Targeting glial β_2-adrenoceptors for immunomodulation & neuroprotection in a rat model of Parkinson's disease

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, except for the behavioural & biochemical data presented in chapter 5., which was carried out by Dr. Justin Yssel.

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Summary

Neuro-inflammation is a key contributor to the pathogenesis of Parkinson's disease (PD). Brain-resident microglia are dynamic cellular effectors of inflammation-mediated neurodegeneration in the Parkinsonian brain. Here we have established & characterised an inflammation-mediated preclinical animal model of experimental PD by direct infusion of the bacterial endotoxin, and TLR4 agonist, lipopolysaccharide (LPS) directly into the SNpc of adult male Wistar rats. Unilateral intra-nigral injection of LPS (10µg/2µl) induced robust Iba1^{+} microglial activation, tyrosine hydroxylase-positive (TH^{+}) dopamine cell loss in the substantia nigra *pars compacta* (SNpc), TH^{+} striatal denervation and nigrostriatal dopamine depletion as verified by high performance liquid chromatography with electrochemical detection (HPLC-ECD), findings which were accompanied by deficits in skilled motor function in the staircase test, forelimb use asymmetry in the cylinder test and bidirectional forelimb akinesia in the stepping test.

Moreover, we simultaneously co-administered the astroglial-selective toxin L-alpha-aminoadipic acid to investigate whether a more populous, yet majorly overlooked glial subtype known as astrocytes contribute towards, or protect against an intra-nigral LPS-induced Parkinsonian state. Here we show that transient GFAP^{+} astrocytic dysfunction limits the severity & delays the progression of intra-nigral LPS-induced Iba1^{+} microgliosis, TH^{+} dopaminergic neurodegeneration, nigrostriatal dopamine loss and ensuing motor dysfunction. Our data demonstrates a role for reactive astrocytes in actively sustaining LPS-induced midbrain microglial activation and contributing towards dopaminergic neuronal loss and experimental Parkinsonism, at least in part via the release of soluble factors such as S100β. Here we propose an indelible neurotoxic role for astrocytic crosstalk with midbrain microglia at the interface of intra-nigral LPS-mediated dopaminergic neuropathology, thus spotlighting astrocytes as dark horses of inflammation-mediated neurodegeneration in the Parkinsonian brain.

To this end we sought to investigate whether targeting the noradrenergic system for anti-inflammatory & neuroprotective effects via glial cell immunomodulation and neurotrophic factor production could protect against LPS-mediated neurotoxicity of the nigrostriatal dopaminergic tract. Twice daily treatment with the noradrenaline reuptake inhibitor atomoxetine (3 mg/kg i.p.) alone or in combination with the α2-adrenoceptor antagonist idazoxan (1 mg/kg i.p.) for 7 days commencing 4 hours post lesioning, attenuates intra-nigral LPS-mediated Iba1^{+} microglial activation & TH^{+} dopaminergic neuronal loss, ameliorates nigrostriatal dopamine depletion and provides partial protection against associated motor deficits. These findings demonstrate that pharmacologically enhancing noradrenergic tone exerts anti-inflammatory effects in the inflamed midbrain, and protects against LPS-mediated neurotoxicity of the nigrostriatal dopaminergic tract, thus facilitating motor improvements at least in part via immunomodulation of nigral microglia.

Akin to the greater susceptibility of the elderly population to the detrimental effects of a bacterial infection, we further examined the impact of superimposing an exposure to a peripheral immune stressor on pre-existing intra-nigral LPS-mediated microglial activation, dopaminergic neurodegeneration & motor dysfunction. Here we show that a sub-toxic, low dose of systemic LPS (250 μg/kg i.p.) exacerbates ongoing TH^+ dopamine cell loss in the SNpc, augmented nerve terminal degeneration in the striatum and exaggerated ensuing motor deficits, findings which were underpinned by an expansion in nigral $Iba1^+$ microgliosis, increases in CD68 expression and elevations in nigral IL-1β production. Moreover treatment with formoterol, a long acting, lipophilic and highly selective β_2-adrenoceptor agonist curtailed microglial activation in the SN and prevented exacerbations in the degeneration of the nigrostriatal dopaminergic tract, thus attenuating the exaggerated deficits in motor function. These findings indicate that an acute episode of a systemic bacterial infection can accelerate neurodegenerative disease progression, whereas pharmacologically targeting β_2-AR's directly could slow/halt the progression of Parkinsonian neuropathology & symptomology in instances where inflammation contributes to disease pathogenesis.

Taken together, the data gathered herein highlights midbrain glia (both microglia & astrocytes) as crucial cellular effectors of immune-mediated dopaminergic neurodegeneration in the Parkinsonian brain, and promotes pharmacologically targeting the CNS noradrenergic system for anti-inflammatory and neurotrophic effects to provide neuroprotection against inflammation-mediated neurotoxicity, nigrostriatal dopamine loss and motor dysfunction.

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It was fun. First and foremost, I am extremely grateful for my supervisor Dr. Andrew Harkin for giving me this opportunity to conduct my PhD research in his laboratory. I feel very fortunate to have had a supervisor as knowledgeable, understanding and assuring as he is. His confidence in my ability to undertake this research, which was challenging at times, was highly influential towards the completion of this body of work and helped provide a research environment that was a pleasure to work in, and further serves as a testament to his experience as a supervisor.

I would like to thank all the members of AH lab both past and present who I had the pleasure of meeting throughout my undergraduate & postgraduate studies, especially Justin Yssel, Jennifer David and Elaine Dempsey. I learned a lot from you guys and was very lucky to share a lab with such friendly and talented people. I must also extend my gratitude to members of the other labs in TCIN which offered guidance & support in the completion of my studies. Of particular note, I would like to thank CC lab, from where I previously learned my craft in certain scientific methods which subsequently proved crucial to the completion of the work presented herein.

I want to extend a massive thank you to all of my students who helped with the experimental work involved and the analysis of data which contributed to the completion of this thesis. Their company throughout the past 3 years made my experience of working in TCIN more enjoyable and spurred on an element of teamwork in my approach to the scientific research conducted which I greatly appreciated. No doubt it's the people that make the place and I've certainly made some great friends over the years. In particular, I would like to thank Feilim Desmond (M.Sc. Pharmacy & Pharmaceutical sciences), Rosa Chiara Goisis (M.Sc. Neuroscience), Ruth Haverty (SS Neuroscience), Caoimhe McNamara (Human health & disease) and I suppose Hannah Sammon (my honorary HHD student!). You guys made me laugh a lot throughout the years!

On a more sombre, yet equally important note, I realise it is a huge privilege to be able to use animals in my research, and I must knowingly acknowledge that the impact & value of my research is afforded purely and undisputedly on the back of the lives of animals born and bred specifically for scientific purposes. To this end I would like to dedicate this thesis to my dog Rambo, a brutish Jack Russell terrier that has brought me and my family years of friendship and joy, and serves as a constant reminder in my mind of the undeniable eminence and value of a man's best friend.

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5-HIAA	5-Hydroxyindole-3-acetic acid
5-HT	Serotonin
6-OHDA	6-Hydroxydopamine
ATP	Adenosine triphosphate
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
BBB	Blood-Brain Barrier
BDNF	Brain derived neurotrophic factor
Camp	Cyclic adenosine monophosphate
CDNF	Cerebral dopamine neurotrophic factor
COX-2	Cyclooxygenase-2
CPu	Caudate Putamen
CSF	Cerebral spinal fluid
DA	Dopamine
DAB	3-3'-Diaminobenzidine
DAergic	Dopaminergic
DOPAC	3,4-Dihydroxyphenyl-acetic acid
GDNF	Glial derived neurotrophic factor
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography

HVA	Homovanillic acid
i.p.	Intraperitoneal
IL-10	Interleukin-10
IL-1ra	Interleukin-1 receptor antagonist
IL-1RI	Interleukin-1 type-1 receptor
IL-1RII	Interleukin-1 type-2 receptor
IL-1β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase
LC	Locus coeruleus
L-DOPA	1,3,4-Dihydroxyphenylamine
LPS	Lipopolysaccharide
MANF	Mesencephalic astrocyte derived neurotrophic factor
MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
MFP	Medial Forebrain Bundle
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NA	Noradrenaline
NAergic	Noradrenergic
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NAT	Noradrenaline Transporter

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
N-Methyl 5-HT	Methylated serotonin
NO	Nitric oxide
NOS	Nitric oxide synthase
NRI	Noradrenaline reuptake inhibitor
PBS	Phosphate buffered saline
PD	Parkinson's disease
PET	Positron emission tomography
PRR	Pathogen recognition receptors
ROS	Reactive oxygen species
RT	Room temperature
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
TGF-β	Transforming growth factor-β
TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VMAT-2	Vesicular monoamine transporter-2
β2-AR	Beta-2 Adrenoceptor

Chapter 1

Introduction

1.1 Parkinson's disease: an age-related neurodegenerative disease

Parkinson's disease is an age-related progressive neurodegenerative syndrome characterised by degeneration of the nigrostriatal dopaminergic system, formation of filamentous intra-neuronal Lewy body inclusions and extrapyramidal motor dysfunction (Braak et al., 2003; Jankovic, 2008). PD is a progressive, age-related neurodegenerative disease that affects 1% of people over the age of 60 worldwide (de Lau and Breteler, 2006). There is no cure, and the current therapies including L-DOPA/carbidopa, dopamine agonists, catechol-O-methyltransferase (COMT), & monoamine oxidase B (MAO-B) inhibitors and dopamine transporter (DAT) blockers are prescribed medications aimed only at providing symptomatic relief. The efficacy of these dopaminergic therapies declines as the disease progresses however, as the extent of neurodegeneration and dopamine loss within the nigrostriatal dopaminergic system advances to a point where the capacity of manipulating the DAergic system with these drug therapies to alleviate Parkinsonian symptomology is no longer effective. Moreover, when patients present themselves in a clinic with motor symptoms indicative of PD, it is estimated that they have already lost 80% of striatal dopamine content (Gibb and Lees, 1988), preceded by a 50% loss of midbrain dopaminergic neurons (German et al., 1989), and are therefore at a very advanced stage of the disease upon diagnosis. Thus, a major unmet clinical need is the development of a neuroprotective strategy to halt disease progression in patients with PD.

The majority of nigrostriatal dopamine-producing cell bodies reside in an area of the midbrain known as the substantia nigra pars compacta (A9), a densely neuromelanin-pigmented neural hub of dopamine neurons medial to the pars reticulata, projecting their axons along the nigrostriatal pathway to the dorsal striatum where they release the neurotransmitter dopamine. Ventral dendrites of dopamine neurons in the SNpc extend deeply into the pars reticulata portion of the substantia nigra, a brain region containing GABAergic inhibitory neurons which influence dopaminergic neurotransmission. In Parkinson's disease there is an estimated 60% loss of dopamine producing neurons in the SNpc and an 80% loss of striatal dopamine content, these pathological manifestations culminating in a severe movement disorder of tremor, rigidity and bradykinesia (Sulzer, 2007; Pasquini et al., 2018).

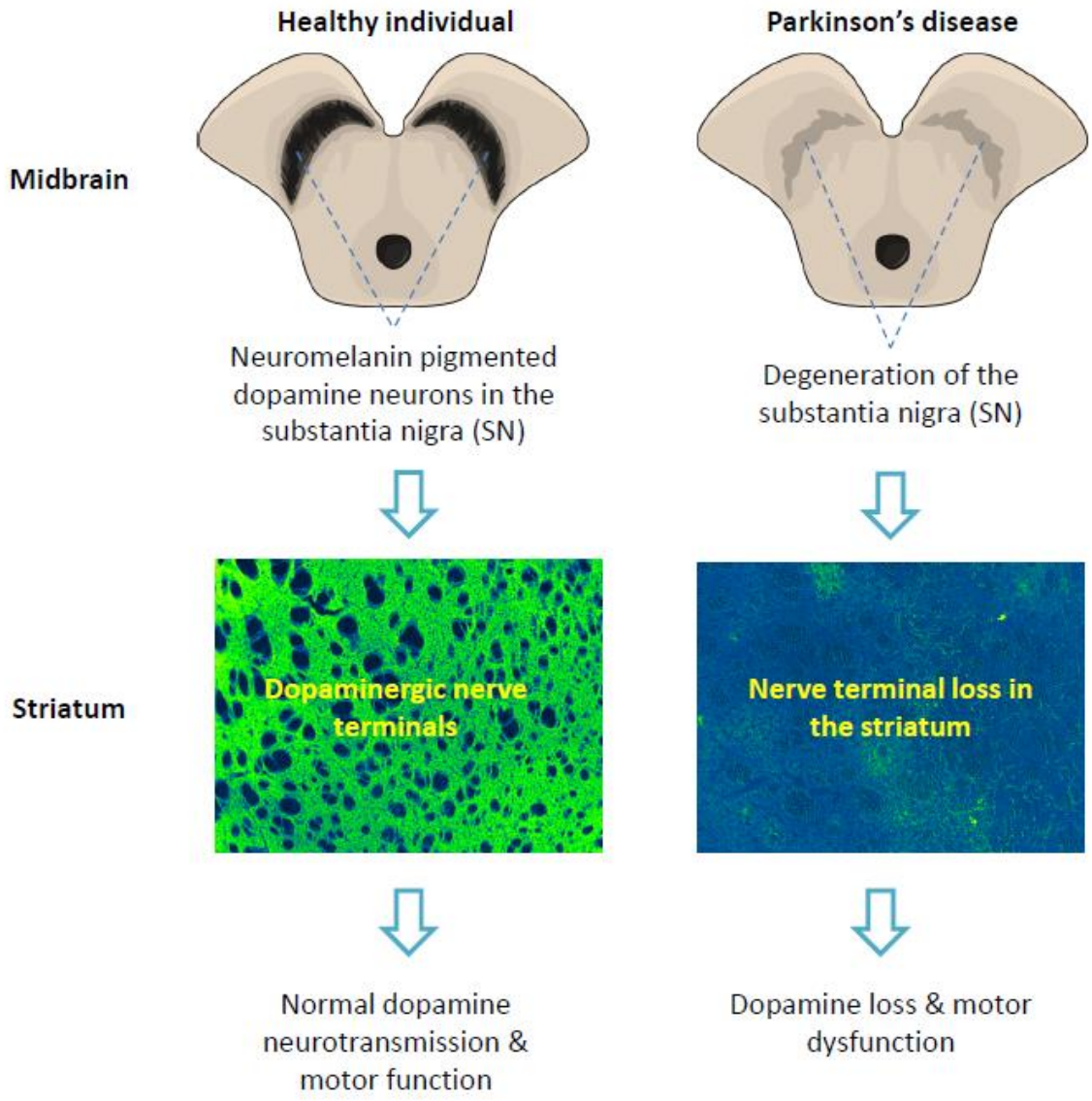


Figure 1.1 Parkinson's disease pathology. The substantia nigra pars compacta (SNpc) is located in the midbrain & contains neuromelanin pigmented dopamine neurons, which project their axons along the nigrostriatal pathway to the striatum where they release dopamine, a neurotransmitter integral to coordinating motor function (left panel). Degeneration of the nigrostriatal dopaminergic system is a salient neuropathological feature of Parkinson's disease. Once dopamine producing cells within the SNpc begin to die, their associated nerve terminals within the striatum consequently degenerate, culminating in a loss of striatal dopamine content and ensuing motor dysfunction in PD patients (right panel).

