1016/j.nurt.2010.05.014>
- Lund, S., Christensen, K. V., Hedtjárn, M., Mortensen, A. L., Hagberg, H., Falsig, J., Hasseldam, H., Schrattenholz, A., Pörzgen, P., & Leist, M. (2006). The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and *in vivo* conditions. *Journal of Neuroimmunology*, 180(1), 71-87. <https://doi.org/10.1016/j.jneuroim.2006.07.007>
- Luo, R., Yao, Y., Chen, Z., & Sun, X. (2025a). An examination of the LPS-TLR4 immune response through the analysis of molecular structures and protein-protein interactions. *Cell Communication and Signaling*, 23(1), 142. <https://doi.org/10.1186/s12964-025-02149-4>
- Luo, Y., Qiao, L., Li, M., Wen, X., Zhang, W., & Li, X. (2025b). Global, regional, national epidemiology and trends of Parkinson's disease from 1990 to 2021: findings from the Global Burden of Disease Study 2021 [Original Research]. *Frontiers in Aging Neuroscience*, Volume 16 - 2024. <https://doi.org/10.3389/fnagi.2024.1498756>
- Lynch, M. A. (2009). The multifaceted profile of activated microglia. *Mol Neurobiol*, 40(2), 139-156. <https://doi.org/10.1007/s12035-009-8077-9>
- Ma, S. Y., Røytgård, M., Rinne, J. O., Collan, Y., & Rinne, U. K. (1997). Correlation between neuromorphometry in the substantia nigra and clinical features in Parkinson's disease using disector counts. *J Neurol Sci*, 151(1), 83-87. [https://doi.org/10.1016/s0022-510x\(97\)00100-7](https://doi.org/10.1016/s0022-510x(97)00100-7)
- Ma, Y., Zhao, X., Li, J., & Shen, Q. (2012). The comparison of different daidzein-PLGA nanoparticles in increasing its oral bioavailability. *Int J Nanomedicine*, 7, 559-570. <https://doi.org/10.2147/ijn.S27641>
- Maas, B. R., Göttgens, I., Tijssen Klasen, H. P., Kapelle, W. M., Radder, D. L., Bloem, B. R., Post, B., de Vries, N. M., & Darweesh, S. K. (2024). Age and gender differences in non-motor symptoms in people with Parkinson's disease. *Frontiers in neurology*, 15, 1339716.
- Machado, A., Herrera, A., Venero, J., Santiago, M., DePablos, R., Villaran, R., Espinosa-Oliva, A., Argüelles, S., Sarmiento, M., & Delgado-Cortés, M. (2011). Inflammatory Animal Model for Parkinson's Disease: The Intranigral Injection of LPS Induced the Inflammatory Process along with the Selective

- Degeneration of Nigrostriatal Dopaminergic Neurons. *International Scholarly Research Notices*, 2011(1), 476158.
- Maher, R., Moreno-Borralló, A., Jindal, D., Mai, B. T., Ruiz-Hernandez, E., & Harkin, A. (2023). Intranasal Polymeric and Lipid-Based Nanocarriers for CNS Drug Delivery. *Pharmaceutics*, 15(3). <https://doi.org/10.3390/pharmaceutics15030746>
- Maiti, B., & Perlmutter, J. S. (2020). A Clinical Trial of Isradipine: What Went Wrong? *Ann Intern Med*, 172(9), 625-626. <https://doi.org/10.7326/m20-1023>
- Maity, S., Abbaspour, R., Nahabedian, D., & Connor, S. A. (2022). Norepinephrine, beyond the Synapse: Coordinating Epigenetic Codes for Memory. *Int J Mol Sci*, 23(17). <https://doi.org/10.3390/ijms23179916>
- Makadia, H. K., & Siegel, S. J. (2011). Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. *Polymers*, 3(3), 1377-1397. <https://www.mdpi.com/2073-4360/3/3/1377>
- Mandal, S., Mandal, S. D., Chuttani, K., Sawant, K. K., & Subudhi, B. B. (2016). Design and evaluation of mucoadhesive microemulsion for neuroprotective effect of ibuprofen following intranasal route in the MPTP mice model. *Drug Development and Industrial Pharmacy*, 42(8), 1340-1350.
- Mandela, P., & Ordway, G. A. (2006). The norepinephrine transporter and its regulation. *Journal of neurochemistry*, 97(2), 310-333.
- Manzanares, D., & Ceña, V. (2020). Endocytosis: The Nanoparticle and Submicron Nanocompounds Gateway into the Cell. *Pharmaceutics*, 12(4). <https://doi.org/10.3390/pharmaceutics12040371>
- Marecki, E. K., Oh, K. W., Knight, P. R., & Davidson, B. A. (2024). Poly(lactic-co-glycolic acid) nanoparticle fabrication, functionalization, and biological considerations for drug delivery. *Biomicrofluidics*, 18(5), 051503. <https://doi.org/10.1063/5.0201465>
- Marien, M. R., Colpaert, F. C., & Rosenquist, A. C. (2004). Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain Res Brain Res Rev*, 45(1), 38-78. <https://doi.org/10.1016/j.brainresrev.2004.02.002>
- Marinelli, L., Ciulla, M., Ritsema, J. A. S., van Nostrum, C. F., Cacciatore, I., Dimmito, M. P., Palmerio, F., Orlando, G., Robuffo, I., Grande, R., Puca, V., & Di Stefano, A. (2022). Preparation, Characterization, and Biological Evaluation of a Hydrophilic Peptide Loaded on PEG-PLGA Nanoparticles. *Pharmaceutics*, 14(9), 1821. <https://www.mdpi.com/1999-4923/14/9/1821>
- Marinova-Mutafchieva, L., Sadeghian, M., Broom, L., Davis, J. B., Medhurst, A. D., & Dexter, D. T. (2009). Relationship between microglial activation and dopaminergic neuronal loss in the substantia nigra: a time course study in a 6-hydroxydopamine model of Parkinson's disease. *J Neurochem*, 110(3), 966-975. <https://doi.org/10.1111/j.1471-4159.2009.06189.x>
- Marinova-Mutafchieva, L., Sadeghian, M., Broom, L., Davis, J. B., Medhurst, A. D., & Dexter, D. T. (2009). Relationship between microglial activation and dopaminergic neuronal loss in the substantia nigra: a time course study in a 6-hydroxydopamine model of Parkinson's disease. *Journal of neurochemistry*, 110(3), 966-975.
- Marsh, S. E., Walker, A. J., Kamath, T., Dissing-Olesen, L., Hammond, T. R., de Soysa, T. Y., Young, A. M. H., Murphy, S., Abdulraouf, A., Nadaf, N., Dufort, C., Walker,

- A. C., Lucca, L. E., Kozareva, V., Vanderburg, C., Hong, S., Bulstrode, H., Hutchinson, P. J., Gaffney, D. J.,...Stevens, B. (2022). Dissection of artifactual and confounding glial signatures by single-cell sequencing of mouse and human brain. *Nature Neuroscience*, 25(3), 306-316. <https://doi.org/10.1038/s41593-022-01022-8>
- Marsili, L., Rizzo, G., & Colosimo, C. (2018). Diagnostic Criteria for Parkinson's Disease: From James Parkinson to the Concept of Prodromal Disease. *Front Neurol*, 9, 156. <https://doi.org/10.3389/fneur.2018.00156>
- Martín-Camacho, U. d. J., Rodríguez-Barajas, N., Sánchez-Burgos, J. A., & Pérez-Larios, A. (2023). Weibull β value for the discernment of drug release mechanism of PLGA particles. *International Journal of Pharmaceutics*, 640, 123017. <https://doi.org/https://doi.org/10.1016/j.ijpharm.2023.123017>
- Martínez Rivas, C. J., Tarhini, M., Badri, W., Miladi, K., Greige-Gerges, H., Nazari, Q. A., Galindo Rodríguez, S. A., Román, R. Á., Fessi, H., & Elaissari, A. (2017). Nanoprecipitation process: From encapsulation to drug delivery. *International Journal of Pharmaceutics*, 532(1), 66-81. <https://doi.org/https://doi.org/10.1016/j.ijpharm.2017.08.064>
- Mavridis, M., Degryse, A. D., Lategan, A. J., Marien, M. R., & Colpaert, F. C. (1991). Effects of locus coeruleus lesions on parkinsonian signs, striatal dopamine and substantia nigra cell loss after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in monkeys: a possible role for the locus coeruleus in the progression of Parkinson's disease. *Neuroscience*, 41(2-3), 507-523. [https://doi.org/10.1016/0306-4522\(91\)90345-o](https://doi.org/10.1016/0306-4522(91)90345-o)
- Mazzarino, M., De La Torre, X., Fiacco, I., Pompei, C., Calabrese, F., & Botrè, F. (2013). A simplified procedure for the analysis of formoterol in human urine by liquid chromatography–electrospray tandem mass spectrometry: Application to the characterization of the metabolic profile and stability of formoterol in urine. *Journal of Chromatography B*, 931, 75-83.
- McAuslan, M. (PhD thesis). *Assessment of treatment with the β 2-adrenoreceptor agonist formoterol on inflammogen LPS-induced cognitive impairment following systemic and direct challenge to the locus coeruleus of rats*
- McCoy, M. K., Martinez, T. N., Ruhn, K. A., Szymkowski, D. E., Smith, C. G., Botterman, B. R., Tansey, K. E., & Tansey, M. G. (2006). Blocking soluble tumor necrosis factor signaling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease. *J Neurosci*, 26(37), 9365-9375. <https://doi.org/10.1523/jneurosci.1504-06.2006>
- McCulloch, P. F., Lahrman, K. A., DelPrete, B., & DiNovo, K. M. (2018). Innervation of the Nose and Nasal Region of the Rat: Implications for Initiating the Mammalian Diving Response. *Front Neuroanat*, 12, 85. <https://doi.org/10.3389/fnana.2018.00085>
- McFarland, N. R., Lee, J. S., Hyman, B. T., & McLean, P. J. (2009). Comparison of transduction efficiency of recombinant AAV serotypes 1, 2, 5, and 8 in the rat nigrostriatal system. *J Neurochem*, 109(3), 838-845. <https://doi.org/10.1111/j.1471-4159.2009.06010.x>
- McGeer, P. L., Itagaki, S., Boyes, B. E., & McGeer, E. G. (1988). Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's

- disease brains. *Neurology*, 38(8), 1285-1291.
<https://doi.org/10.1212/wnl.38.8.1285>
- McMillan, P. J., White, S. S., Franklin, A., Greenup, J. L., Leverenz, J. B., Raskind, M. A., & Szot, P. (2011). Differential response of the central noradrenergic nervous system to the loss of locus coeruleus neurons in Parkinson's disease and Alzheimer's disease. *Brain Res*, 1373, 240-252.
<https://doi.org/10.1016/j.brainres.2010.12.015>
- McNamee, E. N., Griffin, É. W., Ryan, K. M., Ryan, K. J., Heffernan, S., Harkin, A., & Connor, T. J. (2010a). Noradrenaline acting at β -adrenoceptors induces expression of IL-1 β and its negative regulators IL-1ra and IL-1RII, and drives an overall anti-inflammatory phenotype in rat cortex. *Neuropharmacology*, 59(1-2), 37-48.
- McNamee, E. N., Ryan, K. M., Griffin, E. W., González-Reyes, R. E., Ryan, K. J., Harkin, A., & Connor, T. J. (2010b). Noradrenaline acting at central beta-adrenoceptors induces interleukin-10 and suppressor of cytokine signaling-3 expression in rat brain: implications for neurodegeneration. *Brain Behav Immun*, 24(4), 660-671.
<https://doi.org/10.1016/j.bbi.2010.02.005>
- Mehrizi, T. Z., Kafiabad, S. A., & Eshghi, P. (2021). Effects and treatment applications of polymeric nanoparticles on improving platelets' storage time: a review of the literature from 2010 to 2020. *Blood Res*, 56(4), 215-228.
<https://doi.org/10.5045/br.2021.2021094>
- Meissner, W. G. (2012). When does Parkinson's disease begin? From prodromal disease to motor signs. *Revue Neurologique*, 168(11), 809-814.
<https://doi.org/https://doi.org/10.1016/j.neurol.2012.07.004>
- Mejías-Aponte, C. A., Drouin, C., & Aston-Jones, G. (2009). Adrenergic and noradrenergic innervation of the midbrain ventral tegmental area and retrorubral field: prominent inputs from medullary homeostatic centers. *Journal of Neuroscience*, 29(11), 3613-3626.
- Meng, Q., Wang, A., Hua, H., Jiang, Y., Wang, Y., Mu, H., Wu, Z., & Sun, K. (2018). Intranasal delivery of Huperzine A to the brain using lactoferrin-conjugated N-trimethylated chitosan surface-modified PLGA nanoparticles for treatment of Alzheimer's disease. *International Journal of Nanomedicine*, Volume 13, 705-718. <https://doi.org/10.2147/ijn.s151474>
- Meredith, G. E., & Rademacher, D. J. (2011). MPTP mouse models of Parkinson's disease: an update. *J Parkinsons Dis*, 1(1), 19-33. <https://doi.org/10.3233/jpd-2011-11023>
- Miklossy, J., Doudet, D., Schwab, C., Yu, S., McGeer, E., & McGeer, P. (2006). Role of ICAM-1 in persisting inflammation in Parkinson disease and MPTP monkeys. *Experimental neurology*, 197(2), 275-283.
- Mir, M., Tolosa, L., Asensio, V. J., Lladó, J., & Olmos, G. (2008). Complementary roles of tumor necrosis factor alpha and interferon gamma in inducible microglial nitric oxide generation. *J Neuroimmunol*, 204(1-2), 101-109.
<https://doi.org/10.1016/j.jneuroim.2008.07.002>
- Mischley, L. K., Lau, R. C., Shankland, E. G., Wilbur, T. K., & Padowski, J. M. (2017). Phase IIb Study of Intranasal Glutathione in Parkinson's Disease. *J Parkinsons Dis*, 7(2), 289-299. <https://doi.org/10.3233/jpd-161040>

- Mittal, S., Bjørnevik, K., Im, D. S., Flierl, A., Dong, X., Locascio, J. J., Abo, K. M., Long, E., Jin, M., & Xu, B. (2017). β 2-Adrenoreceptor is a regulator of the α -synuclein gene driving risk of Parkinson's disease. *Science*, 357(6354), 891-898.
- Mohan, L. J., McDonald, L., Daly, J. S., & Ramtoola, Z. (2020). Optimising PLGA-PEG Nanoparticle Size and Distribution for Enhanced Drug Targeting to the Inflamed Intestinal Barrier. *Pharmaceutics*, 12(11). <https://doi.org/10.3390/pharmaceutics12111114>
- Molina, J. A., Jiménez-Jiménez, F. J., Ortí-Pareja, M., & Navarro, J. A. (1998). The role of nitric oxide in neurodegeneration. Potential for pharmacological intervention. *Drugs Aging*, 12(4), 251-259. <https://doi.org/10.2165/00002512-199812040-00001>
- Monge-Fuentes, V., Biolchi Mayer, A., Lima, M. R., Geraldles, L. R., Zanotto, L. N., Moreira, K. G., Martins, O. P., Piva, H. L., Felipe, M. S. S., & Amaral, A. C. (2021). Dopamine-loaded nanoparticle systems circumvent the blood–brain barrier restoring motor function in mouse model for Parkinson's Disease. *Scientific Reports*, 11(1), 1-16.
- Mori, K., Ozaki, E., Zhang, B., Yang, L., Yokoyama, A., Takeda, I., Maeda, N., Sakanaka, M., & Tanaka, J. (2002). Effects of norepinephrine on rat cultured microglial cells that express α 1, α 2, β 1 and β 2 adrenergic receptors. *Neuropharmacology*, 43(6), 1026-1034. [https://doi.org/10.1016/s0028-3908\(02\)00211-3](https://doi.org/10.1016/s0028-3908(02)00211-3)
- Morin, T. M., Allan, N., Coutts, J., Hooker, J. M., Langille, M., Metcalfe, A., Thamboo, A., Jackson, J., Sharma, M., Rees, T., Enright, K., & Irving, K. (2024). Laminar Fluid Ejection for Olfactory Drug Delivery: A Proof of Concept Study. *IEEE J Transl Eng Health Med*, 12, 727-738. <https://doi.org/10.1109/jtehm.2024.3503498>
- Morin, T. M., Allan, N., Hooker, J. M., Bernhard, W., Barreto, K., Jett, K. A., Costa Carvalho, J. L., Sarafis, K., Geyer, C. R., Wei, Y., Zhu, L., Li, F., Enright, K., & Irving, K. (2025). Olfactory Drug Delivery in Rodents: Deposition and Pharmacokinetics. *ACS Pharmacol Transl Sci*, 8(7), 2167-2179. <https://doi.org/10.1021/acspstsci.5c00206>
- Morshed, N., Rennie, C., Faria, M., Collins-Praino, L., & Care, A. (2024). Protein Coronas Derived from Cerebrospinal Fluid Enhance the Interactions Between Nanoparticles and Brain Cells. *bioRxiv*, 2024.2005.2031.596763. <https://doi.org/10.1101/2024.05.31.596763>
- Mount, M. P., Lira, A., Grimes, D., Smith, P. D., Faucher, S., Slack, R., Anisman, H., Hayley, S., & Park, D. S. (2007). Involvement of Interferon- γ in Microglial-Mediated Loss of Dopaminergic Neurons. *The Journal of Neuroscience*, 27(12), 3328-3337. <https://doi.org/10.1523/jneurosci.5321-06.2007>
- Müller, W. E., Wang, X., & Schröder, H. C. (2013). Biomedical Inorganic Polymers. *Prog. Mol. Subcell. Biol.*, 54, 65-116.
- Musumeci, T., Di Benedetto, G., Carbone, C., Bonaccorso, A., Amato, G., Lo Faro, M. J., Burgaletto, C., Puglisi, G., Bernardini, R., & Cantarella, G. (2022). Intranasal Administration of a TRAIL Neutralizing Monoclonal Antibody Adsorbed in PLGA Nanoparticles and NLC Nanosystems: An In Vivo Study on a Mouse Model of Alzheimer's Disease. *Biomedicines*, 10(5). <https://doi.org/10.3390/biomedicines10050985>

- Nadarassan, D. K., Chrystyn, H., Clark, B. J., & Assi, K. H. (2007). Validation of high-performance liquid chromatography assay for quantification of formoterol in urine samples after inhalation using UV detection technique. *J Chromatogr B Analyt Technol Biomed Life Sci*, 850(1-2), 31-37. <https://doi.org/10.1016/j.jchromb.2006.10.059>
- Naidoo, V., Mdanda, S., Ntshangase, S., Naicker, T., Kruger, H. G., Govender, T., Naidoo, P., & Baijnath, S. (2019). Brain penetration of ketamine: Intranasal delivery VS parenteral routes of administration. *Journal of psychiatric research*, 112, 7-11.
- Nakao, V. W., Mazucanti, C. H. Y., de Sá Lima, L., de Mello, P. S., de Souza Port's, N. M., Kinoshita, P. F., Leite, J. A., Kawamoto, E. M., & Scavone, C. (2022). Neuroprotective action of α -Klotho against LPS-activated glia conditioned medium in primary neuronal culture. *Scientific Reports*, 12(1), 18884. <https://doi.org/10.1038/s41598-022-21132-4>
- Nance, E. A., Woodworth, G. F., Sailor, K. A., Shih, T. Y., Xu, Q., Swaminathan, G., Xiang, D., Eberhart, C., & Hanes, J. (2012). A dense poly(ethylene glycol) coating improves penetration of large polymeric nanoparticles within brain tissue. *Sci Transl Med*, 4(149), 149ra119. <https://doi.org/10.1126/scitranslmed.3003594>
- Negrini, M., Tomasello, G., Davidsson, M., Fenyi, A., Adant, C., Hauser, S., Espa, E., Gubinelli, F., Manfredsson, F. P., Melki, R., & Heuer, A. (2022). Sequential or Simultaneous Injection of Preformed Fibrils and AAV Overexpression of Alpha-Synuclein Are Equipotent in Producing Relevant Pathology and Behavioral Deficits. *J Parkinsons Dis*, 12(4), 1133-1153. <https://doi.org/10.3233/jpd-212555>
- Neha, B., Ganesh, B., & Preeti, K. (2013). Drug delivery to the brain using polymeric nanoparticles: a review. *International Journal of Pharmaceutical and Life Sciences*, 2(3), 107-132.
- Nema, T., & Bhargava, S. (2024). Intranasal delivery of insulin for the restoration of memory signaling in Alzheimer's disease. *Parkinsonism & related disorders*, 122.
- Neuchat, E. E., Bocklud, B. E., Kingsley, K., Barham, W. T., Luther, P. M., Ahmadzadeh, S., Shekoochi, S., Cornett, E. M., & Kaye, A. D. (2023). The Role of Alpha-2 Agonists for Attention Deficit Hyperactivity Disorder in Children: A Review. *Neurol Int*, 15(2), 697-707. <https://doi.org/10.3390/neurolint15020043>
- Nguyen, V. T., Walker, W. S., & Benveniste, E. N. (1998). Post-transcriptional inhibition of CD40 gene expression in microglia by transforming growth factor-beta. *Eur J Immunol*, 28(8), 2537-2548. [https://doi.org/10.1002/\(sici\)1521-4141\(199808\)28:08<2537::Aid-immu2537>3.0.Co;2-1](https://doi.org/10.1002/(sici)1521-4141(199808)28:08<2537::Aid-immu2537>3.0.Co;2-1)
- Nietz, A. K., Popa, L. S., Streng, M. L., Carter, R. E., Kodandaramaiah, S. B., & Ebner, T. J. (2022). Wide-Field Calcium Imaging of Neuronal Network Dynamics In Vivo. *Biology (Basel)*, 11(11). <https://doi.org/10.3390/biology11111601>
- Niu, H., Wang, Q., Zhao, W., Liu, J., Wang, D., Muhammad, B., Liu, X., Quan, N., Zhang, H., & Zhang, F. (2020). IL-1 β /IL-1R1 signaling induced by intranasal lipopolysaccharide infusion regulates alpha-Synuclein pathology in the olfactory bulb, substantia nigra and striatum. *Brain Pathology*, 30(6), 1102-1118.
- Noh, C., Shin, H. J., Lee, S., Kim, S. I., Kim, Y. H., Lee, W. H., Kim, D. W., Lee, S. Y., & Ko, Y. K. (2020). CX3CR1-Targeted PLGA Nanoparticles Reduce Microglia Activation