1.2 Clinical symptoms of Parkinson's disease

Motor symptoms: Two pathways (direct & indirect) of opposing effects are integral to the basal ganglia circuitry underlying the initiation, coordination and cessation of voluntary movement. Inhibitory inputs from the ''direct pathway'' & excitatory inputs from the ''indirect pathway'' modulate the output activity of the basal ganglia in order to facilitate and suppress motor activity. The ''direct pathway'' connects the striatum to the output nuclei of the basal ganglia; the Globus pallidus interna (GPi) and the substantia nigra pars reticulata (SNr). Neuronal projections along the ''direct pathway'' stem from the putamen, express dopamine D1 receptors and exert an inhibitory effect on neurons within the GPi/SNr. Neuronal projections along the ''indirect pathway'' connect the putamen with the GPi/SNr via synaptic connections with the Globus pallidus externa (GPe) and the subthalamic nucleus (STN). Neuronal projections running from the putamen to GPe, and from GPe to the STN express dopamine D2 receptors and are GABAergic (inhibitory). Conversely, neurons stemming from the STN to the GPi/SNr are glutamatergic (excitatory). Thus, the stimulation of ''indirect pathway'' neurons leads to inhibition of the GPe and consequent disinhibition of the STN and the excitation of the GPi/SNr. This model of the basal ganglia underlies the circuitry for dopamine to modulate glutamatergic influences on corticostriatal neuronal inputs by exerting a bimodal effect on its pathway-specific neurons; activating D1 receptors in the direct pathway & inhibiting D2 receptors in the indirect pathway (Obeso et al., 2000).

In Parkinson's disease, a major dopamine deficiency leads to a decreased excitation of neurons in the direct pathway & a reduced inhibition of neurons of the indirect pathway. Decreased excitation of neurons projecting along the direct pathway annuls the inhibition of the GPi/SNr, and conversely, reduced inhibition of neurons along the indirect pathway leads to overt inhibition of the GPe, disinhibition of the STN and resultant over-excitation of the GPi/SNr. Taken together, the net outcome is an exorbitant activation of output neurons of the basal ganglia consorted by an overt inhibition of motor circuitry, culminating in the abnormal motoric phenotype of a Parkinsonian state. Tremor, rigidity and bradykinesia (slowness of movement) form the cardinal triad of motoric manifestations in Parkinson's disease (Berardelli et al., 2001). The terms akinesia & hypokinesia are often described synonymously with bradykinesia in the literature; Akinesia however, refers to a poverty in spontaneous motor function (i.e. an arm swing whilst walking or a distinct facial expression) whereas hypokinesia refers to that in addition to being slow, patients' movements are also smaller (i.e. the micrographia of a PD patients handwriting). Freezing of gait (FOG) is reportedly an independent motor manifestation of PD that is induced by paroxysmal neuropathology which is not correlated with the pathology that is responsible for causing rigidity, postural instability or bradykinesia in patients (Bartels et al., 2003).

Non-motor symptoms: In terms of neuropsychiatric symptomology, depression, apathy and anxiety are often implicated in prodromal PD. Dysfunction in multiple neurotransmitter

systems have been implicated based on the pathology of the locus coeruleus, SNpc and raphe nuclei. According to the diagnostic and statistical manual of mental disorders-V (DSM-V), about 35% of PD patients suffer an early onset of symptoms of depression, but up to 45% of PD may suffer from depression at a later stage of the disease as well (Postuma et al., 2012). Depression however, is reportedly difficult to define in PD cases as the symptoms are altered to that of pure depression; a heightened degree of irritability and dysphoria, a pessimistic outlook on the future and a sense of guilt are typical of the pathophysiology of depression in PD (Pellicano et al., 2007). Similarly, anxiety disorders coexist with depression and occur in 20-40% of PD patients, comprising general anxiety disorder, agoraphobia, social phobia and panic attacks (Chaudhuri and Schapira, 2009). Sleep disturbances and insomnia are another common prodromal symptom of PD: Studies on transgenic mouse model of PD have shown that disruptions in signalling output from the suprachiasmatic nuclei (SCN), the pacemaker of the circadian system, contributes to sleep disturbances in early PD (Willison et al., 2013). Rapid eye movement behavioural disorder (RBD), defined by violent motor behavioural instances and defensive responses to vocalisation, may be reflected through the patients' movements during sleep in PD cases (Pellicano et al., 2007). In fact, evidence suggests that parasomnias such as REM behavioural disorder is possibly an early marker that antedates the development of Parkinson's disease later in life (Iranzo et al., 2006).

Sensory symptoms, such as olfactory impairments occur frequently in prodromal PD; more than 50% of patients suffer anosmia (inability to perceive odour), and roughly 35% experience a severe form of hyposmia (reduced ability to detect odour) (Postuma et al., 2012). Deficits relating to olfactory identification and discrimination are popular symptoms, along with an increased olfactory threshold, dependent on the severity of damage to dopamine neurons in the olfactory bulb and olfactory nuclei (Pellicano et al., 2007). Mild cognitive impairment in Parkinson's disease (PD-MCI), associated with attention and vigilance deficits, occurs in 30-40% of patients and confers a narrower time frame in succumbing to dementia as the disease progresses (Postuma et al., 2012). Moreover, according to studies by (Tadaiesky et al., 2008), 6-OHDA-induced denervation of dopaminergic nerve terminals in the bilateral striatum confers cognitive impairments in memory-based tasks. In humans, up to 80% of PD patients may be afflicted by dementia in the later stages of the disease, the most prominent risk factors being hallucinations, postural and gait disturbances, old age and mild cognitive impairment (PD-MCI) (Aarsland and Kurz, 2010). According to studies by (Bhalsing et al., 2013) involving 136 Parkinson's disease patients and 172 healthy controls, there was a higher prevalence of restless leg syndrome (RLS) in PD patients (11.9%) relative to controls (2.9%). Rare incidences of abnormal sensations and restlessness in lower back regions of patients have also been reported as variants in RLS. The incidence of RLS and its variants remains idiopathic although it has been suggested that degeneration of dopaminergic neurons of the diencephalon-spinal pathway of the hypothalamus is a key player in RLS susceptibility in PD cases and the impulsive motor impairments that ensues (Wong et al., 2014).

Filamentous alpha-synuclein inclusions termed Lewy bodies and Lewy neurites are a salient neuropathological hallmark feature of the Parkinsonian brain (Grazia Spillantini et al., 1998). Mutations in alpha-synuclein gene (*SNCA*), such as locus duplication or triplication causes familial Parkinson's disease (Singleton et al., 2003; Chartier-Harlin et al., 2004). Based on the stage-dependent variation in the distribution of α-synuclein pathology in Lewy bodies, Braak's staging can provide a basic blueprint for defining phases in sporadic Parkinson's disease progression (Braak et al., 2003). Braak stages I and II propose that the earliest pathological changes are in the olfactory bulb and medulla but as PD progresses to Braak stages III and IV, Lewy body pathology manifests in the midbrain and thereafter in the SNpc (McDowell and Chesselet, 2012). The cardinal motor symptoms of PD are not noticeable in patients until stage IV, thus, the myriad of non-motor symptoms in early PD may be afforded by disturbances that occur within the previous three stages. Assessing individuals with pre-motor PD initially involves screening to try and pick up susceptibility markers (e.g. subtle motor signs, problems with olfaction, RBD) which implicate an increased risk of developing PD. Patients should then be sorted into groups, depending on what combinations of markers if any, they tested positive for. Many will have varying combinations of markers and functional neuroimaging techniques should be carried out to establish the quantity of patients with Parkinson's disease at risk syndrome (PARS) per group (Hagenah and Brüggemann, 2012). The same authors depicted a Parkinson's at risk syndrome (PARS) model on preclinical diagnostic tools for PD (reconstructed below). The proportion of individuals with combinations of PD susceptibility markers should be high in the pre-diagnostic stage but relatively low in the preclinical stage.

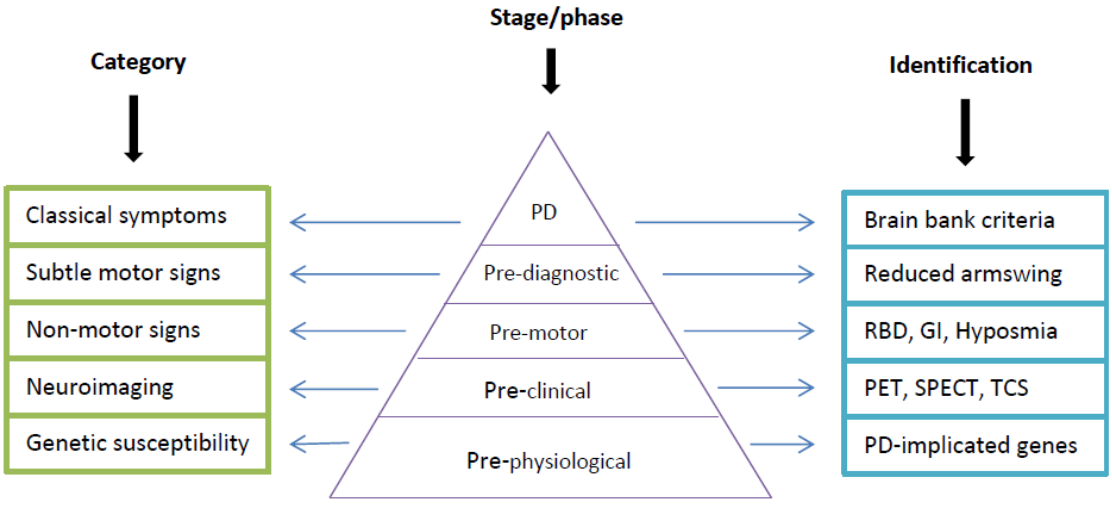


Figure 1.2 Postulated stages preceding the onset of classical motor symptoms in PD. Categorical symptomology relative to the postulated stages are shown on the left whereas specific techniques and conditions that aid in the identification of these stages are labelled on the right. Diagram constructed using Microsoft Word applications, reconstruction was partly adapted from (Hagenah and Brüggemann, 2012).

1.3 Experimental models of Parkinson's disease

Establishing a clinically relevant animal model of PD is necessary in order to assess the efficacy of potentially neuroprotective strategies to treat the human condition. Such models should mimic at least partially, the clinical manifestations of PD patients and replicate key neuropathological features of the disease. Multiple classical toxin-based animal models exist, most popular being the 6-hydroxy-dopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models.

Intra-striatal injection of 6-OHDA induces a retrograde degeneration of dopaminergic axons, commencing at a terminal level in the striatum and culminating in the progressive loss of nigral cell bodies and associated motor impairments (Lee et al., 1996; Cheng et al., 2011). TH^+ denervation within the striatum is seen as early as 6 hours post terminal lesioning with 6-OHDA (28 µg) followed by a pronounced $OX\text{-}42^+$ striatal microgliosis evident from 72 hours onward, culminating in ipsilateral nigral cell loss after 2 weeks (Walsh et al., 2011). Conversely, following systemic administration, MPTP also produces a reliable and reproducible lesion of the nigrostriatal DAergic system (Jackson-Lewis et al., 1995). Depending on the dose of MPTP used, striatal dopamine content is depleted in the region from 40% (14 mg/kg i.p. x 4 times for 1 day) to 90% (20 mg/kg i.p. x 4 times in 1 day) when assessed via HPLC at 7 days post lesioning, findings which were congruent with stable deficits in TH-immunopositive cell bodies in the SNpc and their affiliated nerve terminals in the striatum (Jackson-Lewis and Przedborski, 2007). Thus, both the MPTP and 6-OHDA models of PD are suitable toxins used to induce dopaminergic neuronal death, striatal nerve terminal degeneration dopamine loss and motor deficits (Mori et al., 2005; Decressac et al., 2012). There are disadvantages to the use of these toxins in modelling the pathophysiology of PD however; both induce acute damage to the nigrostriatal DAergic system, their effects are often non-progressive and rarely lead to the generation of Lewy body inclusions, a salient neuropathological feature of Parkinson's disease.

Alpha-synuclein plays a role in maintaining a supply of synaptic vesicles in pre-synaptic terminals (Bendor et al., 2013). In both familial and sporadic cases of Parkinson's disease α-synuclein becomes misfolded and accumulates intra-neuronally leading to the formation of Lewy Body inclusions and ensuing neural degeneration (Spillantini et al., 1998). Given that a missense mutation in the *alpha-synuclein* gene is a major genetic pre-determinant in the development of Parkinson's disease, and that the α-synuclein protein is a prominent constituent of Lewy bodies and Lewy neurites, hallmark neuropathological features of the PD brain (Goedert, 2001), more focus has been put on research into α-synuclein mutant models in recent years to better understand the aetiology and pathogenesis of PD. Interestingly, 60-day old transgenic Drosophila flies expressing the mutant A30P & A53T α-synuclein proteins linked to familial Parkinson's disease exhibit intra-neuronal α-synuclein inclusions, dorsomedial dopaminergic neuronal loss and locomotor disturbances, highlighting a powerful genetic approach to modelling clinical features of PD in a non-

mammalian alternative (Feany and Bender, 2000). Moreover, lentiviral-mediated α-syn expression within the substantia nigra drives selective dopaminergic neurodegeneration, striatal denervation and the accumulation of neuritic pathology in rats (Lo Bianco et al., 2002). Consistent with earlier findings showing that mutations in the α-synuclein gene are identified in autosomal dominant familial PD cases (Polymeropoulos et al., 1997), more recent quantitative genomic analysis of families with Parkinson's disease have demonstrated that genomic duplication or triplication of the α-synuclein gene (*SNCA*) causes Parkinson's disease (Chartier-Harlin et al.; Singleton et al., 2003) highlighting that genetic variability within a single promoter, in this case the SNCA promoter can contribute to the development of PD. Understanding how aberrant, mutant alpha-synuclein behaves & contributes to the development of Parkinsonian neuropathology will prove paramount in the therapeutic outlook of preventing the onset and/or the development of synucleinopathies within the PD brain.

Studies by (Auluck et al., 2002) have shown that interfering with endogenous chaperone activity enhanced α-synuclein-mediated dopaminergic neuronal loss, and that directed augmentation of the molecular chaperone Hsp70 in dopaminergic neurons using the GAL4/UAS expression system suppressed α-synuclein-induced neurotoxicity in Drosophila. Other studies have shown that α-synuclein may exert its toxic effects in multiple models of PD by compromising ER-Golgi vesicular trafficking and that Rab1, a GTPase involved in membrane trafficking protects against αSyn-derived dopaminergic neuronal loss (Cooper et al., 2006). Given dopamine's unstable, auto-oxidative capacity to produce reactive oxygen species (ROS), a rise in cytosolic DA content due to ER-Golgi vesicular trafficking defects may render DA neurons particularly susceptible to oxidative damage. In any case, genomic interference with the α-synuclein gene seems to offer a more dexterous approach to modelling the pathophysiology of PD in order to exploit potential avenues of therapeutic intervention(s).

1.4 Microglia at the cellular crux of inflammatory derived neuronal damage; revisiting classical inflammatory pathways leading to neuronal insult.

Microglia constitute about 12% of cells in the human brain, existing as benign surveillants with dynamic effector functions in the healthy and pathologic CNS (Hanisch and Kettenmann, 2007). Upon activation of these brain resident macrophages, transition from a ''resting'' ramified morphology to an amoeboid state accompanied by an upregulated script of cell surface molecules is observed (Cho et al., 2006), including major histocompatibility (MHC) molecules, complement receptor 3 (CR3), cluster of differentiation 80 (CD80 + CD86), DC-SIGN (CD209), CD14 (Ponomarev et al., 2005), chemokine receptors and several others, as reviewed in (Galea et al., 2007). Within this activated state, immunocompetent microglia may serve to prevent toxic debris accumulation and maintain neuronal viability (Streit, 2002; Streit et al., 2004; Simard et al., 2006) and enhance their survival via release of trophic factors (Morgan et al., 2004). Furthermore, microglia within the mature CNS influence

neural precursor cell fate and are effective mediators of repair by virtue of guiding migratory stem cells to sites of injury/inflammation via the release of soluble factors (Aarum et al., 2003) and are reportedly involved in maintaining hippocampal neurogenesis (Walton et al., 2006; Ziv et al., 2006). Thus, within this state, activated microglia carry out diverse duties essential for neuronal survival. In spite of this, microglial over-activation may lead to copious production of cytotoxic factors including nitric oxide (NO) (Liu et al., 2002), superoxide (Giulian et al., 1993) and TNF-α (Lee et al., 1993) which in turn holds deleterious neurotoxic consequences for neuronal populations (Block et al., 2007a). There is a wide body of literature stapling inflammation as a major contributor to the pathogenesis of neurodegenerative disease, a process in which microglia are at the crux of the issue (Hanisch and Kettenmann, 2007; Saijo et al., 2013).

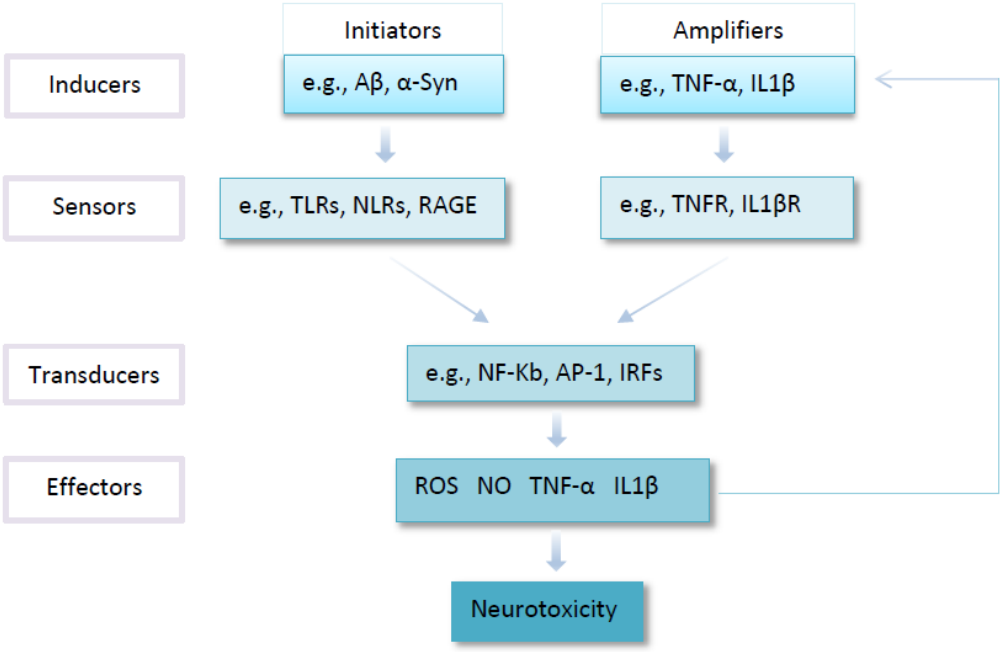


Figure 1.3 Neuroinflammatory signalling cascades leading to neurodegeneration. The release of amyloid beta (Aβ) or alpha synuclein (α-Syn) from damaged/dying neurons (inducers) and the consequent detection by pathogen recognition receptors (Sensors) on microglia results in inflammatory signalling cascades converging on a myriad of transcription factors (Transducers) which upregulate proinflammatory molecules, leading to neurotoxicity (Effectors). Similarly, certain cytokines such as TNF-α can amplify or mitigate a secondary response, leading to prolonged inflammation. Diagram idea partly adopted from (Saijo et al., 2013) and constructed using basic Microsoft Office Word applications.

Studies by (Tang et al., 2013) have shown that prior intra-nigral knockdown of the histone H3K27me3 demethylase Jumonji domain containing 3 (Jmjd3) increases over-activated $Iba1^{+}$ cell counts & exacerbates TH^{+} dopaminergic neuronal loss in the SNpc in response to subsequent MPTP intoxication (4 x 20 mg/kg i.p.). The authors demonstrated that Jmjd3 is essential for the beneficial M2 microglia phenotype, and that suppression of Jmjd3 polarises microglia towards an M1 phenotype, increasing IL-1β, iNOS, IL-6, TNF-α, p-NfκB expression

levels and decreasing the survival rate of ventral midbrain DA neurons *in vitro*. Data derived from the same study revealed that Jmjd3 expression levels were lower in the midbrain of aged mice, accompanied by elevated H3K27me3 levels and an increase in the M1 inflammatory markers iNOS, TNF-α & IL-6, and a suppression in the typical M2 marker Arginase1, thus implying that the aging process itself may be responsible for switching midbrain microglia from an M2 phenotype to a more detrimental M1 phenotype via epigenetic modification of histone H3K27me3, leading to an increased susceptibility of the aging brain to neuronal insult in response to environmental stimuli or genetic predisposition at the onset of Parkinson's disease. Studies by (Tan et al., 2018) using immunohistochemistry, laser capture microdissection & qPCR have shown that nuclear histone deacetylase (HDAC2) mRNA & protein expression is upregulated in nigral $LN3^{+}$ activated microglia & in TH^{+} dopamine neurons of the post mortem PD brain relative to healthy controls and is also increased in immortalised human midbrain microglia in response to LPS stimulation in a dose-dependent manner (most significantly at 5 and 10 μg/ml). Taken in tandem with the above, epigenetic changes in nigral microglia at the interface of DAergic neuronal death, such as DNA methylation & histone modifications may play a role in the pathogenesis of PD by instigating dysregulated microglial overactivation and ensuing inflammation-mediated cytotoxicity.

Moreover, studies by (Du et al., 2018) have shown that genetic knockdown of the Kir6.1/K-ATP channel ($Kir6.1^{+/-}$) inhibits microglial M2 polarisation and promotes an over-activated M1 pro-inflammatory phenotype via the p38/MAPK-Nf-κB signalling pathway, increasing nigral IL-1β, TNF-α, iNOS and CCL3 expression and exacerbating both MPTP- and intra-nigral LPS-induced dopaminergic neuronal loss in the SNpc. Thus, pharmacologically modulating microglial activation states may prove efficacious in ameliorating neurotoxicity of the nigrostriatal dopaminergic system. Indeed, recent studies have shown that pre-treatment with taurine (150 mg/kg i.p.) diminished paraquat (10 mg/kg i.p.) & maneb (30 mg/kg i.p.)-induced accumulation of midbrain α-synuclein oligomers and attenuated progressive dopaminergic neurodegeneration and the associated gait abnormalities in mice via inactivating microglial M1 polarisation, mitigating NADPH oxidase activation & attenuating activation of the Nf-Kb pathway and downregulating iNOS, TNF-α & IL-1β mRNA expression (Che et al., 2018).

1.5 Astrocytes & neurodegeneration: overlooked glia in the pathogenesis of Parkinson's disease?

Astrocytes are a highly populous subpopulation of glia composing 20-40% of the total number of cells in the mammalian brain that ensheath neurons with lamellate distal processes, playing roles in glutamate recycling, growth factor production and inflammatory processes (Adami et al., 2001). Glial cell interactions are at the forefront of neurodegenerative disease progression, particularly in cases where pro-inflammatory events contribute to neuropathology. Indeed, LPS-activated microglia induce the formation

of A1 neurotoxic astrocytes via the secretion of TNF, IL-1α and the first subcomponent of the classical pathway of complement activation C1q (Liddelow et al., 2017). The authors further show that 30-60% of nigral astrocytes exhibit an A1 neurotoxic phenotype (C3 complement protein & glial fibrillary acidic acid (GFAP) colocalization) in the post mortem midbrain of Parkinson's disease patients and that not only do A1's lose the ability to promote neuronal survival and synaptogenesis and to phagocytose debris, but these astrocytes kill axotomized CNS neurons & oligodendrocytes.

Astrocytes are capable of secreting proteins that at high concentrations can be toxic to their neuronal counterparts. S100β is a glial-specific member of the S100 family of calcium binding proteins expressed primarily by mature astrocytes (Zimmer et al., 1995) but also plays a role in regulating the activity of microglia (Adami et al., 2001). S100β protein expression is increased in the CSF and post mortem substantia nigra of Parkinson's disease patients (Sathe et al., 2012). S100β expression levels are upregulated within astrocytes 1 day post MPTP administration (Muramatsu et al., 2003). Moreover, S100β induces apoptosis at high (micromolar) concentrations via its direct activity on neurons in a manner associated with IL-6-mediated neurodegenerative signalling cascades (Yuekui et al., 2000) and/or by activation of microglia (Sorci et al., 2010). S100β-mediated cell death mechanisms are also associated with astroglial iNOS activation and subsequent neuronal exposure to NO (Hu et al., 1997), and with raised intracellular Ca^{2+} levels or caspase-3 expression (Iuvone et al., 2007). Interestingly, genetic ablation of S100β provides substantial protection from MPTP-induced neurotoxicity of dopamine neurons via the RAGE & TNF pathway (Sathe et al., 2012). Taken together, S100β acts as a damage associated molecular pattern (DAMP) in the guise of an alarmin which can initiate and perpetuate inflammatory reactions in Parkinson's disease and overt increases in astroglial S100β levels may therefore be involved in disease pathogenesis.

Most cases of Parkinson's disease are idiopathic, but mutations in 17 genes have also been implicated in the development of PD (Hernandez et al., 2016). Eight of these genes encode proteins that play functional roles in astrocytic neurobiology: *park7, snca, pla2g6, atp13a2, lrrk2, gba, pink1* and *park2*. The expression of *park7* (DJ-1) is upregulated in reactive astrocytes in post mortem PD brains (Bandopadhyay et al., 2004). Knockdown of DJ-1 expression in astrocytes impairs astrocyte-derived protection against rotenone-mediated neurotoxicity *in vitro*, whereas overexpression of DJ-1 within astrocytes augments their neuroprotective capacity (Bandopadhyay et al., 2004). DJ-1 knockout mice (DJ-$1^{-/-}$) demonstrate an enhanced sensitivity to the neurotoxic effects of 6-OHDA on the nigrostriatal dopaminergic system *in vivo,* consistent with the suppressed ability of astrocytes to employ cellular defence mechanisms against 6-OHDA-induced oxidative stress & neurotoxicity (Lev et al., 2013).