- and Pain Behavior in Rats with Spinal Nerve Ligation. *Int J Mol Sci*, 21(10). <https://doi.org/10.3390/ijms21103469>
- Nomoto, M., Yabe, H., Ando, R., Sumiyoshi, Y., Akagi, T., Ise, R., Akiyoshi, Y., Date, C., Nakano, S., & Suzuki, R. (2025). A Proof-of-concept Study of TR-012001, a Nasal Levodopa, in Patients with Parkinson's Disease with OFF Episodes, Without Dopa Decarboxylase Inhibitor (P11-5.009). *Neurology*, 104(7_Supplement_1), 2617. <https://doi.org/doi:10.1212/WNL.0000000000210464>
- Novak, P., Pimentel Maldonado, D. A., & Novak, V. (2019). Safety and preliminary efficacy of intranasal insulin for cognitive impairment in Parkinson disease and multiple system atrophy: A double-blinded placebo-controlled pilot study. *PLoS One*, 14(4), e0214364. <https://doi.org/10.1371/journal.pone.0214364>
- O'Donnell, J. M., & Frazer, A. (1985). Effects of clenbuterol and antidepressant drugs on beta adrenergic receptor/N-protein coupling in the cerebral cortex of the rat. *The Journal of pharmacology and experimental therapeutics*, 234(1), 30-36.
- O'Farrell, K., Fagan, E., Connor, T. J., & Harkin, A. (2017). Inhibition of the kynurenine pathway protects against reactive microglial-associated reductions in the complexity of primary cortical neurons. *Eur J Pharmacol*, 810, 163-173. <https://doi.org/10.1016/j.ejphar.2017.07.008>
- O'Neill, E., & Harkin, A. (2018). Targeting the noradrenergic system for anti-inflammatory and neuroprotective effects: implications for Parkinson's disease. *Neural Regen Res*, 13(8), 1332-1337. <https://doi.org/10.4103/1673-5374.235219>
- O'Neill, E., Yssel, J. D., McNamara, C., & Harkin, A. (2020). Pharmacological targeting of β 2-adrenoceptors is neuroprotective in the LPS inflammatory rat model of Parkinson's disease. *British journal of pharmacology*, 177(2), 282-297.
- O'Reilly, K., O'Farrell, K., Midttun, O., Rakovets, Y., David-Bercholz, J., & Harkin, A. (2021). Kynurenic Acid Protects Against Reactive Glial-associated Reductions in the Complexity of Primary Cortical Neurons. *J Neuroimmune Pharmacol*, 16(3), 679-692. <https://doi.org/10.1007/s11481-020-09976-x>
- O'Sullivan, J. B., Ryan, K. M., Curtin, N. M., Harkin, A., & Connor, T. J. (2009). Noradrenaline reuptake inhibitors limit neuroinflammation in rat cortex following a systemic inflammatory challenge: implications for depression and neurodegeneration. *Int J Neuropsychopharmacol*, 12(5), 687-699. <https://doi.org/10.1017/s146114570800967x>
- O'Sullivan, J. B., Ryan, K. M., Harkin, A., & Connor, T. J. (2010). Noradrenaline reuptake inhibitors inhibit expression of chemokines IP-10 and RANTES and cell adhesion molecules VCAM-1 and ICAM-1 in the CNS following a systemic inflammatory challenge. *J Neuroimmunol*, 220(1-2), 34-42. <https://doi.org/10.1016/j.jneuroim.2009.12.007>
- O'Neill, E., Chiara Goisis, R., Haverty, R., & Harkin, A. (2019). L-alpha-aminoadipic acid restricts dopaminergic neurodegeneration and motor deficits in an inflammatory model of Parkinson's disease in male rats. *Journal of neuroscience research*, 97(7), 804-816.
- Ohtsuki, S., Yamaguchi, H., Kang, Y.-S., Hori, S., & Terasaki, T. (2010). Reduction of L-type amino acid transporter 1 mRNA expression in brain capillaries in a mouse model of Parkinson's disease. *Biological and Pharmaceutical Bulletin*, 33(7), 1250-1252.

- Olanow, C. W., Standaert, D. G., Kieburtz, K., Viegas, T. X., & Moreadith, R. (2020). Once-Weekly Subcutaneous Delivery of Polymer-Linked Rotigotine (SER-214) Provides Continuous Plasma Levels in Parkinson's Disease Patients. *Mov Disord*, 35(6), 1055-1061. <https://doi.org/10.1002/mds.28027>
- Olsson, M., Nikkhah, G., Bentlage, C., & Björklund, A. (1995). Forelimb akinesia in the rat Parkinson model: differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. *Journal of Neuroscience*, 15(12), 5391-5401.
- Onyema, H. N., Berger, M., Musyanovych, A., Bantz, C., Maskos, M., & Freese, C. (2021). Uptake of polymeric nanoparticles in a human induced pluripotent stem cell-based blood-brain barrier model: Impact of size, material, and protein corona. *Biointerphases*, 16(2), 021004.
- Ouchi, Y., Yoshikawa, E., Sekine, Y., Futatsubashi, M., Kanno, T., Ogusu, T., & Torizuka, T. (2005). Microglial activation and dopamine terminal loss in early Parkinson's disease. *Ann Neurol*, 57(2), 168-175. <https://doi.org/10.1002/ana.20338>
- Ouzzine, M., Gulberti, S., Ramalanjaona, N., Magdalou, J., & Fournel-Gigleux, S. (2014). The UDP-glucuronosyltransferases of the blood-brain barrier: their role in drug metabolism and detoxication [Review]. *Frontiers in cellular neuroscience*, Volume 8 - 2014. <https://doi.org/10.3389/fncel.2014.00349>
- Öztürk, A. A., Yenilmez, E., & Özarda, M. G. (2019). Clarithromycin-Loaded Poly (Lactic-co-glycolic Acid) (PLGA) Nanoparticles for Oral Administration: Effect of Polymer Molecular Weight and Surface Modification with Chitosan on Formulation, Nanoparticle Characterization and Antibacterial Effects. *Polymers (Basel)*, 11(10). <https://doi.org/10.3390/polym11101632>
- Pacelli, C., Giguère, N., Bourque, M. J., Lévesque, M., Slack, R. S., & Trudeau, L. (2015). Elevated Mitochondrial Bioenergetics and Axonal Arborization Size Are Key Contributors to the Vulnerability of Dopamine Neurons. *Curr Biol*, 25(18), 2349-2360. <https://doi.org/10.1016/j.cub.2015.07.050>
- Papadopoulou, V., Kosmidis, K., Vlachou, M., & Macheras, P. (2006). On the use of the Weibull function for the discernment of drug release mechanisms. *Int J Pharm*, 309(1-2), 44-50. <https://doi.org/10.1016/j.ijpharm.2005.10.044>
- Paredes-Rodriguez, E., Vegas-Suarez, S., Morera-Herreras, T., De Deurwaerdere, P., & Miguelez, C. (2020). The Noradrenergic System in Parkinson's Disease. *Front Pharmacol*, 11, 435. <https://doi.org/10.3389/fphar.2020.00435>
- Patel, T., Zhou, J., Piepmeyer, J. M., & Saltzman, W. M. (2012). Polymeric nanoparticles for drug delivery to the central nervous system. *Advanced drug delivery reviews*, 64(7), 701-705.
- Patterson, J. R., Hirst, W. D., Howe, J. W., Russell, C. P., Cole-Strauss, A., Kemp, C. J., Duffy, M. F., Lamp, J., Umstead, A., Kubik, M., Stoll, A. C., Vega, I. E., Steece-Collier, K., Chen, Y., Campbell, A. C., Nezich, C. L., Glajch, K. E., & Sortwell, C. E. (2022). Beta2-adrenoreceptor agonist clenbuterol produces transient decreases in alpha-synuclein mRNA but no long-term reduction in protein. *npj Parkinson's Disease*, 8(1), 61. <https://doi.org/10.1038/s41531-022-00322-x>
- Peng, Y., Jiang, D.-y., Yao, S.-y., Zhang, X., Kazuo, S., Liu, J., Du, M.-q., Lin, L.-x., Chen, Q., & Jin, H. (2025). Gene-modified animal models of Parkinson's disease. *Experimental neurology*, 390, 115287. <https://doi.org/10.1016/j.expneurol.2025.115287>

- Perkušić, M., Nižić Nodilo, L., Ugrina, I., Špoljarić, D., Jakobušić Brala, C., Pepić, I., Lovrić, J., Safundžić Kučuk, M., Trenkel, M., Scherließ, R., Zadravec, D., Kalogjera, L., & Hafner, A. (2023). Chitosan-Based Thermogelling System for Nose-to-Brain Donepezil Delivery: Optimising Formulation Properties and Nasal Deposition Profile. *Pharmaceutics*, 15(6). <https://doi.org/10.3390/pharmaceutics15061660>
- Perry, V. H., & Holmes, C. (2014). Microglial priming in neurodegenerative disease. *Nat Rev Neurol*, 10(4), 217-224. <https://doi.org/10.1038/nrneurol.2014.38>
- Peter, I., Dubinsky, M., Bressman, S., Park, A., Lu, C., Chen, N., & Wang, A. (2018). Anti-Tumor Necrosis Factor Therapy and Incidence of Parkinson Disease Among Patients With Inflammatory Bowel Disease. *JAMA Neurol*, 75(8), 939-946. <https://doi.org/10.1001/jamaneurol.2018.0605>
- Peterson, I. L., Liktors-Busa, E., Karlage, K. L., Young, S. J., Scholpa, N. E., Schnellmann, R. G., & Largent-Milnes, T. M. (2024). Formoterol dynamically alters endocannabinoid tone in the periaqueductal gray inducing headache. *The Journal of Headache and Pain*, 25(1), 200.
- Pham, K. Y., Khanal, S., Bohara, G., Rimal, N., Song, S. H., Nguyen, T. T. K., Hong, I. S., Cho, J., Kang, J. S., Lee, S., Choi, D. Y., & Yook, S. (2025). HDAC6 inhibitor-loaded brain-targeted nanocarrier-mediated neuroprotection in methamphetamine-driven Parkinson's disease. *Redox Biol*, 79, 103457. <https://doi.org/10.1016/j.redox.2024.103457>
- Phạm, T. L., Yin, Y., Kwon, H. H., Shin, N., Kim, S. I., Park, H., Shin, J., Shin, H. J., Hwang, J. A., Song, H. J., Kim, S. R., Lee, J. H., Hwang, P. T. J., Jun, H. W., & Kim, D. W. (2020). miRNA 146a-5p-loaded poly(d,l-lactic-co-glycolic acid) nanoparticles impair pain behaviors by inhibiting multiple inflammatory pathways in microglia. *Nanomedicine (Lond)*, 15(11), 1113-1126. <https://doi.org/10.2217/nnm-2019-0462>
- Plotegher, N., Gratton, E., & Bubacco, L. (2014). Number and Brightness analysis of alpha-synuclein oligomerization and the associated mitochondrial morphology alterations in live cells. *Biochim Biophys Acta*, 1840(6), 2014-2024. <https://doi.org/10.1016/j.bbagen.2014.02.013>
- Pober, J. S., & Sessa, W. C. (2007). Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology*, 7(10), 803-815.
- Ponomarev, E. D., Shriver, L. P., Maresz, K., & Dittel, B. N. (2005). Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. *J Neurosci Res*, 81(3), 374-389. <https://doi.org/10.1002/jnr.20488>
- Portela, L. C. P. N., Cahú, T. B., Bezerra, T. S., Santos, D. K. D. d. N., Sousa, G. F., Portela, R. W. S., Melo, C. M. L., & Bezerra, R. d. S. (2022). Biocompatibility and immunostimulatory properties of fish collagen and shrimp chitosan towards peripheral blood mononuclear cells (PBMCs). *International Journal of Biological Macromolecules*, 210, 282-291. <https://doi.org/https://doi.org/10.1016/j.ijbiomac.2022.05.018>
- Pott Godoy, M. C., Tarelli, R., Ferrari, C. C., Sarchi, M. I., & Pitossi, F. J. (2008). Central and systemic IL-1 exacerbates neurodegeneration and motor symptoms in a model of Parkinson's disease. *Brain*, 131(Pt 7), 1880-1894. <https://doi.org/10.1093/brain/awn101>