α-synuclein (*SNCA*) expression is high in neurons but quite low in astrocytes (Solano et al., 2000), albeit α-synuclein inclusions have been found in astrocytes as well as neurons (Braak et al., 2007) implying that astrocytes sequester α-synuclein released from neurons. Indeed, α-synuclein aggregates are directly transmissible from neurons to astrocytes, leading to astroglial inflammatory responses which may contribute to the development of pathologic

synucleinopathies such as Parkinson's disease (Braidy et al., 2013; Rannikko et al., 2015). The localisation of endocytosed α-synuclein within the astrocyte lysosome implies that astrocytes play a key role in removing & degrading this protein and may promote a favourable environment for neurons to thrive in (Lee et al., 2010). Importantly, in a transgenic mouse line where mutant *SNCA* was overexpressed using an astrocyte-specific promoter, a widespread pre-symptomatic astrogliosis, increased aggregated & truncated α-synuclein expression, a downregulation of glutamate transporters (GLAST & GLT-1), abnormal AQP4 localisation (involved in water transport and BBB function), microglial activation, dopaminergic neuronal loss in the SNpc (60.5%) & motor impairments occurred (Gu et al., 2010). The study indicated that astrocytes uptake α-synuclein but when protein concentrations are high extracellularly (i.e. they reach a certain threshold), this eventually leads to the development of α-synuclein aggregates within astrocytes themselves, at which point they lose regulatory functions (recycling of glutamate & BBB integrity) and a more serious pathology develops.

Mutations in *LRRK2* are the most common genetic root of PD and confer a phenotype similar to late onset idiopathic Parkinson's disease (Trinh et al., 2014). LRRK2 is expressed in neurons microglia and astrocytes and is heavily involved in the autophagy-lysosome pathway in astrocytes (Manzoni et al., 2013). LRRK2 regulates the number, size and function of lysosomes in astrocytes and mutation of LRRK2 induces enlarged & dysfunctional lysosomes, a reduction in lysosomal pH and an increase in the lysosomal ATPase *ATP13A2,* a gene linked to PD-related syndrome (Kufor-Rakeb syndrome) in murine and human LRRK2 G2019S carriers (Henry et al., 2015). Moreover, genetic ablation of LRRK2 abrogates immune cell infiltration & dopamine cell loss in the SNpc induced by α-synuclein overexpression or LPS exposure, thus implicating LRRK2 in the pathogenesis of PD (Daher et al., 2014).

The majority of PD-related mutations in *PINK1* are loss of function mutations linked to recessive Parkinsonism (Koyano and Matsuda, 2015). *PINK1* encodes PTEN-induced putative kinase 1 (PINK1), a protein involved in mitophagy, a process involving the selective degradation of damaged mitochondria. PINK1 plays an integral role in the development of GFAP-positive astrocytes and *PINK1*-KO mice have reduced numbers of astrocytes compared to WT controls (Choi et al., 2016). Astrocyte proliferation is markedly reduced in PINK1-deficient postnatal astrocyte cultures along with mitochondrial health and ATP production (Choi et al., 2013). The resultant deficiency in astrocyte proliferation and cell numbers in mutant *PINK1* carriers may have major consequences for the neuroprotective capacity of astrocytes and general brain homeostasis.

PARKIN is encoded by the *PARK2* gene and is also implicated in the process of mitophagy (Koyano and Matsuda, 2015). α-synuclein aggregates have been found within astrocytes in the post mortem brains of patients with autosomal recessive juvenile Parkinsonism linked to *PARK2* mutations (Hayashi et al., 2000). PARK2-KO glial cultures have less astrocytes, reduced level of proliferation, a reduced neurotrophic capacity, increased damaged mitochondria and limited GSH secretion, indicating that astroglial dysfunction is strongly associated with parkin mutations (Solano et al., 2008). Interestingly, parkin is reportedly

involved in astroglial inflammatory responses as well; IL-1β decreases parkin expression levels in primary mouse astrocytes whereas TNF-α upregulates parkin expression (Khasnavis and Pahan, 2014). Remarkably, the same author's found that treatment with cinnamon powder (*Cinnamonum verum*) upregulates sodium benzoate (NaB; metabolic product of cinnamon powder) in the blood and brain of mice and protects against MPTP-induced neurotoxicity of nigrostriatal dopaminergic neurons via upregulating Parkin & DJ-1 protein expression within the substantia nigra.

Thus, given their A1-associated neurotoxic phenotype, their potential to produce overt levels of soluble mediators (e.g. S100β & IL-6) and their PD-associated gene & protein expression profiles linked to the onset and progression of neuronal loss, pharmacologically targeting reactive astrocytes is a currently overlooked, yet ripe prospect to sway reactive gliosis from a neurotoxic arm towards an anti-inflammatory and neurotrophic phenotype (e.g. BDNF, CDNF, GDNF & MANF production) in the treatment of Parkinson's disease.

1.6 Noradrenaline; an organic fertilizer of the brain with anti-inflammatory properties.

Noradrenaline can affect a wide array of microglial functions through adrenergic signalling. Cultured rat microglia express mRNA encoding α_{1a}-, α_{2a}-, β_1- and β_2-ARs and have a higher expression of β_2-AR than any other cell type in the brain (Mori et al., 2002). Stimulation of astrocytic β_2-AR's, and the subsequent increase in cAMP/PKA, may also provide neuroprotection through secretion of neurotrophic factors. For example, the application of noradrenaline to astroglial cell cultures induced the expression of NGF (Furukawa et al., 1989), BDNF (Jurič et al., 2006) and NT-3 (Mele et al., 2010); which were chiefly mediated by α_1 and/or β-adrenoceptor activation. GDNF also promoted the survival of rodent midbrain dopaminergic cultures, increasing their differentiation and proliferation as assessed by cell body size, neurite outgrowth, and increased dopamine uptake (Lin et al., 1993c). NA may also increase astrocytic anti-inflammatory cytokine and chemokine production including IL-10, GM-CSF and sTNF-R1 (Braun et al., 2014). While generally considered a pro-inflammatory factor, GM-CSF may also confer neuroprotection. For example, GM-CSF injection in mice attenuated nigral dopaminergic cell loss following exposure to the environmental toxin paraquat, either alone or following microglial priming with LPS (Mangano et al., 2011).

Locus coeruleus (LC) neuronal activity oscillates with the sleep-wake cycle and levels of cortical vigilance (Aston-Jones and Bloom, 1981). Given that increases in coerulean activity forecasts sleep to wakefulness, the residing consensus staples the LC with a seminal role in inducting and regulating cortical arousal (Berridge, 2008). Cells of the LC can project rostrally and mediate arousal in forebrain regions (Aston-Jones and Cohen, 2005), and recent studies involving optogenetic manipulation of LC neurons has demonstrated that this pontine nucleus exerts an essential role in the sleep-wakefulness cycle and is finely tuned to modulate cortical arousal (Carter et al., 2010). Reversible reduction in LC-NAergic activity induced by bilateral clonidine (an α_2-receptor agonist) administration within the prefrontal cortex (PFC) impairs cognitive function during visuospatial reaction time trials of attention

(Mair et al., 2005). Moreover, optimal levels of NA are required within the PFC for selective attention and working memory trials in aged non-human primates, as local administration of clonidine to presynaptic sites (which inhibits NA release and weakens NAergic tone) impaired performance, whereas clonidine at postsynaptic sites enhanced cognitive performance (Arnsten and Goldman-Rakic, 1985).

Behavioural studies involving the hole board procedure in rodents have been used to drive phasic bursts of LC activity in order to demonstrate the role of β-adrenergic receptors in promoting hippocampal long term potentiation (LTP) (Neuman and Harley, 1983; Uzakov et al., 2005). Moreover, recent studies in rodents have demonstrated that electrical stimulation of the LC enhances LTP of hippocampal-PFC synapses that are putatively associated with offline long-term memory consolidation and that hippocampal-PFC LTP is suppressed by rodents pre-treated with DSP4 (50 mg/kg i.p.) (Lim et al., 2010). The LC-NAergic system does not act alone in reinforcing synaptic plasticity however, as NA has also been shown to work in concert with other neuromodulators such as acetylcholine within the medial septum and basal lateral nucleus of the amygdala to strengthen LTP within the dentate gyrus (Bergado et al., 2007). Consistent with these findings demonstrating the role of NA in modulating synaptic plasticity, studies by (Gelinas et al., 2008) reveal that β-AR's gate the cAMP-PKA cascade to facilitate LTP and hippocampal dependent memory formation in response to noradrenergic signalling. These findings are relevant given that a large number of Parkinson's disease patients suffer from mild cognitive impairment (PD-MCI) which predicts the development of dementia, which can occur in up to 80% of patients over the long-term course of the disease (Litvan et al., 2012).

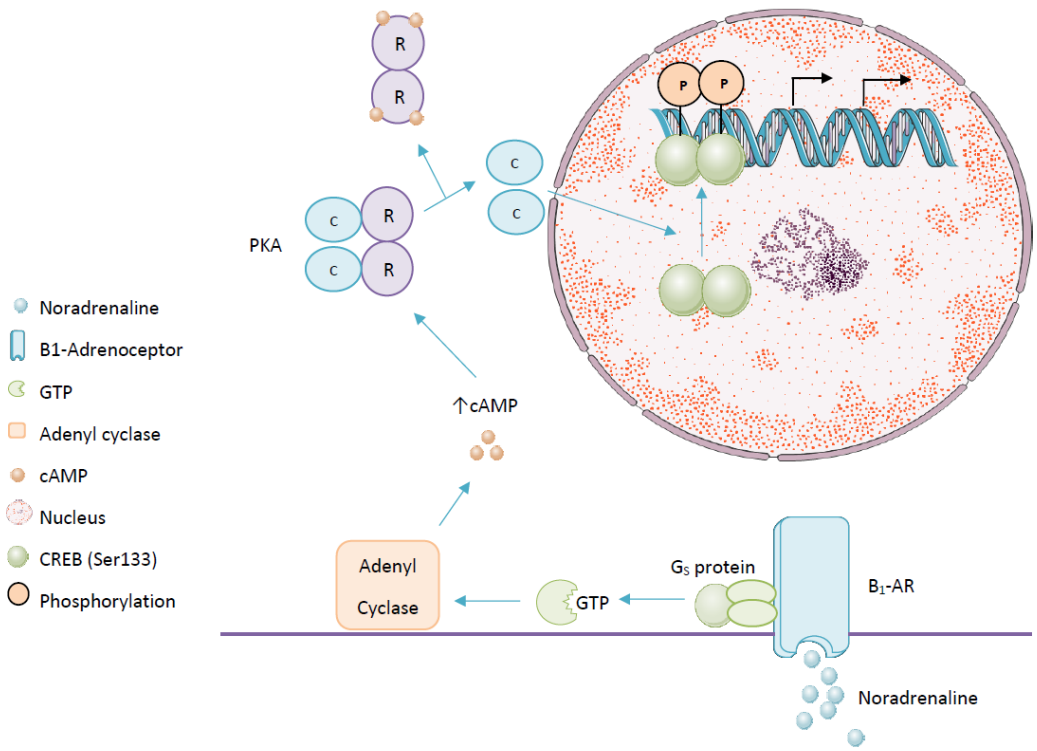


Figure 1.4 Noradrenergic signalling and hippocampal-dependent memory retrieval. Noradrenergic stimulation of B_1-adrenergic receptors (B_1-AR) activates the intracellular cAMP-protein kinase A cascade leading to activation of the transcription factor cAMP-responsive element binding protein (CREB), which enters the nucleus, leading to transcription of synaptic effector proteins (black arrows) and thus facilitating long term potentiation (LTP) and the formation of long-term memory. Diagram adapted from (Sara, 2009) and constructed using basic Microsoft Office Word applications.

The activation of this cAMP-PKA cascade is deemed integral to NA-mediated long-term memory consolidation as interruptions in any step leading to PKA activation has previously been shown to inhibit long lasting LTP (Huang et al., 2006). The notion that the NAergic system plays an integral role in memory retrieval is supported by studies in dopamine-β-hydroxylase knockout ($D\beta H^{-/-}$) mice. It has been shown that $D\beta H^{-/-}$ mice were capable of learning a contextual fear conditioning (CFC) task but display deficits in hippocampal-dependent memory retention 48hr post training. Moreover, administration of propranolol (beta-blocker) prior to the CFC task or spatial learning diminished memory retrieval 24hr post training, but not at 1 hr or 1 week after training, thus implying that the noradrenergic system is pertinent to retrieval of recent, but not distant memories (Murchison et al., 2004).

1.7 Reconciling noradrenergic signalling & central immune homeostasis in the pathologic CNS

Noradrenaline, reactive microgliosis, and self-perpetuating neurotoxicity

Microglia are classified as brain resident macrophages of haematopoietic origin, constituting 5-20% of total rodent glial cells (Lawson et al., 1990) and existing as benign surveillants with dynamic effector functions in the healthy and pathologic CNS (Hanisch and Kettenmann, 2007). Microglia constantly screen CNS tissue (Davalos et al., 2005b), influence hippocampal synaptogenesis (Marín-Teva et al., 2004) and play a causative role in annulling excess glutamate-induced neurotoxicity via GLT-1 mediated glutamate uptake (Persson et al., 2005). Prolonged activation of these glia however gives credence to the unwarranted rapport of reactive microgliosis and self-perpetuating neurotoxicity, a hallmark feature of pathological inflammatory states in neurodegenerative disease (Qin et al., 2004; Kettenmann et al., 2011). Microglia have long since been stapled at the cellular crux of inflammatory derived neuronal damage, as over-reactive microgliosis leads to the copious production of cytotoxic factors including nitric oxide (NO) (Liu et al., 2002), superoxide (Giulian et al., 1993), and TNF-α (Lee et al., 1993) holding deleterious consequences for neuronal populations (Block et al., 2007b).