- Pourtalebi Jahromi, L., Ghazali, M., Ashrafi, H., & Azadi, A. (2020). A comparison of models for the analysis of the kinetics of drug release from PLGA-based nanoparticles. *Heliyon*, 6(2), e03451. <https://doi.org/10.1016/j.heliyon.2020.e03451>
- Priya, S., Desai, V. M., & Singhvi, G. (2023). Surface Modification of Lipid-Based Nanocarriers: A Potential Approach to Enhance Targeted Drug Delivery. *ACS Omega*, 8(1), 74-86. <https://doi.org/10.1021/acsomega.2c05976>
- Provenzano, R., Schiller, B., Rao, M., Coyne, D., Brenner, L., & Pereira, B. J. (2009). Ferumoxytol as an intravenous iron replacement therapy in hemodialysis patients. *Clin J Am Soc Nephrol*, 4(2), 386-393. <https://doi.org/10.2215/cjn.02840608>
- Pugazhenth, S., Zhang, Y., Bouchard, R., & Mahaffey, G. (2013). Induction of an inflammatory loop by interleukin-1 β and tumor necrosis factor- α involves NF- κ B and STAT-1 in differentiated human neuroprogenitor cells. *PLoS One*, 8(7), e69585. <https://doi.org/10.1371/journal.pone.0069585>
- Purves, D., Augustine, G.J., Fitzpatrick, D., et al. (2001). *Neuroscience* (6th edition. ed.). Sinauer Associates.
- Pustulka, K. M., Wohl, A. R., Lee, H. S., Michel, A. R., Han, J., Hoye, T. R., McCormick, A. V., Panyam, J., & Macosko, C. W. (2013). Flash nanoprecipitation: particle structure and stability. *Mol Pharm*, 10(11), 4367-4377. <https://doi.org/10.1021/mp400337f>
- Qian, L., Wu, H.-m., Chen, S.-H., Zhang, D., Ali, S. F., Peterson, L., Wilson, B., Lu, R.-B., Hong, J.-S., & Flood, P. M. (2011). β 2-adrenergic receptor activation prevents rodent dopaminergic neurotoxicity by inhibiting microglia via a novel signaling pathway. *The Journal of Immunology*, 186(7), 4443-4454.
- Qin, H., Wilson, C. A., Lee, S. J., Zhao, X., & Benveniste, E. N. (2005). LPS induces CD40 gene expression through the activation of NF- κ B and STAT-1 α in macrophages and microglia. *Blood*, 106(9), 3114-3122. <https://doi.org/10.1182/blood-2005-02-0759>
- Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., Knapp, D. J., & Crews, F. T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia*, 55(5), 453-462.
- Qin, X. Y., Zhang, S. P., Cao, C., Loh, Y. P., & Cheng, Y. (2016). Aberrations in Peripheral Inflammatory Cytokine Levels in Parkinson Disease: A Systematic Review and Meta-analysis. *JAMA Neurol*, 73(11), 1316-1324. <https://doi.org/10.1001/jamaneurol.2016.2742>
- Rahmanian-Devin, P., Baradaran Rahimi, V., & Askari, V. R. (2021). Thermosensitive Chitosan- β -Glycerophosphate Hydrogels as Targeted Drug Delivery Systems: An Overview on Preparation and Their Applications. *Adv Pharmacol Pharm Sci*, 2021, 6640893. <https://doi.org/10.1155/2021/6640893>
- Rampado, R., Crotti, S., Caliceti, P., Pucciarelli, S., & Agostini, M. (2020). Recent Advances in Understanding the Protein Corona of Nanoparticles and in the Formulation of “Stealthy” Nanomaterials [Review]. *Frontiers in Bioengineering and Biotechnology*, Volume 8 - 2020. <https://www.frontiersin.org/journals/bioengineering-and-biotechnology/articles/10.3389/fbioe.2020.00166>

- Rani, N., Kaushik, A., Kardam, S., Kag, S., Raj, V. S., Ambasta, R. K., & Kumar, P. (2024). Reimagining old drugs with new tricks: Mechanisms, strategies and notable success stories in drug repurposing for neurological diseases. *Prog Mol Biol Transl Sci*, 205, 23-70. <https://doi.org/10.1016/bs.pmbts.2024.03.029>
- Rao, J. P., & Geckeler, K. E. (2011). Polymer nanoparticles: Preparation techniques and size-control parameters. *Progress in Polymer Science*, 36(7), 887-913. <https://doi.org/https://doi.org/10.1016/j.progpolymsci.2011.01.001>
- Rasheed, N. O. A., El Sayed, N. S., & El-Khatib, A. S. (2018). Targeting central $\beta 2$ receptors ameliorates streptozotocin-induced neuroinflammation via inhibition of glycogen synthase kinase3 pathway in mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 86, 65-75.
- Rassu, G., Soddu, E., Cossu, M., Gavini, E., Giunchedi, P., & Dalpiaz, A. (2016). Particulate formulations based on chitosan for nose-to-brain delivery of drugs. A review. *Journal of Drug Delivery Science and Technology*, 32, 77-87.
- Rattanaseth, P., Katewongsa, K., Intuyod, K., Pinlaor, S., Thanan, R., & Sakonsinsiri, C. (2025). Poly(d,l-lactide-co-glycolide) Nanoparticles Encapsulating Doxorubicin for Improved Treatment in Cholangiocarcinoma and Drug-Resistant Cells. *ACS Appl Bio Mater*, 8(7), 6055-6065. <https://doi.org/10.1021/acsabm.5c00628>
- Reekes, T. H., Higginson, C. I., Ledbetter, C. R., Sathivadivel, N., Zweig, R. M., & Disbrow, E. A. (2020). Sex specific cognitive differences in Parkinson disease. *npj Parkinson's Disease*, 6(1), 7.
- Remy, P., Doder, M., Lees, A., Turjanski, N., & Brooks, D. (2005). Depression in Parkinson's disease: loss of dopamine and noradrenaline innervation in the limbic system. *Brain*, 128(Pt 6), 1314-1322. <https://doi.org/10.1093/brain/awh445>
- Ritger, P. L., & Peppas, N. A. (1987). A simple equation for description of solute release II. Fickian and anomalous release from swellable devices. *Journal of Controlled Release*, 5(1), 37-42. [https://doi.org/https://doi.org/10.1016/0168-3659\(87\)90035-6](https://doi.org/https://doi.org/10.1016/0168-3659(87)90035-6)
- Rodgers, E. S., Elliott, C. T., Po, A. L. W., Mackie, D. P., Scott, E. M., & Kreuter, J. (1997). The potential of polymeric nanoparticles to increase the immunogenicity of the hapten clenbuterol. *Food and Agricultural Immunology*, 9(3), 159-166. <https://doi.org/10.1080/09540109709354946>
- Romero-Miguel, D., Lamanna-Rama, N., Casquero-Veiga, M., Gómez-Rangel, V., Desco, M., & Soto-Montenegro, M. L. (2021). Minocycline in neurodegenerative and psychiatric diseases: An update. *Eur J Neurol*, 28(3), 1056-1081. <https://doi.org/10.1111/ene.14642>
- Rommelfanger, K. S., Edwards, G. L., Freeman, K. G., Liles, L. C., Miller, G. W., & Weinshenker, D. (2007). Norepinephrine loss produces more profound motor deficits than MPTP treatment in mice. *Proc Natl Acad Sci U S A*, 104(34), 13804-13809. <https://doi.org/10.1073/pnas.0702753104>
- Rommelfanger, K. S., Weinshenker, D., & Miller, G. W. (2004). Reduced MPTP toxicity in noradrenaline transporter knockout mice. *J Neurochem*, 91(5), 1116-1124. <https://doi.org/10.1111/j.1471-4159.2004.02785.x>
- Rudrapal, M., J. Khairnar, S., & G. Jadhav, A. (2020). Drug Repurposing (DR): An Emerging Approach in Drug Discovery. In. IntechOpen. <https://doi.org/10.5772/intechopen.93193>

- Ruiz-Picazo, A., Gonzalez-Alvarez, I., Bermejo, M., & Gonzalez-Alvarez, M. (2024). New mathematical model from dynamic dissolution rate tests. *Eur J Pharm Sci*, 201, 106864. <https://doi.org/10.1016/j.ejps.2024.106864>
- Ružena, Č., Jindra, V., & Renáta, H. (2020). Chirality of β 2-agonists. An overview of pharmacological activity, stereoselective analysis, and synthesis. *Open Chemistry*, 18(1), 628-647. <https://doi.org/doi:10.1515/chem-2020-0056>
- Ryall, J. G., Sillence, M. N., & Lynch, G. S. (2006). Systemic administration of beta2-adrenoceptor agonists, formoterol and salmeterol, elicit skeletal muscle hypertrophy in rats at micromolar doses. *Br J Pharmacol*, 147(6), 587-595. <https://doi.org/10.1038/sj.bjp.0706669>
- Ryan, K. J., Griffin, É., Yssel, J. D., Ryan, K. M., McNamee, E. N., Harkin, A., & Connor, T. J. (2013). Stimulation of central β 2-adrenoceptors suppresses NF κ B activity in rat brain: a role for I κ B. *Neurochemistry international*, 63(5), 368-378.
- Ryan, K. M., Boyle, N. T., Harkin, A., & Connor, T. J. (2020). Dexamethasone attenuates inflammatory-mediated suppression of β (2)-adrenoceptor expression in rat primary mixed glia. *J Neuroimmunol*, 338, 577082. <https://doi.org/10.1016/j.jneuroim.2019.577082>
- Ryan, K. M., Griffin, É. W., Ryan, K. J., Tanveer, R., Vanattou-Saifoudine, N., McNamee, E. N., Fallon, E., Heffernan, S., Harkin, A., & Connor, T. J. (2016). Clenbuterol activates the central IL-1 system via the β 2-adrenoceptor without provoking inflammatory response related behaviours in rats. *Brain, behavior, and immunity*, 56, 114-129. <https://doi.org/https://doi.org/10.1016/j.bbi.2016.02.023>
- Ryan, K. M., & Harkin, A. (2020). Regulation of β (2)-adrenoceptors in brain glia: implications for neuroinflammatory and degenerative disorders. *Neural Regen Res*, 15(11), 2035-2036. <https://doi.org/10.4103/1673-5374.282255>
- Saha, P., Kathuria, H., & Pandey, M. M. (2023). Intranasal nanotherapeutics for brain targeting and clinical studies in Parkinson's disease. *Journal of Controlled Release*, 358, 293-318. <https://doi.org/https://doi.org/10.1016/j.jconrel.2023.04.021>
- Sahin, A., Esendagli, G., Yerlikaya, F., Caban-Toktas, S., Yoyen-Ermis, D., Horzum, U., Aktas, Y., Khan, M., Couvreur, P., & Capan, Y. (2017). A small variation in average particle size of PLGA nanoparticles prepared by nanoprecipitation leads to considerable change in nanoparticles' characteristics and efficacy of intracellular delivery. *Artif Cells Nanomed Biotechnol*, 45(8), 1657-1664. <https://doi.org/10.1080/21691401.2016.1276924>
- Saldiva, P. H., Parada, M. A., Macchione, M., Paiva, P. S., Guimarães, E. T., Lorenzi, G., Martins, M. A., Montes, G. S., Balbani, A. P., & King, M. (1995). Nasal mucus clearance in rats: differences with sex and phase of the oestrous cycle. *J Appl Toxicol*, 15(4), 289-295. <https://doi.org/10.1002/jat.2550150410>
- Salem, H. F., Ali, A. A., Rabea, Y. K., El-Ela, F. I. A., & Khallaf, R. A. (2023). Optimization and appraisal of chitosan-grafted PLGA nanoparticles for boosting pharmacokinetic and pharmacodynamic effect of duloxetine HCl using box-benken design. *Journal of Pharmaceutical Sciences*, 112(2), 544-561.
- Sánchez-López, E., Ettcheto, M., Egea, M. A., Espina, M., Cano, A., Calpena, A. C., Camins, A., Carmona, N., Silva, A. M., Souto, E. B., & García, M. L. (2018). Memantine loaded PLGA PEGylated nanoparticles for Alzheimer's disease: in

- vitro and in vivo characterization. *J Nanobiotechnology*, 16(1), 32.
<https://doi.org/10.1186/s12951-018-0356-z>
- Sasaki, H., Kamimura, H., Shiobara, Y., Esumi, Y., Takaichi, M., & Yokoshima, T. (1982). Disposition and metabolism of formoterol fumarate, a new bronchodilator, in rats and dogs. *Xenobiotica*, 12(12), 803-812.
<https://doi.org/10.3109/00498258209038952>
- Sasaki, M., Shibata, E., Tohyama, K., Takahashi, J., Otsuka, K., Tsuchiya, K., Takahashi, S., Ehara, S., Terayama, Y., & Sakai, A. (2006). Neuromelanin magnetic resonance imaging of locus ceruleus and substantia nigra in Parkinson's disease. *Neuroreport*, 17(11), 1215-1218.
<https://doi.org/10.1097/01.wnr.0000227984.84927.a7>
- Saura, J. (2007). Microglial cells in astroglial cultures: a cautionary note. *Journal of neuroinflammation*, 4(1), 26. <https://doi.org/10.1186/1742-2094-4-26>
- Schallert, T., Fleming, S. M., Leasure, J. L., Tillerson, J. L., & Bland, S. T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology*, 39(5), 777-787.
- Schwab, R. S., England, A. C., Jr., Poskanzer, D. C., & Young, R. R. (1969). Amantadine in the Treatment of Parkinson's Disease. *JAMA*, 208(7), 1168-1170.
<https://doi.org/10.1001/jama.1969.03160070046011>
- Schwarz, S. T., Xing, Y., Tomar, P., Bajaj, N., & Auer, D. P. (2017). In Vivo Assessment of Brainstem Depigmentation in Parkinson Disease: Potential as a Severity Marker for Multicenter Studies. *Radiology*, 283(3), 789-798.
<https://doi.org/10.1148/radiol.2016160662>
- Sears, M. R. (2002). Adverse effects of β_2 -agonists. *Journal of Allergy and Clinical Immunology*, 110(6), S322-S328. <https://doi.org/10.1067/mai.2002.129966>
- Segovia, S., & Guillamón, A. (1993). Sexual dimorphism in the vomeronasal pathway and sex differences in reproductive behaviors. *Brain Res Brain Res Rev*, 18(1), 51-74. [https://doi.org/10.1016/0165-0173\(93\)90007-m](https://doi.org/10.1016/0165-0173(93)90007-m)
- Semkova, I., Schilling, M., Henrich-Noack, P., Rami, A., & Kriegstein, J. (1996). Clenbuterol protects mouse cerebral cortex and rat hippocampus from ischemic damage and attenuates glutamate neurotoxicity in cultured hippocampal neurons by induction of NGF. *Brain Res*, 717(1-2), 44-54.
[https://doi.org/10.1016/0006-8993\(95\)01567-1](https://doi.org/10.1016/0006-8993(95)01567-1)
- Sevrioukova, I. F., & Poulos, T. L. (2012). Structural and mechanistic insights into the interaction of cytochrome P4503A4 with bromoergocryptine, a type I ligand. *J Biol Chem*, 287(5), 3510-3517. <https://doi.org/10.1074/jbc.M111.317081>
- Shah, P., Dubey, P., Vyas, B., Kaul, A., Mishra, A. K., Chopra, D., & Patel, P. (2021a). Lamotrigine loaded PLGA nanoparticles intended for direct nose to brain delivery in epilepsy: pharmacokinetic, pharmacodynamic and scintigraphy study. *Artificial Cells, Nanomedicine, and Biotechnology*, 49(1), 511-522.
<https://doi.org/10.1080/21691401.2021.1939709>
- Shah, P., Dubey, P., Vyas, B., Kaul, A., Mishra, A. K., Chopra, D., & Patel, P. (2021b). Lamotrigine loaded PLGA nanoparticles intended for direct nose to brain delivery in epilepsy: pharmacokinetic, pharmacodynamic and scintigraphy study. *Artif Cells Nanomed Biotechnol*, 49(1), 511-522.
<https://doi.org/10.1080/21691401.2021.1939709>

- Sharaf, N. S., Shetta, A., Elhalawani, J. E., & Mamdouh, W. (2021). Applying Box-Behnken Design for Formulation and Optimization of PLGA-Coffee Nanoparticles and Detecting Enhanced Antioxidant and Anticancer Activities. *Polymers (Basel)*, 14(1). <https://doi.org/10.3390/polym14010144>
- Sharma, D., Sharma, R. K., Sharma, N., Gabrani, R., Sharma, S. K., Ali, J., & Dang, S. (2015). Nose-to-brain delivery of PLGA-diazepam nanoparticles. *AAPS PharmSciTech*, 16(5), 1108-1121.
- Sharma, M., Arbabzada, N., & Flood, P. M. (2019). Mechanism underlying β 2-AR agonist-mediated phenotypic conversion of LPS-activated microglial cells. *J Neuroimmunol*, 332, 37-48. <https://doi.org/10.1016/j.jneuroim.2019.03.017>
- Sharma, M., & Flood, P. M. (2018). β -arrestin2 regulates the anti-inflammatory effects of Salmeterol in lipopolysaccharide-stimulated BV2 cells. *Journal of Neuroimmunology*, 325, 10-19. <https://doi.org/10.1016/j.jneuroim.2018.10.001>
- Sharma, N., Sharma, S., & Nehru, B. (2017). Curcumin protects dopaminergic neurons against inflammation-mediated damage and improves motor dysfunction induced by single intranigral lipopolysaccharide injection. *Inflammopharmacology*, 25(3), 351-368. <https://doi.org/10.1007/s10787-017-0346-z>
- Sharma, S., Tyagi, A., & Dang, S. (2023). Nose to Brain Delivery of Transferrin conjugated PLGA nanoparticles for clonidine. *International Journal of Biological Macromolecules*, 252, 126471.
- Shen, J., & Burgess, D. J. (2013). In Vitro Dissolution Testing Strategies for Nanoparticulate Drug Delivery Systems: Recent Developments and Challenges. *Drug Deliv Transl Res*, 3(5), 409-415. <https://doi.org/10.1007/s13346-013-0129-z>
- Sheppard, O., Coleman, M. P., & Durrant, C. S. (2019). Lipopolysaccharide-induced neuroinflammation induces presynaptic disruption through a direct action on brain tissue involving microglia-derived interleukin 1 beta. *J Neuroinflammation*, 16(1), 106. <https://doi.org/10.1186/s12974-019-1490-8>
- Shetab Boushehri, M. A., Abdel-Mottaleb, M. M. A., Béduneau, A., Pellequer, Y., & Lamprecht, A. (2018). A nanoparticle-based approach to improve the outcome of cancer active immunotherapy with lipopolysaccharides. *Drug Deliv*, 25(1), 1414-1425. <https://doi.org/10.1080/10717544.2018.1469684>
- Shin, H. J., Kim, I. S., Choi, S. G., Lee, K., Park, H., Shin, J., Kim, D., Beom, J., Yi, Y. Y., Gupta, D. P., Song, G. J., Chung, W. S., Lee, C. J., & Kim, D. W. (2024). Rejuvenating aged microglia by p16(ink4a)-siRNA-loaded nanoparticles increases amyloid- β clearance in animal models of Alzheimer's disease. *Mol Neurodegener*, 19(1), 25. <https://doi.org/10.1186/s13024-024-00715-x>
- Sholl, D. A. (1953). Dendritic organization in the neurons of the visual and motor cortices of the cat. *J Anat*, 87(4), 387-406.
- Simola, N., Morelli, M., & Carta, A. R. (2007). The 6-hydroxydopamine model of Parkinson's disease. *Neurotox Res*, 11(3-4), 151-167. <https://doi.org/10.1007/bf03033565>
- Simón-Vázquez, R., Tsapis, N., Lorscheider, M., Rodríguez, A., Calleja, P., Mousnier, L., de Miguel Villegas, E., González-Fernández, Á., & Fattal, E. (2022). Improving dexamethasone drug loading and efficacy in treating arthritis through a

- lipophilic prodrug entrapped into PLGA-PEG nanoparticles. *Drug Deliv Transl Res*, 12(5), 1270-1284. <https://doi.org/10.1007/s13346-021-01112-3>
- Skrzypczak-Wiercioch, A., & Sałat, K. (2022). Lipopolysaccharide-induced model of neuroinflammation: mechanisms of action, research application and future directions for its use. *Molecules*, 27(17), 5481.
- Smajić, S., Prada-Medina, C. A., Landoulsi, Z., Ghelfi, J., Delcambre, S., Dietrich, C., Jarazo, J., Henck, J., Balachandran, S., Pachchek, S., Morris, C. M., Antony, P., Timmermann, B., Sauer, S., Pereira, S. L., Schwamborn, J. C., May, P., Grünewald, A., & Spielmann, M. (2022). Single-cell sequencing of human midbrain reveals glial activation and a Parkinson-specific neuronal state. *Brain*, 145(3), 964-978. <https://doi.org/10.1093/brain/awab446>
- Smeyne, R. J., & Jackson-Lewis, V. (2005). The MPTP model of Parkinson's disease. *Molecular Brain Research*, 134(1), 57-66. <https://doi.org/10.1016/j.molbrainres.2004.09.017>
- So, H. C., Chau, C. K., Lau, A., Wong, S. Y., & Zhao, K. (2019). Translating GWAS findings into therapies for depression and anxiety disorders: gene-set analyses reveal enrichment of psychiatric drug classes and implications for drug repositioning. *Psychol Med*, 49(16), 2692-2708. <https://doi.org/10.1017/s0033291718003641>
- Sola, P., Garikapati, K. K., Krishnamurthy, P. T., & Kumari, M. (2023). Polysorbate 80 surface modified SLNs of formoterol suppress SNCA gene and mitochondrial oxidative stress in mice model of Parkinson's disease. *Scientific Reports*, 13(1), 19942. <https://doi.org/10.1038/s41598-023-46511-3>
- Song, S., Jiang, L., Oyarzabal, E. A., Wilson, B., Li, Z., Shih, Y. I., Wang, Q., & Hong, J. S. (2019a). Loss of Brain Norepinephrine Elicits Neuroinflammation-Mediated Oxidative Injury and Selective Caudo-Rostral Neurodegeneration. *Mol Neurobiol*, 56(4), 2653-2669. <https://doi.org/10.1007/s12035-018-1235-1>
- Song, S., Liu, J., Zhang, F., & Hong, J.-S. (2020). Norepinephrine depleting toxin DSP-4 and LPS alter gut microbiota and induce neurotoxicity in α -synuclein mutant mice. *Scientific Reports*, 10(1), 15054. <https://doi.org/10.1038/s41598-020-72202-4>
- Song, S., Wang, Q., Jiang, L., Oyarzabal, E., Riddick, N. V., Wilson, B., Moy, S. S., Shih, Y. I., & Hong, J. S. (2019b). Noradrenergic dysfunction accelerates LPS-elicited inflammation-related ascending sequential neurodegeneration and deficits in non-motor/motor functions. *Brain Behav Immun*, 81, 374-387. <https://doi.org/10.1016/j.bbi.2019.06.034>
- Soo Choi, H., Liu, W., Misra, P., Tanaka, E., Zimmer, J. P., Ito, B., Bawendi, M. G., & Frangioni, J. V. (2007). Renal clearance of quantum dots. *Nature Biotechnology*, 25(10), 1165-1170. <https://doi.org/10.1038/nbt1340>
- Sousa de Almeida, M., Susnik, E., Drasler, B., Taladriz-Blanco, P., Petri-Fink, A., & Rothen-Rutishauser, B. (2021). Understanding nanoparticle endocytosis to improve targeting strategies in nanomedicine. *Chem Soc Rev*, 50(9), 5397-5434. <https://doi.org/10.1039/d0cs01127d>
- Spindler, L. M., Feuerhake, A., Ladel, S., Günday, C., Flamm, J., Günday-Türel, N., Türel, E., Tovar, G. E. M., Schindowski, K., & Gruber-Traub, C. (2021). Nano-in-Micro-Particles Consisting of PLGA Nanoparticles Embedded in Chitosan Microparticles via Spray-Drying Enhances Their Uptake in the Olfactory Mucosa