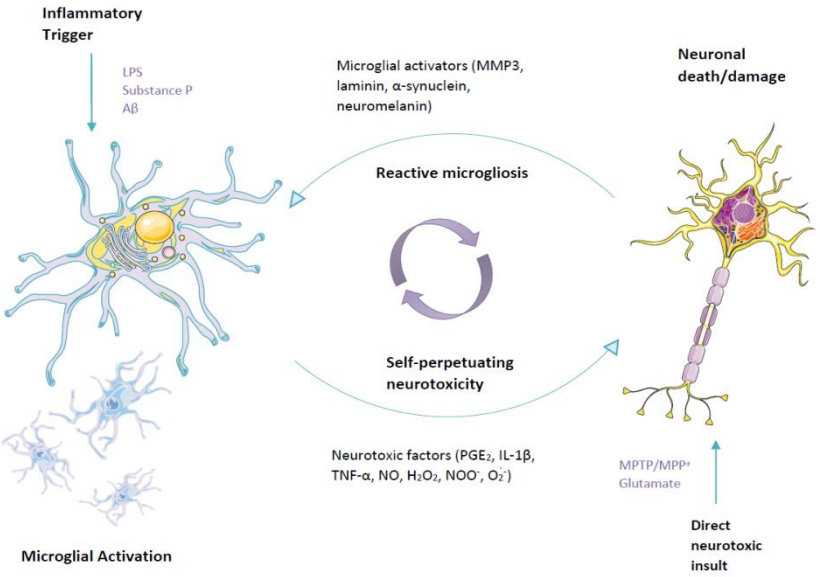


Figure 1.5 Microglial activators and neurotoxic factors; adjuvants to neurotoxicity. Microglial recognition of inflammatory stimuli (e.g. LPS, substance P, Aβ) drives the production of neurotoxic factors (PGE2, IL-1β, TNF-α, NO etc.) leading to neuronal injury / death. Subsequent release of microglial activators (e.g. MMP3, laminin, α-synuclein e.t.c.) from damaged / dying neurons activate microglia and a cycle of self-perpetuating neurotoxicity ensues. Alternatively, direct neurotoxic insult (e.g. via MPTP/MPP+, glutamate) provokes neuronal damage/death and contributes to driving pathologic CNS insult. Diagram partially adapted from (Block et al., 2007a) and created using Microsoft Office Word applications.

The locus coeruleus (LC), lying within the outermost layer of the pontine tegmentum, is the primary source hub of noradrenergic cell bodies in the CNS, their neuronal projections innervating multiple central brain regions, including the substantia nigra (SN) and the striatum (Gesi et al., 2000). At autopsy, neuronal cell bodies are reduced to a greater extent in the LC (63%) than in the substantia nigra of Parkinson's disease patients and in the nucleus basalis in Alzheimer's disease patients (Zarow et al., 2003b). Given that degeneration of the LC-noradrenergic system is concomitant with dopaminergic neurodegeneration in PD patients, it's evident that disturbances in CNS noradrenaline (NA) levels due to LC noradrenergic cell loss, as occurs in Parkinson's disease can exacerbate PD-related neuropathology (Mavridis et al., 1991), whilst pharmacological agents that increase extra-synaptic NA bioavailability may provide neuroprotection.

Given the inherent role of the LC in regulating brain immune homeostasis via modulating microglial functions through noradrenergic signalling (Jiang et al., 2015), LC degeneration and ensuing cortical NA deficiencies are well documented to negatively influence neurodegenerative disease pathology *in vivo*. N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4) has been used pre-clinically to induce selective lesions of the LC-noradrenergic system. Sixteen months post DSP4 treatment (2 X 50 mg/kg

i.p.) degeneration of the LC promoted elevations in iNOS & peroxynitrite in LC-projection areas and hippocampal plaque deposits in 10 month old APP23-tg AD mice (Heneka et al., 2006). Data derived from TH-immunostaining from the same study revealed that APP23-tg DSP4-treated mice exhibited a 50-60% loss of LC neurons, and displayed heightened working memory deficits in the radial arm maze test relative to non- NA-depleted controls, thus implying that prior NAergic deficits could be a predisposing factor to enhanced AD-like pathology and cognitive decline. Moreover, noradrenergic depletion reduces $CD11b^{+}$ microglial phagocytosis of Aβ by 70% and suppresses recruitment of these glia to hippocampal Aβ plaque deposits in NA-depleted APP-tg mice relative to controls with intact NAergic systems, findings which were ameliorated by pharmacological intervention with the NA-precursor L-threo-DOPS (Heneka et al., 2010). More recent studies on 3 month old APP/PS1-tg mice demonstrate that DSP4-induced degeneration of the LC leads to a severe diminution in coerulean and cortical NAT expression levels commencing as early as 4.5 months, findings which were accompanied by enhanced cerebral amyloidosis and spatial memory deficits at 6.5 months of age relative to APP/PS1 mice with intact NAergic systems, highlighting an aggravating effect of noradrenergic deficiency on amyloidosis and cognitive dysfunction over time (Jardanhazi-Kurutz et al., 2010). Moreover, further studies demonstrating the pathological and cognitive effects of NA deficiency in AD models show that at 9 months of age, chronic DSP4 treatment (5 mg/kg i.p. every 2 weeks) leads to a 5-fold increase in cortical Aβ plaque load within APP-tg mice (Kalinin et al., 2007) and that $D\beta H^{-/-}$/APP/PS1 double mutant mice lacking NA, exhibit exacerbations in spatial memory deficits relative to APP/PS1 mice with normal NAergic systems (Hammerschmidt et al., 2013).

Lesioning of the LC-NAergic system lowers basal levels of the NF-κB inhibitory proteins IκBα & IκBβ and cortical HSP70 expression, leading to an ensuing precipitation in $A\beta_{1\text{-}42}$ induced iNOS and microglial IL-1β expression within the frontal cortex of DSP4 (2 x 50 µg/kg i.p.) treated rats (Heneka et al., 2003). The same study shows that co-injection of peroxisome proliferator activated receptor (PPARγ) agonists restored IκBα and IκBβ expression and concurrently reduced cortical inflammatory responses to aggregated Aβ. These findings are consistent with studies demonstrating that pre-treatment of primary rat cortical neurons with NA (10 µmol/L) attenuates Aβ-induced neurotoxicity whilst concomitantly raising glutathione production, but not in the presence of a PPAR antagonist (Madrigal et al., 2007). Moreover, similar to studies showing that NA increases astrocytic IκB expression, β_2-agonists have previously been shown to exert their anti-inflammatory effects via the IκB/NF-κB pathway *in vitro*, and inhibit LPS induced TNF-α and IL-8 production from human pro-monocytic THP-1 cells (Farmer and Pugin, 2000c). More recent findings have demonstrated that submicromolar concentrations of NA suppress microglial IBA-1 up-regulation in response to LPS in a β_2-AR-independent fashion via the inhibition of NADPH oxidase-mediated superoxide production, highlighting microglial NADPH oxidase as a potential target in mediating the extra-synaptic anti-inflammatory actions of NA in maintaining brain immune homeostasis (Jiang et al., 2015).

1.8 Targeting the CNS noradrenergic system for anti-inflammatory and neurotrophic effects to combat neurodegeneration; insights From the current literature

Noradrenaline is hypothesised to play a bi-modal neuroprotective role in the brain in a variety of neurodegenerative disease states via its interactions with glial cells, particularly by down-regulating microglial pro-inflammatory gene expression (Dello Russo et al., 2004b; Jiang et al., 2015), and also by promoting a neurotrophic effect in the brain via astrocytic growth factor production in a β-adrenergic dependent manner (Culmsee et al., 1999a; Culmsee et al., 1999b). Pre-treatment with NA protects primary rat hippocampal cells against $A\beta_{1\text{-}42}$ and $A\beta_{25\text{-}35}$ mediated toxicity via the production of NGF and BDNF downstream of β-adrenergic signalling (Counts and Mufson, 2010). Moreover, in the multiple sclerosis field, CNS noradrenaline deficiency exacerbates EAE, whereas dual treatment with the noradrenaline reuptake inhibitor (NRI) atomoxetine (20 mg/kg i.p.) and L-threo-DOPS (400 mg/kg s.c.) administered three times weekly improves EAE clinical scores in NA-depleted mice (Simonini et al., 2010). Similarly, the SNRI venlafaxine suppresses CD3, CD8, IL-12 p40, TNF-α, IFN-γ, CCL2 and RANTES gene transcripts in the CNS lesions of an experimental adoptive myelin-specific T-cell model of EAE, whilst concomitantly upregulating BDNF expression in the inflamed spinal cord of these animals (Vollmar et al., 2009).

The severe loss of noradrenergic cell bodies (up to 80%) in the locus coeruleus in the early stages of Parkinson's diseae progression, even before the clinical manifestation of motor impairments in human patients therefore, is likely to have a major effect on disease progression. Forfeiting the innate immunomodulatory potential of noradrenaline in vulnerable projection areas such as the midbrain, which is densely populated with activated microglia, particularly in the vicinity of degenerating nigral dopamine neurons, is likely to facilitate uncontrolled pro-inflammatory reactions that contribute to the neurodegenerative processes that occur along the nigrostriatal tract in Parkinson's disease. Similarly, the relative paucity of nigral astrcoytes lends to a depleted cellular source of neurotrophic support to damaged/dying dopamine neurons. The greater the extent of LC cell loss, the greater the magnitude of noradrenaline depletion in the SN and striatum, which in turn restricts adequate *de novo* growth factor synthesis and release and promotes this brain region as a hotspot for dopaminergic neurodegeneration. As of such, pharmacologically augmenting central noradrenaline bioavailability, or mimicking its endogenous effects on β_2-adrenoceptors for example could restore the immunomodulatory and neurotrophic deficit and forstall or prevent further neurodegeneration.

In the Parkinson's disease field, pre-treatment with submicromolar concentrations of noradrenaline attenuates LPS-induced microglial activation, and NADPH oxidase-derived superoxide production and nitric oxide release from rat primary mesencephalic neuron/glia cultures (Jiang et al., 2015). Moreover, either genetic deletion of the noradrenaline transporter (NAT) or pharmacological blockade with the NRI nisoxetine (2 x 5mg/kg i.p.) confers neuroprotection against MPTP-induced Parkinsonism in mice (Rommelfanger et al.,

2004). In line with this, the α_2-adrenoceptor agonists clonidine and UK 14304 inhibit apomorphine-induced rotational asymmetry in response to unilateral medial forebrain bundle infusion of 6-OHDA (Chopin et al., 1999). In contrast, the blockade of α_2-adrenoceptors exerts an ameliorative effect on L-DOPA-induced dyskinesia's in MPTP Parkinsonian mice and nonhuman primates (Grondin et al., 2000a; Archer and Fredriksson, 2003). Similarly, the neuroprotective effect of 28-day treatment with the α2 adrenoceptor antagonist dexefaroxan (0.63 mg/kg i.p.) on devascularisation-induced degeneration of cholinergic neurons in the nucleus basalis was coincident with persistent NGF production in areas surrounding the cortical infarct (Debeir et al., 2004). It is possible that dexefaroxan-induced α_2-AR blockade enhances noradrenergic tone, leading to the activation of postsynaptic astrocytic β-adrenoceptors and enhancement of their reactivity in response to brain injury (Junker et al., 2002), culminating towards amplified NGF production (Lu et al., 1991);(Semkova et al., 1996b) and the protection of basal forebrain cholinergic neurons. The selective serotonin-noradrenaline reuptake inhibitor (SNRI) venlafaxine has previously been reported to exert immunomodulatory effects in an astroglia-microglia co-culture model by suppressing astrocytic IL-6 and IFN-γ secretion and reversing the morphological phenotype of activated microglia to resting state (Vollmar et al., 2008).

Taken together these findings fit in line with the theory that noradrenaline exerts a biphasic neuroprotective role in the brain via the inhibition of pro-inflammatory mediator release and stimulating growth factor production from glial cells. Previous data from our laboratory has shown that stimulation of CNS β_2-adrenoceptors with clenbuterol (0.5 mg/kg i.p.) suppresses NfκB activity and ameliorates expression of the NfκB-inducible genes TNFα and ICAM-1 in response to central injection of bacterial LPS (1 μg/5μl; icv), whilst concurrently elevating the temporal expression of the NfκB-inhibitory protein IκBα (Ryan et al., 2013). Moreover, noradrenaline negatively regulates the IL-1 system in glial cells via upregulating IL-1Ra and the IL-1RII decoy receptor *in vitro* (McNamee et al., 2010a) and *in vivo* (McNamee et al., 2010c), and raises CNS expression levels of the broad spectrum anti-inflammatory cytokine IL-10 and its downstream signalling molecule SOCS-3 in a β-adrenoceptor-dependent manner (McNamee et al., 2010b). Similarly, enhancing noradrenergic tone ameliorates LPS (250 μg/kg; i.p.) induced increases in cortical IL-1β, TNF-α, iNOS, CD11b and CD40 gene expression (O'Sullivan et al., 2009), and decreases the elevated expression of the chemokines RANTES and IP-10, as well as the cell adhesion molecules ICAM-1 and VCAM-1 in the CNS following systemic inflammatory insult (O'Sullivan et al., 2010). Thus, both noradrenaline augmentation strategies and β_2-adrenoceptor stimulation drive an anti-inflammatory phenotype in the CNS and may be of great therapeutic value in conditions where inflammation contributes to neuropathology.