- [Original Research]. *Frontiers in pharmacology*, Volume 12 - 2021. <https://doi.org/10.3389/fphar.2021.732954>
- Sprenger, T., Viana, M., & Tassorelli, C. (2018). Current Prophylactic Medications for Migraine and Their Potential Mechanisms of Action. *Neurotherapeutics*, 15(2), 313-323. <https://doi.org/10.1007/s13311-018-0621-8>
- Sridhar, V., Tiwari, A., Wairkar, S., Gupta, G. L., & Gaud, R. (2020). Pramipexole thermosensitive nasal gel for experimental parkinsonism in rats. *Journal of Drug Delivery Science and Technology*, 59, 101954. <https://doi.org/https://doi.org/10.1016/j.jddst.2020.101954>
- Sridhar, V., Wairkar, S., Gaud, R., Bajaj, A., & Meshram, P. (2018). Brain targeted delivery of mucoadhesive thermosensitive nasal gel of selegiline hydrochloride for treatment of Parkinson's disease. *J Drug Target*, 26(2), 150-161. <https://doi.org/10.1080/1061186x.2017.1350858>
- Srinivasan, J., & Schmidt, W. J. (2003). Potentiation of parkinsonian symptoms by depletion of locus coeruleus noradrenaline in 6-hydroxydopamine-induced partial degeneration of substantia nigra in rats. *Eur J Neurosci*, 17(12), 2586-2592. <https://doi.org/10.1046/j.1460-9568.2003.02684.x>
- Straat, M. E., Hoekx, C. A., van Velden, F. H., Arias-Bouda, L. M. P., Dumont, L., Blondin, D. P., Boon, M. R., Martinez-Tellez, B., & Rensen, P. C. (2023). Stimulation of the beta-2-adrenergic receptor with salbutamol activates human brown adipose tissue. *Cell Reports Medicine*, 4(2).
- Suk, J. S., Xu, Q., Kim, N., Hanes, J., & Ensign, L. M. (2016). PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Adv Drug Deliv Rev*, 99(Pt A), 28-51. <https://doi.org/10.1016/j.addr.2015.09.012>
- Sun, H., Dan, X., Chen, H., Feng, J., Wang, W., Ye, Q., Liu, Y., Liu, C., Chen, S., Chan, P. . (2025). Efficacy and safety of Rotigotine Extended-Release Microspheres in Early Parkinson's Disease [abstract]. *Mov Disord.*, 40.
- Sun, J., Wang, L., Bao, H., Premi, S., Das, U., Chapman, E. R., & Roy, S. (2019). Functional cooperation of α -synuclein and VAMP2 in synaptic vesicle recycling. *Proc Natl Acad Sci U S A*, 116(23), 11113-11115. <https://doi.org/10.1073/pnas.1903049116>
- Sun, R., Chen, Y., Pei, Y., Wang, W., Zhu, Z., Zheng, Z., Yang, L., & Sun, L. (2024). The drug release of PLGA-based nanoparticles and their application in treatment of gastrointestinal cancers. *Heliyon*, 10(18), e38165. <https://doi.org/https://doi.org/10.1016/j.heliyon.2024.e38165>
- Surmeier, D. J. (2018). Determinants of dopaminergic neuron loss in Parkinson's disease. *Febs j*, 285(19), 3657-3668. <https://doi.org/10.1111/febs.14607>
- Suwijn, S. R., van Boheemen, C. J., de Haan, R. J., Tissingh, G., Booij, J., & de Bie, R. M. (2015). The diagnostic accuracy of dopamine transporter SPECT imaging to detect nigrostriatal cell loss in patients with Parkinson's disease or clinically uncertain parkinsonism: a systematic review. *EJNMMI Res*, 5, 12. <https://doi.org/10.1186/s13550-015-0087-1>
- Suzuki, Y., & Ishihara, H. (2021). Difference in the lipid nanoparticle technology employed in three approved siRNA (Patisiran) and mRNA (COVID-19 vaccine) drugs. *Drug Metab Pharmacokinet*, 41, 100424. <https://doi.org/10.1016/j.dmpk.2021.100424>

- Swetledge, S., Carter, R., Stout, R., Astete, C. E., Jung, J. P., & Sabliov, C. M. (2021). Stability and ocular biodistribution of topically administered PLGA nanoparticles. *Scientific Reports*, 11(1), 12270. <https://doi.org/10.1038/s41598-021-90792-5>
- Tagliaferro, P., & Burke, R. E. (2016). Retrograde Axonal Degeneration in Parkinson Disease. *J Parkinsons Dis*, 6(1), 1-15. <https://doi.org/10.3233/jpd-150769>
- Tai, W., Ye, X., Bao, X., Zhao, B., Wang, X., & Zhang, D. (2013). Inhibition of Src tyrosine kinase activity by squamosamide derivative FLZ attenuates neuroinflammation in both in vivo and in vitro Parkinson's disease models. *Neuropharmacology*, 75, 201-212.
- Takeda, A., Sawashita, J., & Okada, S. (1998). Manganese concentration in rat brain: manganese transport from the peripheral tissues. *Neuroscience Letters*, 242(1), 45-48. [https://doi.org/https://doi.org/10.1016/S0304-3940\(98\)00006-8](https://doi.org/https://doi.org/10.1016/S0304-3940(98)00006-8)
- Tamás, A., Lubics, A., Szalontay, L., Lengvári, I., & Reglődi, D. (2005). Age and gender differences in behavioral and morphological outcome after 6-hydroxydopamine-induced lesion of the substantia nigra in rats. *Behavioural brain research*, 158(2), 221-229.
- Tamashiro, T. T., Dalgard, C. L., & Byrnes, K. R. (2012). Primary microglia isolation from mixed glial cell cultures of neonatal rat brain tissue. *J Vis Exp*(66), e3814. <https://doi.org/10.3791/3814>
- Tang, S., Wang, A., Yan, X., Chu, L., Yang, X., Song, Y., Sun, K., Yu, X., Liu, R., Wu, Z., & Xue, P. (2019). Brain-targeted intranasal delivery of dopamine with borneol and lactoferrin co-modified nanoparticles for treating Parkinson's disease. *Drug Deliv*, 26(1), 700-707. <https://doi.org/10.1080/10717544.2019.1636420>
- Tanna, V., Vora, A., Shah, P., Nair, A. B., Shah, J., & Sawarkar, S. P. (2024). PLGA Nanoparticles Based Mucoadhesive Nasal In Situ Gel for Enhanced Brain Delivery of Topiramate. *AAPS PharmSciTech*, 25(7), 205. <https://doi.org/10.1208/s12249-024-02917-4>
- Tansey, M. G., & Goldberg, M. S. (2010). Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. *Neurobiol Dis*, 37(3), 510-518. <https://doi.org/10.1016/j.nbd.2009.11.004>
- Tansey, M. G., Wallings, R. L., Houser, M. C., Herrick, M. K., Keating, C. E., & Joers, V. (2022). Inflammation and immune dysfunction in Parkinson disease. *Nature Reviews Immunology*, 22(11), 657-673. <https://doi.org/10.1038/s41577-022-00684-6>
- Tao, J., Chow, S. F., & Zheng, Y. (2019). Application of flash nanoprecipitation to fabricate poorly water-soluble drug nanoparticles. *Acta Pharm Sin B*, 9(1), 4-18. <https://doi.org/10.1016/j.apsb.2018.11.001>
- Teng, Y. D., Choi, H., Huang, W., Onario, R. C., Frontera, W. R., Snyder, E. Y., & Sabharwal, S. (2006). Therapeutic effects of clenbuterol in a murine model of amyotrophic lateral sclerosis. *Neuroscience Letters*, 397(1), 155-158. <https://doi.org/https://doi.org/10.1016/j.neulet.2005.12.007>
- Thijssen, E., den Heijer, J. M., Puibert, D., van Brummelen, E. M. J., Naranda, T., & Groeneveld, G. J. (2022). Safety and pharmacokinetics of multiple dosing with inhalable apomorphine (AZ-009), and its efficacy in a randomized crossover study in Parkinson's disease patients. *Parkinsonism Relat Disord*, 97, 84-90. <https://doi.org/10.1016/j.parkreldis.2022.02.014>

- Thorne, R. G., & Frey, W. H., 2nd. (2001). Delivery of neurotrophic factors to the central nervous system: pharmacokinetic considerations. *Clin Pharmacokinet*, 40(12), 907-946. <https://doi.org/10.2165/00003088-200140120-00003>
- Tian, L., Shang, Y., Chen, R., Bai, R., Chen, C., Inthavong, K., & Tu, J. (2019). Correlation of regional deposition dosage for inhaled nanoparticles in human and rat olfactory. *Particle and Fibre Toxicology*, 16(1), 6. <https://doi.org/10.1186/s12989-019-0290-8>
- Timmerman, R., Burm, S. M., & Bajramovic, J. J. (2018). An Overview of in vitro Methods to Study Microglia. *Front Cell Neurosci*, 12, 242. <https://doi.org/10.3389/fncel.2018.00242>
- Todd, L., Palazzo, I., Suarez, L., Liu, X., Volkov, L., Hoang, T. V., Campbell, W. A., Blackshaw, S., Quan, N., & Fischer, A. J. (2019). Reactive microglia and IL1 β /IL-1R1-signaling mediate neuroprotection in excitotoxin-damaged mouse retina. *Journal of neuroinflammation*, 16(1), 118. <https://doi.org/10.1186/s12974-019-1505-5>
- Tomás-Camardiel, M., Venero, J. L., De Pablos, R., Rite, I., Machado, A., & Cano, J. (2004). In vivo expression of aquaporin-4 by reactive microglia. *Journal of neurochemistry*, 91(4), 891-899.
- Tong, G.-F., Qin, N., & Sun, L.-W. (2017). Development and evaluation of Desvenlafaxine loaded PLGA-chitosan nanoparticles for brain delivery. *Saudi pharmaceutical journal*, 25(6), 844-851.
- Topol, E. J. (2019). High-performance medicine: the convergence of human and artificial intelligence. *Nat Med*, 25(1), 44-56. <https://doi.org/10.1038/s41591-018-0300-7>
- Torrente, D., Su, E. J., Schielke, G. P., Warnock, M., Mann, K., & Lawrence, D. A. (2023). Opposing effects of β -2 and β -1 adrenergic receptor signaling on neuroinflammation and dopaminergic neuron survival in α -synuclein-mediated neurotoxicity. *Journal of neuroinflammation*, 20(1), 56.
- Tseng, Y.-F., Lin, H.-C., Chao, J. C.-J., Hsu, C.-Y., & Lin, H.-L. (2021). Calcium Channel blockers are associated with reduced risk of Parkinson's disease in patients with hypertension: A population-based retrospective cohort study. *Journal of the neurological sciences*, 424, 117412.
- Tu, D., Gao, Y., Yang, R., Guan, T., Hong, J.-S., & Gao, H.-M. (2019). The pentose phosphate pathway regulates chronic neuroinflammation and dopaminergic neurodegeneration. *Journal of neuroinflammation*, 16(1), 255.
- Tyndall, C. (PhD thesis). *Investigating microglial and neuronal cellular responses to alphasynuclein preformed fibril and adeno-associated viral vector delivery to the rat substantia nigra and to primary cortical rat cell cultures*
- Ubrich, N., Bouillot, P., Pellerin, C., Hoffman, M., & Maincent, P. (2004). Preparation and characterization of propranolol hydrochloride nanoparticles: a comparative study. *Journal of Controlled Release*, 97(2), 291-300. <https://doi.org/https://doi.org/10.1016/j.jconrel.2004.03.023>
- Udompornpitak, K., Bhunyakarnjanarat, T., Saisorn, W., Manipunttee, C., Plengplang, K., Sittichaitaweekul, S., Jenphatanapong, P., Udomkarnjananun, S., Kaewduangduen, W., Ariya-anandech, K., Samaeng, A., Insin, N., Ritprajak, P., & Leelahavanichkul, A. (2023). Polymeric Particle BAM15 Targeting Macrophages Attenuates the Severity of LPS-Induced Sepsis: A Proof of