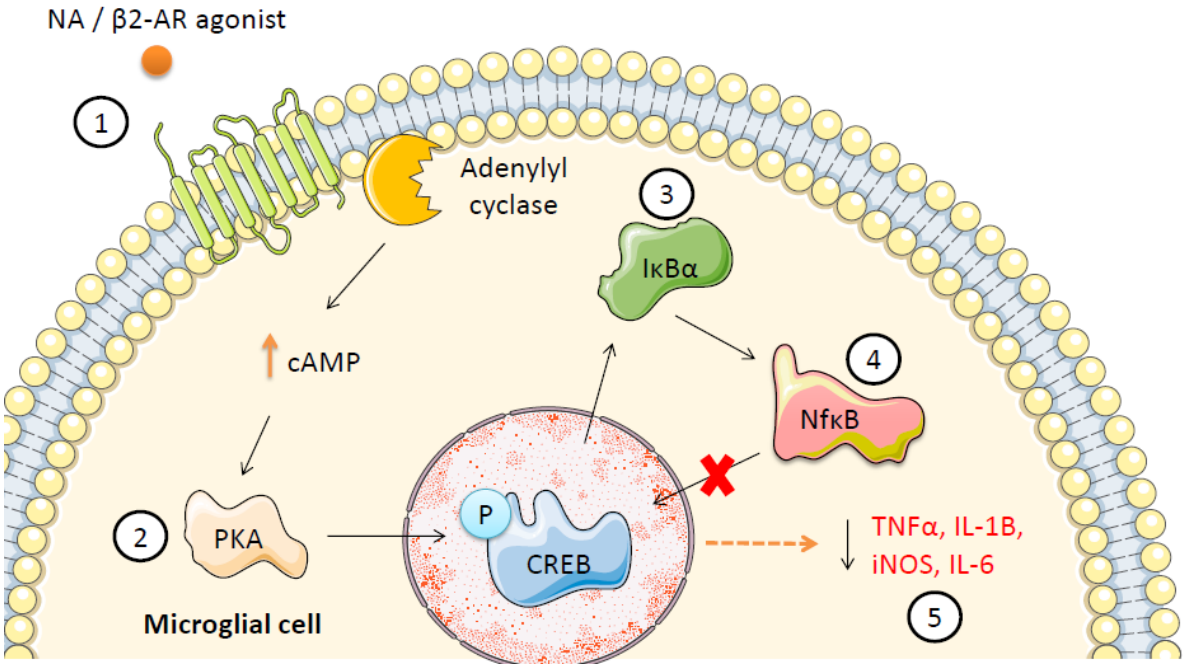


Figure 1.6 Proposed anti-inflammatory mechanism of action of noradrenaline / β_2-AR agonists on nigral microglia in the inflamed substantia nigra; a molecular signalling pathway towards neuroprotection. Stimulation of TLR4 receptors on microglia with LPS triggers a signalling cascade which leads to the phosphorylation and subsequent degradation of IκBα, NfκB nuclear translocation and ensuing increases in pro-inflammatory gene expression. In the current schematic, stimulation of the β2-AR with endogenous NA or with a β2-AR agonist (e.g. clenbuterol/formoterol) may suppress NFκB transcriptional activity via the following pathway: (1) Stimulation of glial β2-ARs activates adenylyl cyclase which raises intracellular cAMP leading to (2) activation of protein kinase A (PKA) which in turn phosphorylates CREB and (3) induces de novo synthesis of the NfκB inhibitory protein IκBα (and possibly preventing its phosphorylation), thus stabilizing cytosolic levels of IκBα which (4) inhibits transcriptional activity of NfκB by preventing its translocation into the nucleus, ultimately (5) decreasing pro-inflammatory gene expression.

1.9 Objectives of the thesis

Neuroinflammation is a major contributor to the progressive loss of dopamine neurons in Parkinson's disease. The use of non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen has been shown to lower the risk of Parkinson's disease in humans (Chen et al., 2005) and to provide neuroprotection in the MPTP mouse model of PD (Teismann and Ferger, 2001), thus indicating that anti-inflammatory pharmacological strategies could be implemeted to delay the onset and indeed progression of the disease.

The loss of noradrenergic cell bodies in the locus coeruleus is an early pathological feature of Parkinson's disease. Noradrenaline concentrations are subsequently depleted in multiple brain regions including the midbrain (containing the SN) and the striatum. Given the putative anti-inflammatory and neurotrophic role of noradrenaline via activation of β_2-adrenoceptors, it is thought that the degeneration of the LC-noradrenergic system could accelerate PD progression, whereas pharmacological intervention strategies aimed at enhancing extra-synaptic noradrenaline

bioavailability or mimicking its endogenous effects on β_2-AR's could provide neuroprotection and slow disease progression.

Studies examining the role of the noradrenergic system in Parkinson's disease has been relatively sparse in the literature to date, likely due to the prioritised attention attributed to the severe pathology along the nigrostriatal dopaminergic tract. The aim of the current project is to assess the anti-inflammatory & neuroprotective potential of enhancing noradrenergic tone (e.g. using the NRI atomoxetine) or targeting glial β_2-adrenoceptors directly (e.g. using the β_2-AR agonist formoterol) in the intra-nigral LPS model of Parkinson's disease.

The hypothesis is that intra-nigral LPS will induce robust microglial activation, dopaminergic neurodegeneration, nigrostriatal dopamine depletion and stable motor deficits in rats. Pharmacologically targeting the noradrenergic system however may inhibit microglial activation and attenuate the loss of dopamine neurons, potentially protecting against motor impairments. Specifically, the main objectives were:

(1) Establish and characterise an inflammatory-based rat model of Parkinson's disease induced by a unilateral intra-nigral injection of the potent inflammagen & TLR4 agonist lipopolysaccharide (LPS). Here we aim to assess the impact of LPS on motor function, glial cell activation and the integrity of the nigrostriatal dopaminergic system.

(2) Assess the role of astrocytes in contributing towards or protecting against LPS-induced PD-related neuropathology & motor dysfunction. Here we aim to assess the impact of astrocytic dysfunction on LPS-mediated neuroinflammation, neurodegeneration and motor deficits.

(3) Assess the impact of enhancing central noradrenergic tone to combat LPS-induced microglial activation and nigrostriatal neurodegeneration. Here we will investigate whether treatment with the noradrenaline reuptake inhibitor atomoxetine and/or the α_2-adrenoceptor antagonist idazoxan can elicit anti-inflammatory and neuroprotective effects in the intra-nigral LPS model of PD.

(4) Pharmacologically target glial β_2-AR's directly for anti-inflammatory and neurotrophic effects in the LPS model of PD. Here we will examine whether treatment with the β_2-AR agonist formoterol can either prevent or restore damage to the nigrostriatal dopaminergic system and attenuate LPS-mediated motor impairments.

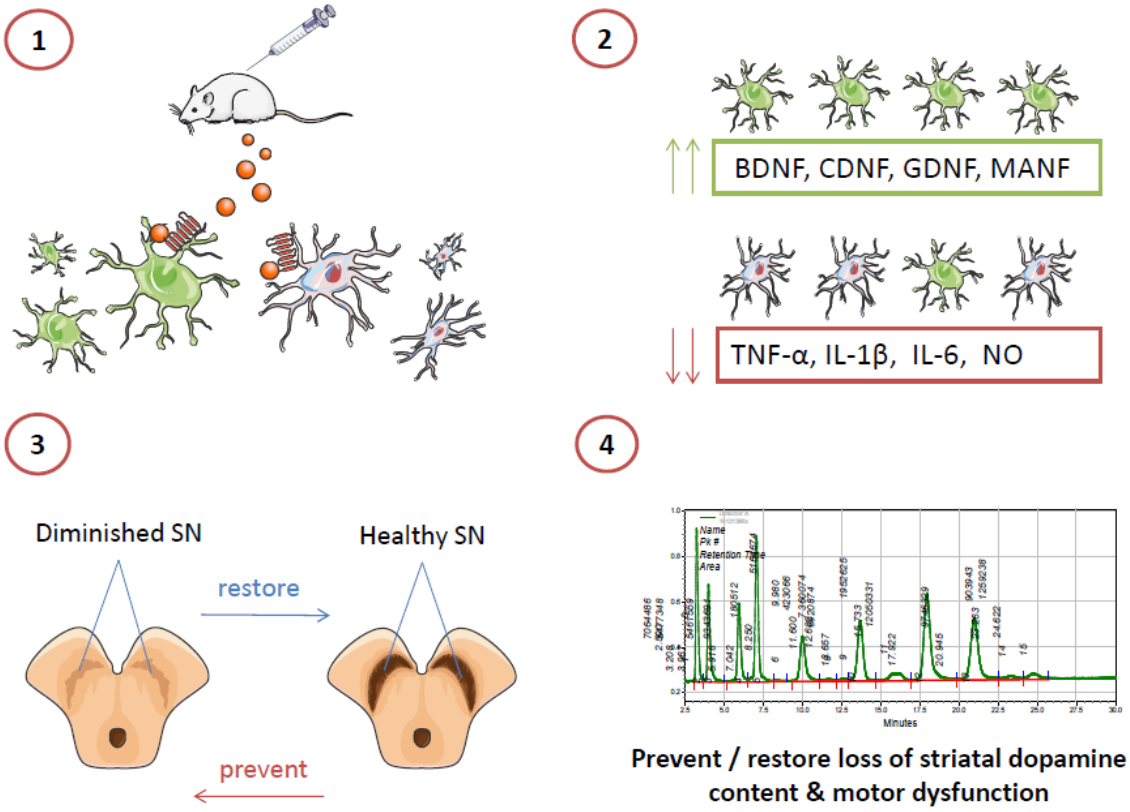


Figure 1.7 Envisaged steps towards neuroprotection (1) Treatment with an NRI (e.g. Atomoxetine) to enhance central NAergic tone or a β_2-AR agonist (e.g. Formoterol) to stimulate glial β_2-AR's directly (2) Promoting a neurotrophic response from CNS astrocytes and suppressing microglial activation & pro-inflammatory mediator production downstream from β_2-AR stimulation. (3) The production of anti-inflammatory mediators & trophic support to exert a neuroprotective effect and either restore or prevent damage to SNpc dopamine producing neurons. (4) Prevent / restore loss of striatal dopamine content & improve motor function.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Animals

Male Wistar Han rats, aged 6-8 weeks old	Comparative medicine, TCD
Standard rat chow	Comparative medicine, TCD

2.1.2 Stereotaxic surgery

Isoflo® (Isoflurane)	Comparative medicine, TCD
MSS-3 Isoflurane Vaporizer	MSS International Ltd.
Betadine	Medlock Medical, Ltd
EMLA cream	Comparative medicine, TCD
Xylocaine Spray (lidocaine)	Comparative medicine, TCD
LACRI-LUBE Eye ointment	Comparative medicine, TCD
Micro drill	
Dental drill bit (0.7 mm)	
Hamilton® Neuros (7002 KH SYR) syringe	Hamilton, Switzerland
Replacement needles (Neuros syringe NRS75 5.0 µl (33/20/3)	Hamilton, Switzerland
Tissue adhesive (Surgibond)	Comparative medicine, TCD

2.1.3 Behavioural testing

Staircase (Model 80300)	Campden Instruments, LTD.
Handycam (HDR CX330)	SONY®
Glass cylinder (20 cm diameter / 50 cm high)	
Syringe needles (26 G x 13 mm) (BD Ireland)	Comparative medicine, TCD
Syringes, plastic (1 ml) (BD Ireland)	Comparative medicine, TCD

2.1.4 Experimental treatments

Lipopolysaccharide (LPS) L2630-10MG (*Escherichia coli* 0111:B4)	Sigma-Aldrich, Ireland
L-alpha-aminoadipic acid (L-AAA) DL-2-Aminoadipic acid A0637-1G	Sigma-Aldrich, Ireland
Atomoxetine hydrochloride 100MG Cat HY-17385/CS-1085	Sigma-Aldrich, Ireland
Idazoxan hydrochloride (I6138-100MG)	Sigma-Aldrich, Ireland
Formoterol hemifumarate (Cat. No. 1448)	Tocris Bioscience, UK

2.1.5 Laboratory machines and equipment

HettichZentrifugenMikro® 22R refrigerated centrifuge	SORVALL®
HPLC with electrochemical detection (Shimadzu L-ECD-6 A)	SHIMADZU
Microscope digital camera (Olympus DP72)	OLYMPUS
Sonicator (Branson Sonifier150)	BRANSON
Perfusion pump	Gilson®

2.1.6 Laboratory chemicals and reagents

Absolute Ethanol (EtOH)	Hazmat, TCD
Chromium (III) potassium sulfate ($Kcr(SO_4)_2$)	Fisher Scientific, Ireland
Deionised water	TCIN
Ethylene Glycol	Sigma-Aldrich, Ireland
Gelatin	Sigma-Aldrich, Ireland
Hydrochloric acid (HCl)	Sigma-Aldrich, Ireland
Isopentane (2-methylbutane)	VWR, Ireland
Medical Oxygen (O_2)	BOC, Ireland
Methanol	Sigma-Aldrich, Ireland
Paraformaldehyde (PFA)	Sigma-Aldrich, Ireland
Sodium chloride (NaCl)	Sigma-Aldrich, Ireland
Sodium hydroxide (NaOH)	Sigma-Aldrich, Ireland
Sodium dihydrogen phosphate (NaH_2PO_4)	Sigma-Aldrich, Ireland

Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich, Ireland
Sucrose	Sigma-Aldrich, Ireland
Tissue-tek OCT compound	Sakura, Ireland
Urethane	Sigma-Aldrich, Ireland

2.1.7 Laboratory plastics and consumables

Collection tubes (15 ml)	
Coverslips (glass; 13 mm)	VWR International
Falcon tubes (15 ml & 50 ml), sterile	Sarstedt, Ireland
Eppendorf tubes (2 ml)	Sarstedt, Ireland
Glass coverslips 22 mm x 60 mm	Fisher Scientific, Ireland
Glass inserts	Fisher Scientific, Ireland
Glass screw top vials	Labquip Ltd., Ireland
Laboratory roll	Housekeeping, TCD
Microscope slides	Fisher Chemical, U.K
Microtubes (2 ml)	Sarstedt, Ireland
Microtome blades (c35 type)	Lab. Instr. & Supply, Ireland
Parafilm laboratory rolls	Sarstedt, Ireland
Pasteur pipettes (3.5 ml)	Sarstedt, Ireland
Pipette tips (10µl, 200µl, 1000µl), filter and non-filter	Sarstedt, Ireland
Petri dishes (92 mm)	Sarstedt, Ireland
Scalpels, disposable, sterile (Swann-Morton)	Fisher Scientific, Ireland

2.1.8 HPLC reagents

1-3-4-Dihydroxyphenylamine (L-DOPA)	Sigma-Aldrich, Ireland
3,4-Dihydroxyphenyl-acetic acid (DOPAC)	Sigma-Aldrich, Ireland
5-Hydroxyindole-3-acetic acid (5-HIAA)	Sigma-Aldrich, Ireland
Citric acid monohydrate (0.1M)	Sigma-Aldrich, Ireland
Dopamine (DA)	Sigma-Aldrich, Ireland
Ethylene diaminetetraacetic acid disodium (0.1 M)	Sigma-Aldrich, Ireland
Homovanillic acid (HVA)	Sigma-Aldrich, Ireland
HPLC Grade methanol	Fisher Scientific, Ireland
HPLC Grade water	Pharmacy, TCD
Methylated serotonin (N-Methyl-5-HT)	Sigma-Aldrich, Ireland

Noradrenaline (NA)	Sigma-Aldrich, Ireland
Octane-1-sulfonic acid (1.4 mM)	Sigma-Aldrich, Ireland
Serotonin (5-HT)	Sigma-Aldrich, Ireland
Sodium dihydrogen phosphate (0.1 M)	Sigma-Aldrich, Ireland
Sodium Hydroxide (NaOH)	Sigma-Aldrich, Ireland

2.1.9 Immunohistochemistry

3,3'-diaminobenzidine (DAB)	Sigma-Aldrich, Ireland
Anti-Iba1 (ab5076; polyclonal goat)	Abcam, UK
Anti-tyrosine hydroxylase (AB152; polyclonal rabbit)	Millipore, UK
Anti-tyrosine hydroxylase (MAB318; monoclonal mouse)	Millipore, UK
Anti-NOS2 (iNOS) (sc-651; polyclonal rabbit)	Santa Cruz Biotechnology
Anti-Nitrotyrosine (sc-32757; monoclonal mouse)	Santa Cruz Biotechnology
Anti-Glial fibrillary acidic protein (Z0334; polyclonal rabbit)	Agilent technologies, UK
Anti-IL-1β (ab9722; polyclonal rabbit)	Abcam, UK
Anti-S100B (ab868; polyclonal rabbit)	Abcam, UK
DPX mounting medium	Fisher Scientific, Ireland
Elite Vectastain© ABC kit (containing Mouse IgG, Rabbit IgG or Goat IgG secondary antibodies)	Vector Laboratories, UK
Alexa Fluor™ 488 goat anti-rabbit (A11008)	Invitrogen
Hydrogen peroxide (30%)	Sigma-Aldrich, Ireland
Normal Goat Serum (NGS)	Sigma-Aldrich, Ireland
Normal Horse Serum (NHS)	Sigma-Aldrich, Ireland
Normal Rabbit Serum (NRS)	Sigma-Aldrich, Ireland
Triton X-100	Sigma-Aldrich, Ireland
Xylenes	Sigma-Aldrich, Ireland

2.2 Methods

2.2.1 Animals

Male Wistar rats, aged 6-8 weeks (200-250g) adequately sized to fit into the stereotactic apparatus were obtained from the Comparative Medicine Unit (CMU, TCD) and allowed to habituate to the animal housing facilities for at least 1 week prior to any experimental procedures. There is a higher incidence of Parkinson's disease in men than in women (approximately 2:1 ratio), hence we decide to use male rats exclusively. Animals were housed in groups of 2 per cage in hard-bottomed polypropylene cages with stainless steel wire tops and wood shaving used as bedding. Animals were kept in climate-controlled rooms (21^{o}C with relative humidity levels of 50%), on a 12:12 hour light/dark cycle (lights on at 08:00). Throughout the experiment animals were allowed food and water *ad libitum.* All behavioral testing was conducted between 09:00 and 18:00. The experimental protocols involved were in compliance with the European directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2.2 Stereotaxic surgery

All surgical procedures were performed using aseptic technique under isoflurane anesthesia (induction at 5% and maintenance at 2%) in 1.5 L/min O_2. Briefly, rat heads were shaved, they placed in a stereotactic frame, and betadine anti-septic was applied to the incision area. The pre-operative analgesic EMLA cream or the topical anaesthetic xylocaine (lidocaine) spray was applied on to the incision area and Lacrilube was applied to their eyelids to minimize dryness during surgical procedures. Once under the sufficient plane of anesthesia, a small midline incision was made, the connective tissue removed and a burr hole was drilled in the appropriate location using bregma as a coordinate reference. The substantia nigra (SN) received either LPS (10 µg/2 µl; 0.89% sterile saline O111:B4 purified by phenol extraction) or vehicle (2 µl sterile saline). The coordinates for SNpc infusion were as follows: anteroposterior (AP) -5.3 mm, mediolateral (ML) ± 2.0 mm and dorsoventral (DV) -8.5 mm. For all injections a Hamilton® Neuros syringe was used and LPS or vehicle solution (0.89% sterile saline) were delivered at a rate of 1 µl/min. The needle was left in place for a further 4 minutes to allow diffusion of LPS or vehicle into the targeted brain region before being slowly withdrawn over a course of 1-2 min to limit efflux up the needle tract. Post-surgical procedures, their wounds were sealed with surgibond tissue adhesive and each rat was single-housed temporarily to aid recovery from anesthesia. The injection site was confirmed post-mortem via visualization of the needle injection tract from dorsoventral (DV) -8.5 mm whilst the SN was serially sectioned on the cryostat.

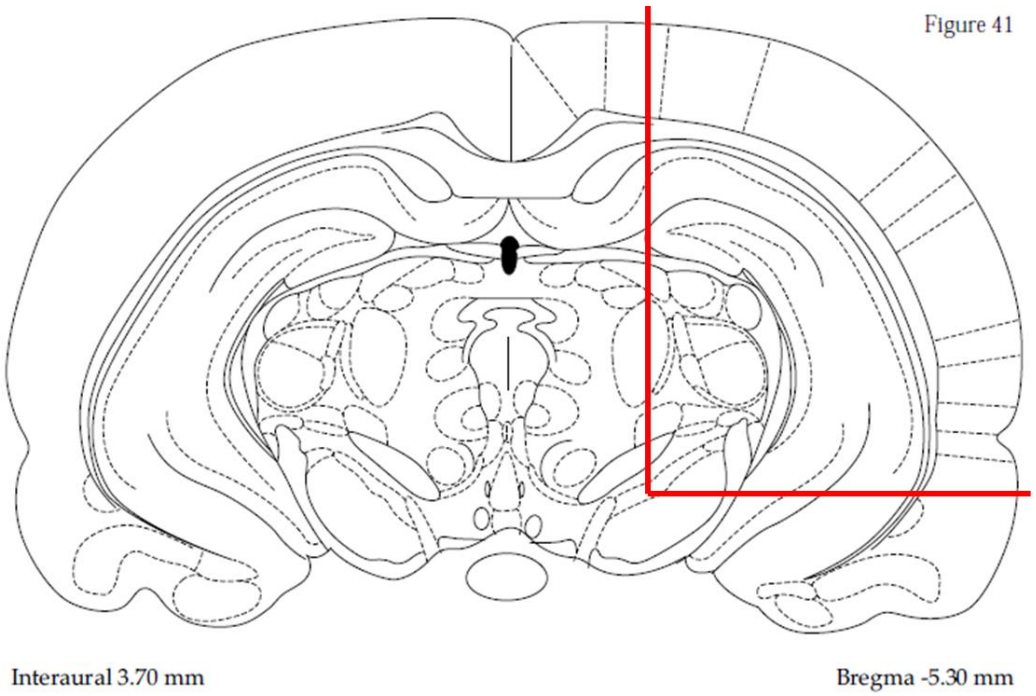


Figure 2.1. Schematic of unilateral intra-nigral injection of LPS (10 µg/2 µl; 0.89% sterile saline) or vehicle (2 µl 0.89% sterile saline). Co-ordinates for unilateral intra-nigral LPS or vehicle injection in the SNpc: AP -5.3, ML ±2.2, DV -8.2, using bregma as a stereotactic coordinate reference.

2.2.3 Drug preparation

All drugs were injected intraperitoneally in an injection volume of 1ml/kg. Atomoxetine (3mg/kg i.p.) and Idazoxan (1mg/kg i.p.) were prepared in 0.89% sterile saline solution. Formoterol hemifumerate was prepared in 1% DMSO. All drugs were prepared freshly on the day and kept in dark containers to maintain potency.

2.2.4 Behavioral testing

Rats underwent multiple rounds of behavioural testing in order to assess motor function following intra-nigral injection of LPS or sterile saline (vehicle control). The first session recorded baseline motor skills and confirmed adequate skilled forepaw performance in the staircase test (6 days prior to surgery), and then at least two rounds of post-lesion testing (days 7 & 14) to demonstrate the impact of lesioning the nigrostriatal tract with LPS/vehicle on three different aspects of motor function: skilled motor function (staircase test), forelimb kinesis (stepping test) & forelimb use asymmetry (cylinder test).

2.2.4.1 Staircase test

The staircase test was conducted as a measurement of skilled motor function. The apparatus consists of (1) an entrance chamber (20 cm long x 11.5 cm wide x 11 cm high) with a clear Plexiglas hinged lid and a series of adjacent metal bars for a floor, (2) a reaching chamber adjoining the entrance chamber (16.5 cm long x 6 cm wide x 11 cm high) made of clear Plexiglas, (3) a central platform within the reaching chamber (16.5 cm long x 2.5 cm wide x 5.5 cm high), and (4) a staircase of 7 steps on either side of the platform. On each step there was a shallow groove in which two pellets (Coco Pops®) were placed. The number of retrievals was determined by counting the number of food pellets remaining at the end of the trial. Additionally ipsilateral and contralateral retrievals were recorded for later analysis of forelimb function. These films were analyzed to determine three parameters; (1) the number of successful reaching attempts made, (2) the number of unsuccessful attempts made and (3) the number of pellets eaten using the tongue. Following this data collection the 'success rate' was calculated for each limb. The success rate was taken to be the number of successful reaching attempts expressed as a percentage of total attempts made. The total number of attempts was determined by adding the number of successful and unsuccessful attempts and subtracting the number of pellets eaten using the tongue.

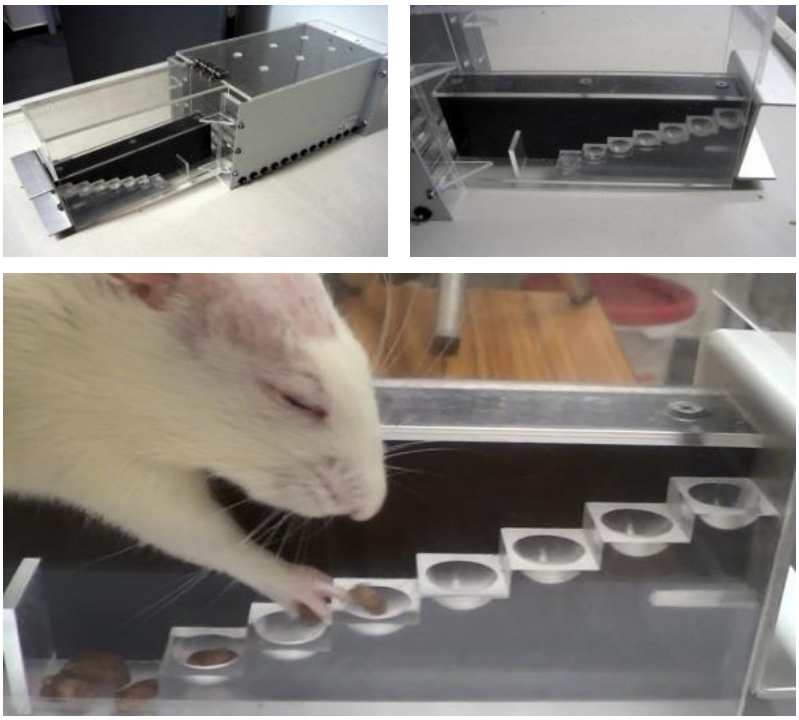


Figure 2.2. Staircase test of skilled motor function. Rats were placed in the staircase test apparatus and their behaviour was video-recorded for 10 minutes. The contralateral limb success rate was taken to be the number of successful reaching attempts expressed as a percentage of total attempts made.

2.2.4.2 Stepping test

The stepping test was used as a measurement of forelimb akinesia. Rats were held by an experimenter with both hands, immobilizing 3 limbs of the animal (2 hind limbs and 1 forelimb) leaving the remaining forelimb unrestrained. Holding the rat perpendicular to the tables' edge, with the free paw touching the table, the rat was directed sideways (90cm in 5 seconds) and the number of adjusting steps making contact with the tabletop was recorded in both the forehand and backhand direction, for each limb. On test day each rat was given one practice trial in both directions for each forelimb. Additionally, rats underwent habituation to the handling technique each day for 3 days prior to the first test session. The number of steps made by the contralateral limb was expressed as a percentage of the total number of steps made by both limbs in that direction.

Figure 2.3 Stepping test of forelimb akinesia. Rats were held by an experimenter (with both hind limbs and one forelimb restrained) and directed across a table top with the unrestrained forelimb in contact with the surface of the table. The number of adjusting steps made in the forehand & backhand direction was recorded for each limb. As an indicator of forelimb kinesis, the number of steps made by the contralateral limb was expressed as a percentage of the total number of steps made by both limbs in that direction.

2.2.4.3 Cylinder test

The cylinder test was used as a test for forelimb use asymmetry. Rats were placed in a clear glass cylinder (18.5 cm diameter x 50 cm high) for 5 min. The cylinder is sufficiently high to prevent the rats from reaching the top and wide enough to allow an approximate 2 cm gap from the base of the tail and the cylinder wall when the rat is on all fours. When placed in the cylinder, rats will engage in exploratory behavior and rear, making contact with the wall of the cylinder with their forepaws. Two observers recorded the number of wall and floor placements upon rearing and subsequent landing. A wall placement was considered to be the first contact with the wall of the cylinder after rearing and a floor placement was considered to be the first contact with the floor after a wall placement. Each placement was recorded as either 'left limb', 'right limb' or 'simultaneous', if both paws contacted at the same time. The number of contralateral limb placements were expressed as a percentage of total placements for both wall and floor contacts on the cylinder. Additionally, rats were excluded from this test if they failed to rear more than 5 times.

Figure 2.4. Cylinder test of asymmetric limb use. Rats were placed in the cylinder and exploratory behaviour was monitored for 5 minutes. To assess forelimb use asymmetry, the number of contralateral limb placements was expressed as a percentage of total placements for both wall and floor contacts on the cylinder.

2.2.4.4 Amphetamine-induced rotation test

The amphetamine-induced rotation test was conducted as an indirect behavioural measure to assess the extent of a dopaminergic lesion. At 2 weeks post-surgical procedures, rats were injected with d-amphetamine (5 mg/kg i.p.) and placed in their home cage (55 cm long x 35 cm wide x 25 cm high). The experimenter then recorded their rotational behaviour for 40 minutes, commencing 5 minutes after the d-amphetamine administration. Results are expressed as ipsilateral and contralateral net turns per minute.