- Concept for Specific Immune Cell-Targeted Therapy. *Pharmaceutics*, 15(12), 2695. <https://www.mdpi.com/1999-4923/15/12/2695>
- Ungerstedt, U. (1968). 6-hydroxy-dopamine induced degeneration of central monoamine neurons. *European Journal of Pharmacology*, 5(1), 107-110. [https://doi.org/https://doi.org/10.1016/0014-2999\(68\)90164-7](https://doi.org/https://doi.org/10.1016/0014-2999(68)90164-7)
- Ungerstedt, U., & Arbuthnott, G. W. (1970). Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res*, 24(3), 485-493. [https://doi.org/10.1016/0006-8993\(70\)90187-3](https://doi.org/10.1016/0006-8993(70)90187-3)
- Valencia, P. M., Farokhzad, O. C., Karnik, R., & Langer, R. (2012). Microfluidic technologies for accelerating the clinical translation of nanoparticles. *Nat Nanotechnol*, 7(10), 623-629. <https://doi.org/10.1038/nnano.2012.168>
- Vallorz, E. L., Blohm-Mangone, K., Schnellmann, R. G., & Mansour, H. M. (2021). Formoterol PLGA-PEG Nanoparticles Induce Mitochondrial Biogenesis in Renal Proximal Tubules. *Aaps j*, 23(4), 88. <https://doi.org/10.1208/s12248-021-00619-4>
- Vallorz, E. L., Encinas-Basurto, D., Schnellmann, R. G., & Mansour, H. M. (2022). Design, Development, Physicochemical Characterization, and In Vitro Drug Release of Formoterol PEGylated PLGA Polymeric Nanoparticles. *Pharmaceutics*, 14(3). <https://doi.org/10.3390/pharmaceutics14030638>
- Van der Perren, A., Van den Haute, C., & Baekelandt, V. (2015). Viral vector-based models of Parkinson's disease. *Curr Top Behav Neurosci*, 22, 271-301. https://doi.org/10.1007/7854_2014_310
- Van Kralingen, C., Kho, D. T., Costa, J., Angel, C. E., & Graham, E. S. (2013). Exposure to Inflammatory Cytokines IL-1 β and TNF α Induces Compromise and Death of Astrocytes; Implications for Chronic Neuroinflammation. *PLoS One*, 8(12), e84269. <https://doi.org/10.1371/journal.pone.0084269>
- Vanden-Hehir, S., Cairns, S. A., Lee, M., Zoupi, L., Shaver, M. P., Brunton, V. G., Williams, A., & Hulme, A. N. (2019). Alkyne-Tagged PLGA Allows Direct Visualization of Nanoparticles In Vitro and Ex Vivo by Stimulated Raman Scattering Microscopy. *Biomacromolecules*, 20(10), 4008-4014. <https://doi.org/10.1021/acs.biomac.9b01092>
- Vautier, S., Lacomblez, L., Chacun, H., Picard, V., Gimenez, F., Farinotti, R., & Fernandez, C. (2006). Interactions between the dopamine agonist, bromocriptine and the efflux protein, P-glycoprotein at the blood-brain barrier in the mouse. *Eur J Pharm Sci*, 27(2-3), 167-174. <https://doi.org/10.1016/j.ejps.2005.09.009>
- Vazey, E. M., & Aston-Jones, G. (2012). The emerging role of norepinephrine in cognitive dysfunctions of Parkinson's disease. *Front Behav Neurosci*, 6, 48. <https://doi.org/10.3389/fnbeh.2012.00048>
- Vekaria, H. J., Hubbard, W. B., Scholpa, N. E., Spry, M. L., Gooch, J. L., Prince, S. J., Schnellmann, R. G., & Sullivan, P. G. (2020). Formoterol, a β 2-adrenoreceptor agonist, induces mitochondrial biogenesis and promotes cognitive recovery after traumatic brain injury. *Neurobiology of disease*, 140, 104866. <https://doi.org/https://doi.org/10.1016/j.nbd.2020.104866>
- Venkatpurwar, V. P., Rhodes, S., Oien, K. A., Elliott, M. A., Tekwe, C. D., Jørgensen, H. G., & Kumar, M. N. V. R. (2015). Drug- not carrier-dependent haematological and

- biochemical changes in a repeated dose study of cyclosporine encapsulated polyester nano- and micro-particles: Size does not matter. *Toxicology*, 330, 9-18. <https://doi.org/https://doi.org/10.1016/j.tox.2015.01.017>
- Veronesi, M. C., Alhamami, M., Miedema, S. B., Yun, Y., Ruiz-Cardozo, M., & Vannier, M. W. (2020). Imaging of intranasal drug delivery to the brain. *Am J Nucl Med Mol Imaging*, 10(1), 1-31.
- Verstraelen, P., Van Dyck, M., Verschuuren, M., Kashikar, N. D., Nuydens, R., Timmermans, J.-P., & De Vos, W. H. (2018). Image-Based Profiling of Synaptic Connectivity in Primary Neuronal Cell Culture [Review]. *Frontiers in Neuroscience*, Volume 12 - 2018. <https://www.frontiersin.org/journals/neuroscience/articles/10.3389/fnins.2018.00389>
- Virdi, G. S., Choi, M. L., Evans, J. R., Yao, Z., Athauda, D., Strohbuecker, S., Nirujogi, R. S., Wernick, A. I., Pelegriana-Hidalgo, N., Leighton, C., Saleeb, R. S., Kopach, O., Alrashidi, H., Melandri, D., Perez-Lloret, J., Angelova, P. R., Sylantyev, S., Eaton, S., Heales, S.,...Gandhi, S. (2022). Protein aggregation and calcium dysregulation are hallmarks of familial Parkinson's disease in midbrain dopaminergic neurons. *NPJ Parkinsons Dis*, 8(1), 162. <https://doi.org/10.1038/s41531-022-00423-7>
- Von Coelln, R., Thomas, B., Savitt, J. M., Lim, K. L., Sasaki, M., Hess, E. J., Dawson, V. L., & Dawson, T. M. (2004). Loss of locus coeruleus neurons and reduced startle in parkin null mice. *Proc Natl Acad Sci U S A*, 101(29), 10744-10749. <https://doi.org/10.1073/pnas.0401297101>
- Wagner, S., Zensi, A., Wien, S. L., Tschickardt, S. E., Maier, W., Vogel, T., Worek, F., Pietrzik, C. U., Kreuter, J., & Von Briesen, H. (2012). Uptake Mechanism of ApoE-Modified Nanoparticles on Brain Capillary Endothelial Cells as a Blood-Brain Barrier Model. *PLoS One*, 7(3), e32568. <https://doi.org/10.1371/journal.pone.0032568>
- Wang, A., Since, M., Dallemagne, P., & Rochais, C. (2025). Implication of Central β (2) Adrenergic Receptor for the Development of Novel Drugs Against Alzheimer's Disease. *Arch Pharm (Weinheim)*, 358(4), e2400750. <https://doi.org/10.1002/ardp.202400750>
- Wang, A., Zhong, G., Ying, M., Fang, Z., Chen, Y., Wang, H., Wang, C., Liu, C., & Guo, Y. (2024). Inhibition of NLRP3 inflammasome ameliorates LPS-induced neuroinflammatory injury in mice via PINK1/Parkin pathway. *Neuropharmacology*, 257, 110063. <https://doi.org/10.1016/j.neuropharm.2024.110063>
- Wang, C., Ward, M. E., Chen, R., Liu, K., Tracy, T. E., Chen, X., Xie, M., Sohn, P. D., Ludwig, C., Meyer-Franke, A., Karch, C. M., Ding, S., & Gan, L. (2017a). Scalable Production of iPSC-Derived Human Neurons to Identify Tau-Lowering Compounds by High-Content Screening. *Stem Cell Reports*, 9(4), 1221-1233. <https://doi.org/10.1016/j.stemcr.2017.08.019>
- Wang, F., Yang, Z., Liu, M., Tao, Y., Li, Z., Wu, Z., & Gui, S. (2020). Facile nose-to-brain delivery of rotigotine-loaded polymer micelles thermosensitive hydrogels: In vitro characterization and in vivo behavior study. *Int J Pharm*, 577, 119046. <https://doi.org/10.1016/j.ijpharm.2020.119046>

- Wang, J., Li, Y., Huang, Z., Wan, W., Zhang, Y., Wang, C., Cheng, X., Ye, F., Liu, K., Fei, G., Zeng, M., & Jin, L. (2018). Neuromelanin-sensitive magnetic resonance imaging features of the substantia nigra and locus coeruleus in de novo Parkinson's disease and its phenotypes. *Eur J Neurol*, 25(7), 949-e973. <https://doi.org/10.1111/ene.13628>
- Wang, J. T., Lin, D. H., Qin, L. F., Wen, Z., & Guo, G. P. (2013). [Transport of mPEG-PLGA nanoparticles across the rat nasal mucosa]. *Yao Xue Xue Bao*, 48(5), 752-758.
- Wang, S., Yu, Y., Wang, A., Duan, X., Sun, Y., Wang, L., Chu, L., Lv, Y., Cui, N., Fan, X., Sha, C., Xu, L., & Sun, K. (2022). Temozolomide hexadecyl ester targeted plga nanoparticles for drug-resistant glioblastoma therapy via intranasal administration. *Front Pharmacol*, 13, 965789. <https://doi.org/10.3389/fphar.2022.965789>
- Wang, X. H., Xie, X., Luo, X. G., Shang, H., & He, Z. Y. (2017b). Inhibiting purinergic P2X7 receptors with the antagonist brilliant blue G is neuroprotective in an intranigral lipopolysaccharide animal model of Parkinson's disease. *Mol Med Rep*, 15(2), 768-776. <https://doi.org/10.3892/mmr.2016.6070>
- Wang, Y. Y., Lai, S. K., Suk, J. S., Pace, A., Cone, R., & Hanes, J. (2008). Addressing the PEG mucoadhesivity paradox to engineer nanoparticles that "slip" through the human mucus barrier. *Angew Chem Int Ed Engl*, 47(50), 9726-9729. <https://doi.org/10.1002/anie.200803526>
- Wavikar, P., Pai, R., & Vavia, P. (2017). Nose to Brain Delivery of Rivastigmine by In Situ Gelling Cationic Nanostructured Lipid Carriers: Enhanced Brain Distribution and Pharmacodynamics. *J Pharm Sci*, 106(12), 3613-3622. <https://doi.org/10.1016/j.xphs.2017.08.024>
- Webster, R., Didier, E., Harris, P., Siegel, N., Stadler, J., Tilbury, L., & Smith, D. (2007). PEGylated proteins: evaluation of their safety in the absence of definitive metabolism studies. *Drug Metab Dispos*, 35(1), 9-16. <https://doi.org/10.1124/dmd.106.012419>
- Wen, P., & Ren, C. (2024). Research progress on intranasal treatment for Parkinson's disease. *Neuroprotection*, 2(2), 79-99. <https://doi.org/10.1002/nep3.42>
- Wilton, D. K., Dissing-Olesen, L., & Stevens, B. (2019). Neuron-Glia Signaling in Synapse Elimination. *Annu Rev Neurosci*, 42, 107-127. <https://doi.org/10.1146/annurev-neuro-070918-050306>
- Wischke, C., & Schwendeman, S. P. (2008). Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. *International Journal of Pharmaceutics*, 364(2), 298-327. <https://doi.org/10.1016/j.ijpharm.2008.04.042>
- Woerman, A. L., & Luk, K. C. (2024). Are Preformed Fibrils a Model of Parkinson's Disease? *J Parkinsons Dis*, 14(6), 1095-1103. <https://doi.org/10.3233/jpd-240228>
- Woolf, Z., Stevenson, T. J., Lee, K., Highet, B., Macapagal Foliaki, J., Ratiu, R., Rustenhoven, J., Correia, J., Schweder, P., Heppner, P., Weinert, M., Coppieters, N., Park, T., Montgomery, J., Smith, A. M., & Dragunow, M. (2025). In vitro models of microglia: a comparative study. *Scientific Reports*, 15(1), 15621. <https://doi.org/10.1038/s41598-025-99867-z>
- Wu, J., Wei, W., Wang, L. Y., Su, Z. G., & Ma, G. H. (2007). A thermosensitive hydrogel based on quaternized chitosan and poly(ethylene glycol) for nasal drug delivery

- system. *Biomaterials*, 28(13), 2220-2232.
<https://doi.org/10.1016/j.biomaterials.2006.12.024>
- Wu, Q., Karthivashan, G., Nakhaei-Nejad, M., Anand, B. G., Giuliani, F., & Kar, S. (2022). Native PLGA nanoparticles regulate APP metabolism and protect neurons against β -amyloid toxicity: Potential significance in Alzheimer's disease pathology. *International Journal of Biological Macromolecules*, 219, 1180-1196.
<https://doi.org/https://doi.org/10.1016/j.ijbiomac.2022.08.148>
- Wu, X., Lu, Y., Dong, Y., Zhang, G., Zhang, Y., Xu, Z., Culley, D. J., Crosby, G., Marcantonio, E. R., Tanzi, R. E., & Xie, Z. (2012). The inhalation anesthetic isoflurane increases levels of proinflammatory TNF- α , IL-6, and IL-1 β . *Neurobiol Aging*, 33(7), 1364-1378.
<https://doi.org/10.1016/j.neurobiolaging.2010.11.002>
- Yan, X., Xu, L., Bi, C., Duan, D., Chu, L., Yu, X., Wu, Z., Wang, A., & Sun, K. (2018). Lactoferrin-modified rotigotine nanoparticles for enhanced nose-to-brain delivery: LESA-MS/MS-based drug biodistribution, pharmacodynamics, and neuroprotective effects. *International Journal of Nanomedicine*, Volume 13, 273-281. <https://doi.org/10.2147/ijn.s151475>
- Yan, Y., Ning, J., Cheng, X., Lv, Q., Teng, S., & Wang, W. (2023). Rapid and High-Throughput Determination of Sixteen β -agonists in Livestock Meat Using One-Step Solid-Phase Extraction Coupled with UHPLC-MS/MS. *Foods*, 12(1), 76.
<https://www.mdpi.com/2304-8158/12/1/76>
- Yang, M., Jin, L., Wu, Z., Xie, Y., Zhang, P., Wang, Q., Yan, S., Chen, B., Liang, H., Naman, C. B., Zhang, J., He, S., Yan, X., Zhao, L., & Cui, W. (2021). PLGA-PEG Nanoparticles Facilitate In Vivo Anti-Alzheimer's Effects of Fucoxanthin, a Marine Carotenoid Derived from Edible Brown Algae. *Journal of Agricultural and Food Chemistry*, 69(34), 9764-9777. <https://doi.org/10.1021/acs.jafc.1c00569>
- Yeh, C. H., Hsieh, L. P., Lin, M. C., Wei, T. S., Lin, H. C., Chang, C. C., & Hsing, C. H. (2018). Dexmedetomidine reduces lipopolysaccharide induced neuroinflammation, sickness behavior, and anhedonia. *PLoS One*, 13(1), e0191070. <https://doi.org/10.1371/journal.pone.0191070>
- Yoon, M. S., Lee, J. M., Jo, M. J., Kang, S. J., Yoo, M. K., Park, S. Y., Bong, S., Park, C. S., Park, C. W., Kim, J. S., Han, S. B., Lee, H. J., & Shin, D. H. (2025). Dual-Drug Delivery Systems Using Hydrogel-Nanoparticle Composites: Recent Advances and Key Applications. *Gels*, 11(7). <https://doi.org/10.3390/gels11070520>
- Yssel, J. D., O'Neill, E., Nolan, Y. M., Connor, T. J., & Harkin, A. (2018). Treatment with the noradrenaline re-uptake inhibitor atomoxetine alone and in combination with the α 2-adrenoceptor antagonist idazoxan attenuates loss of dopamine and associated motor deficits in the LPS inflammatory rat model of Parkinson's disease. *Brain, behavior, and immunity*, 69, 456-469.
- Zahoor, I., Shafi, A., & Haq, E. (2018). Pharmacological Treatment of Parkinson's Disease. In (pp. 129-144). Codon Publications.
<https://doi.org/10.15586/codonpublications.parkinsonsdisease.2018.ch7>
- Zarow, C., Lyness, S. A., Mortimer, J. A., & Chui, H. C. (2003). Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. *Arch Neurol*, 60(3), 337-341.
<https://doi.org/10.1001/archneur.60.3.337>

- Zesiewicz, T. A., Strom, J. A., Borenstein, A. R., Hauser, R. A., Cimino, C. R., Fontanet, H. L., Cintron, G. B., Staffetti, J. F., Dunne, P. B., & Sullivan, K. L. (2004). Heart failure in Parkinson's disease: analysis of the United States medicare current beneficiary survey. *Parkinsonism Relat Disord*, 10(7), 417-420. <https://doi.org/10.1016/j.parkreldis.2004.04.001>
- Zhang, W., Mehta, A., Tong, Z., Esser, L., & Voelcker, N. H. (2021a). Development of Polymeric Nanoparticles for Blood-Brain Barrier Transfer-Strategies and Challenges. *Adv Sci (Weinh)*, 8(10), 2003937. <https://doi.org/10.1002/advs.202003937>
- Zhang, X., Zhu, X., Huang, L., Chen, Z., Wang, Y., Liu, Y., Pan, R., & Lv, L. (2023a). Nano-encapsulated tanshinone IIA in PLGA-PEG-COOH inhibits apoptosis and inflammation in cerebral ischemia/reperfusion injury. *Green Processing and Synthesis*, 12(1). <https://doi.org/doi:10.1515/gps-2022-8156>
- Zhang, Y., Guo, P., Ma, Z., Lu, P., Kebebe, D., & Liu, Z. (2021b). Combination of cell-penetrating peptides with nanomaterials for the potential therapeutics of central nervous system disorders: a review. *J Nanobiotechnology*, 19(1), 255. <https://doi.org/10.1186/s12951-021-01002-3>
- Zhang, Y., Sun, C., Zhang, Q., Deng, Y., Hu, X., & Chen, P. (2020). Intranasal delivery of Paclitaxel encapsulated nanoparticles for brain injury due to Glioblastoma. *Journal of Applied Biomaterials & Functional Materials*, 18, 2280800020977170.
- Zhang, Y., Zhang, H., Zhao, F., Jiang, Z., Cui, Y., Ou, M., Mei, L., & Wang, Q. (2023b). Mitochondrial-targeted and ROS-responsive nanocarrier via nose-to-brain pathway for ischemic stroke treatment. *Acta Pharmaceutica Sinica B*, 13(12), 5107-5120. <https://doi.org/https://doi.org/10.1016/j.apsb.2023.06.011>
- Zhang, Z., Niu, K., Huang, T., Guo, J., Xarbat, G., Gong, X., Gao, Y., Liu, F., Cheng, S., Su, W., Yang, F., Liu, Z., Ginhoux, F., & Zhang, T. (2025). Microglia depletion reduces neurodegeneration and remodels extracellular matrix in a mouse Parkinson's disease model triggered by α -synuclein overexpression. *npj Parkinson's Disease*, 11(1), 15. <https://doi.org/10.1038/s41531-024-00846-4>
- Zhao, Y., Huang, Y., Cao, Y., & Yang, J. (2024). Astrocyte-Mediated Neuroinflammation in Neurological Conditions. *Biomolecules*, 14(10), 1204. <https://www.mdpi.com/2218-273X/14/10/1204>
- Zhao, Y. F., Qiong, Z., Zhang, J. F., Lou, Z. Y., Zu, H. B., Wang, Z. G., Zeng, W. C., Kai, Y., & Xiao, B. G. (2018). The Synergy of Aging and LPS Exposure in a Mouse Model of Parkinson's Disease. *Aging Dis*, 9(5), 785-797. <https://doi.org/10.14336/ad.2017.1028>
- Zheng, Y., Cui, L., Lu, H., Liu, Z., Zhai, Z., Wang, H., Shao, L., Lu, Z., Song, X., & Zhang, Y. (2024). Nose to Brain: Exploring the Progress of Intranasal Delivery of Solid Lipid Nanoparticles and Nanostructured Lipid Carriers. *Int J Nanomedicine*, 19, 12343-12368. <https://doi.org/10.2147/ijn.S497480>
- Zhi, K., Raji, B., Nookala, A. R., Khan, M. M., Nguyen, X. H., Sakshi, S., Pourmotabbed, T., Yallapu, M. M., Kochat, H., Tadrous, E., Pernell, S., & Kumar, S. (2021). PLGA Nanoparticle-Based Formulations to Cross the Blood-Brain Barrier for Drug Delivery: From R&D to cGMP. *Pharmaceutics*, 13(4). <https://doi.org/10.3390/pharmaceutics13040500>

- Zhou, L., Tu, J., Fang, G., Deng, L., Gao, X., Guo, K., Kong, J., Lv, J., Guan, W., & Yang, C. (2018). Combining PLGA Scaffold and MSCs for Brain Tissue Engineering: A Potential Tool for Treatment of Brain Injury. *Stem Cells Int*, 2018, 5024175. <https://doi.org/10.1155/2018/5024175>
- Zielińska, A., Eder, P., Rannier, L., Cardoso, J. C., Severino, P., Silva, A. M., & Souto, E. B. (2022). Hydrogels for Modified-release Drug Delivery Systems. *Curr Pharm Des*, 28(8), 609-618. <https://doi.org/10.2174/1381612828666211230114755>
- Zweers, M. L. T., Engbers, G. H. M., Grijpma, D. W., & Feijen, J. (2004). In vitro degradation of nanoparticles prepared from polymers based on dl-lactide, glycolide and poly(ethylene oxide). *Journal of Controlled Release*, 100(3), 347-356. <https://doi.org/https://doi.org/10.1016/j.jconrel.2004.09.008>