Figure 2.5 Amphetamine-induced rotation test. Rats were injected with d-amphetamine (5 mg/kg i.p.) and their home cage activity was recorded for 40 minutes. The extent of ipsiversive rotational behaviour towards the lesioned hemisphere in response to amphetamine challenge is taken as an indirect measure of the extent of a dopaminergic lesion.

2.2.5 Biogenic amine analysis by High Performance Liquid Chromatography coupled to electrochemical detection (HPLC-ECD)

2.2.5.1 Preparation of HPLC mobile phase and standards

HPLC mobile phase buffer (0.1M citric acid monohydrate, 0.1M sodium dihydrogen phosphate, 1.4mM octane-1-sulfonic acid, 0.1M ethylene diaminetetraacetic acid disodium and 10% (v/v) methanol) was prepared using HPLC grade water. The pH was adjusted to 2.8 by the addition of 5M NaOH. Neurochemical standards of the biogenic amines were also prepared as standard calibration points and to assess retention times. A solution consisting of standard amines noradrenaline (NA), 1,3,4- dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenethylamine (DA), 5-hydroxyindole-3- acetic acid (5-HIAA), homovanillic acid (HVA) 5-hydroxytryptamine (5-HT) and N-methyl-5-HT (internal standard) were individually dissolved in 10ml of HPLC buffer to produce 10 mg/ml concentrations. These were further diluted in mobile phase buffer to yield a 10ml standard amine mixture containing 2ng/20µl of each monoamine.

2.2.5.2 Tissue Preparation for HPLC

Animals were sacrificed 15 days following stereotactic surgery. Brains were quickly removed and the striatum and midbrain (containing the SN) were hand-dissected on dry ice using Palkovits and Brownstein brain atlas for reference. Samples were weighed and transferred to 1 ml eppendorf tubes containing 500µl of ice cold homogenizing buffer (HPLC mobile phase containing 2 ng/ 20µl N-methyl-5-HT) before being homogenized by sonification, centrifuged (15,000 rpm @ 20 min at 4°C) and frozen at -80^{o}C.

2.2.5.3 HPLC analysis of rat brain biogenic amines

100µl of supernatant from each sample was transferred to glass HPLC assay vials and placed into a HPLC system consisting of a high pressure isocratic pump, sample auto-injector valve and a C18 reverse phase column coupled to an electrochemical detector. 10µl sample was auto-injected and pumped through the column at a flow rate of 0.8 ml/min. A standard mixture of amines was run after every fourth sample to recalibrate the system and minimize any drift that occurred in amine retention times during sampling. Neurotransmitter concentrations were quantified by electrochemical detection and resulting chromatograms were generated using a Merck Hitachi D-2000 integrator. Inclusion of the internal standard in each sample allowed for correction of processing losses. These data, together with the brain tissue weights, were used to calculate the concentration of neurotransmitter in each sample. Results are expressed in terms of neurotransmitter (ng) per wet weight of tissue (g).

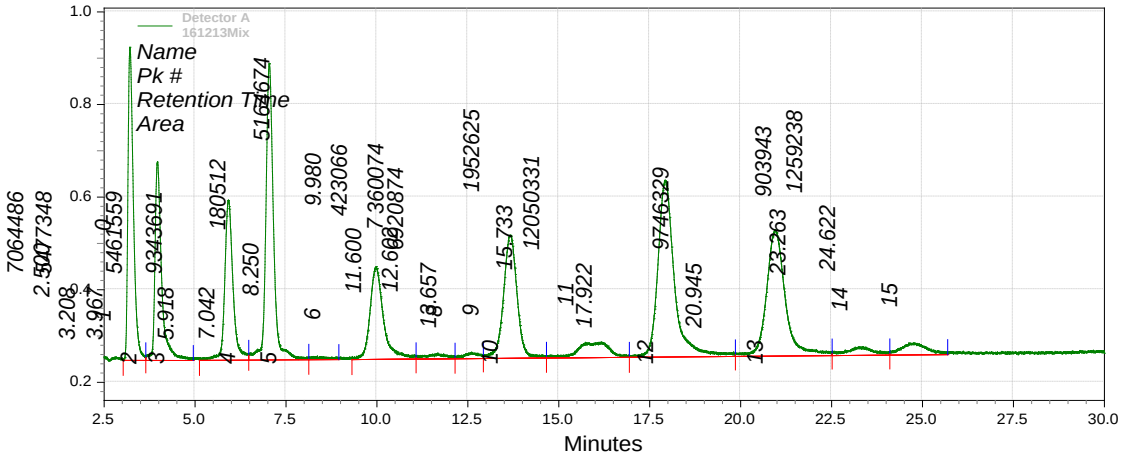


Figure 2.6. Sample chromatogram of retention times and peak heights from a standard mix of biogenic amines and their metabolites. Monoamine retention times from left to right are as follows: Noradrenaline (NA) (3.21 min), L-DOPA (3.97 min), DOPAC (5.92 min), Dopamine (DA) (7.04 min), 5-HIAA (9.98 min), HVA (13.66 min), 5-HT (17.92 min), N-methyl-5-HT (20.95 min).

2.2.6 Immunohistochemistry

2.2.6.1 Tissue processing for immunostaining

Rats were terminally anaesthetized with urethane (5.6M). A thoracotomy was performed to expose the beating heart and an incision was made in the right ventricle. The left ventricle was pierced with a needle which was attached to the tubing of the perfusion pump and clamped in place. The rat was transcardially perfused with ice-cold PBS for 10 min followed by 4% paraformaldehyde (PFA) for 10 min. Once perfusion was complete, animals were decapitated, brains removed and kept overnight in 4% PFA (4°C) before being cryoprotected in 30% sucrose solution for 72 h (4 °C). Brains were then quickly frozen in isopentane on dry-ice and stored at -80°C. On the day of slicing, brains were embedded in O.C.T and 30 µm coronal sections through the SNpc and striatum were cut using a cryostat. Nigral and striatal tissue were placed into collection tubes containing freezing solution (75 g sucrose, 75 ml ethylene glycol made up to 250 ml with PBS) and then stored at -80°C.

2.2.6.2 Immunohistochemical analysis

On the day of staining, free-floating slices were initially rinsed 3 times in PBS for 5min per wash and then incubated in 3% H_2O_2 in 20% MeOH for 20min at room temperature (RT) to block endogenous peroxidase activity. A summary of the different immunohistochemistry protocols are outlined below (primary antibody dilutions were ascertained from recommended dilution factors obtained from individual datasheets provided by manufacturer online):

Pre-treatment	Blocking agent	Primary Ab	Dilution	Secondary Ab
5% Triton (20min @ RT)	10% NHS (45min @ RT)	Mouse anti-TH	1/2000	Biotinylated Horse anti-mouse
5% Triton (20min @ RT)	10% NRS (45min @ RT)	Goat anti-Iba1	1/2000	Biotinylated Rabbit anti-goat
2% Triton (20min @ RT)	10% NGS (45min @ RT)	Rabbit anti-GFAP	1/1000	Alexa 488 Goat anti-rabbit
0.1% Triton	20% NGS (45min @ RT)	Rabbit anti-IL-1β	1/200	
2% Triton (20min @ RT)	20% NGS (45min @ RT)	Rabbit anti-S100B	1/1000	Alexa 488 Goat anti-rabbit
0.3% Triton (20min @ RT)	10% NGS (45min @ RT)	Rabbit anti-iNOS	1/300	Biotinylated Goat anti-rabbit
0.3% Triton (20min @ RT)	10% NHS (45min @ RT)	Mouse anti-3-NT	1/300	Biotinylated Horse anti-mouse

Table 2.1. Summary of IHC protocols.

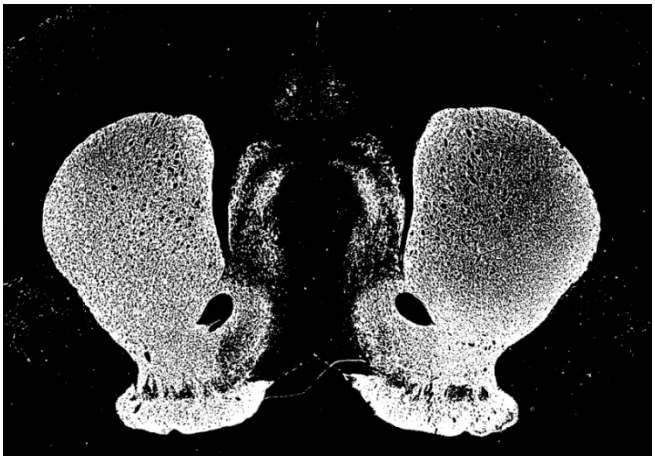
The Avidin biotin complex (ABC) method was used to visualise staining using peroxidase as enzyme, hydrogen peroxide as substrate and 3,3-Diaminobenzidine (DAB) as chromagen. The DAB reaction consisted of the following for free floating immunostaining: 1ml of DAB was diluted in 20ml of PBS and 6µl of 30% H_2O_2 was added last, just prior to commencing the DAB reaction. Slices were subsequently mounted onto gelatin-coated slides and left to dry overnight at RT. The next day slides were desalted in d.H_2O (2min), then dehydrated in a series of ascending alcohols 70% EtOH → 90% EtOH → 100% EtOH (2min each) and cleared in xylenes (1min). Coverslips were applied using DPX mounting medium and were left to dry overnight.

2.2.6.3 Image analysis

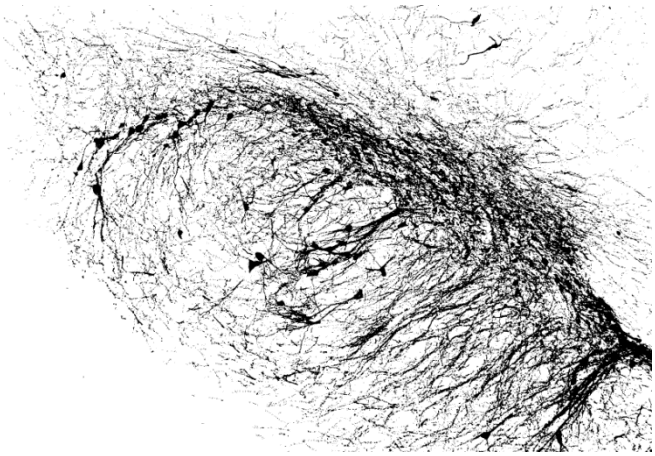
Images were taken using an Olympus DRP72 camera mounted on an Olympus BX51 normal light microscope and analyzed using Image J software (Image J v1.48x; NIH, US). Images of

the substantia nigra and striatum were taken at 1.25x, 10x, 20x or 40x magnification. Images were analysed using imagej software. The percentage area of TH^+ and $Iba1^+$ dopamine & microglial cells respectively were assessed in the SN (AP -4.80 mm to -6.04 mm from bregma) and striatum (AP 1.60 mm to -0.92 mm from bregma). Images were converted to 8-bit, a threshold of consistent value between the hemispheres and treatment groups was set to delineate positive staining from background staining and a border was drawn around the SNpc and striatum. The percentage area of TH-immunoreactivity (TH-ir) or iba1 immunoreactivity (Iba1-ir) was analysed following subtraction of non-stained background (pixel values which fell below that of the set threshold, see examples below).

(A)



(B)



(C)

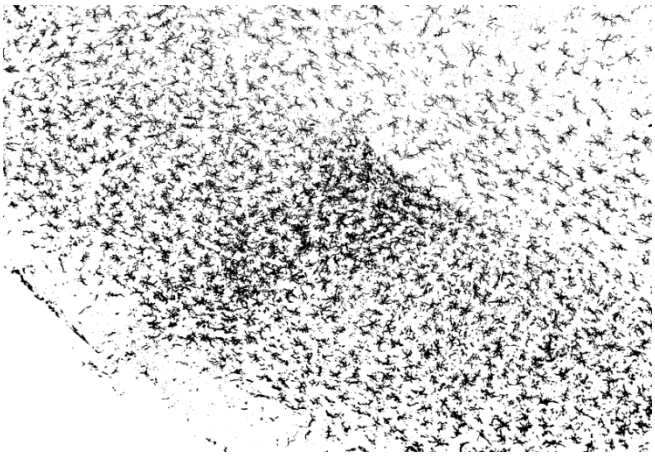


Figure 2.7. Immunohistochemical analysis of post mortem nigral & striatal tissue sections. Sample TH^+ striatal (A) & nigral (B) immunohistochemical analysis. Nigral $Iba1^+$ microglia are shown in (C).

2.2.7 Statistical analysis

Data was analysed using GraphPad prism 7 software. Statistical comparisons were performed using either a Student's *t*-test or analysis of variance (ANOVA) as indicated in the individual results chapters. If significant differences were observed following a Student's *t*-test or ANOVA, the data was further analysed using a Bonferroni *post-hoc* test or a Newman-Keuls test as in chapter 5. A value of $P<0.05$ was considered statistically significant. Results are expressed as mean ± standard error of the mean. The Bonferroni *post-hoc* test was used because it is a stringent multiple comparisons correction that should be used when testing multiple hypotheses; it's necessary because the more tests you run, the more likely it is that you will get a statistically significant result. The Bonferroni *post hoc* test limits the possibility of getting a statistically significant result when testing multiple hypotheses at the same time in an ANOVA, in other words it restricts the possibility of generating false positive results.

Chapter 3: Behavioural results were analysed by a two-way repeated measures ANOVA followed by a *post hoc* Bonferroni. Post-mortem immunohistochemistry and HPLC results were analysed by a student's two-tailed unpaired t-test and *post hoc* Bonferroni.

Chapter 4: Behavioural and immunohistochemistry results were analysed by three-way ANOVA with *post hoc* Bonferroni. HPLC results were analysed by two-way ANOVA with *post hoc* Bonferroni.

Chapter 5*: For behavioural results, data were analysed via a two-way repeated measures ANOVA followed by a *post hoc* Newman-Keuls test. HPLC results were analysed by a two-way ANOVA with *post hoc* Newman-Keuls. Immunohistochemistry results were analysed via a three-way ANOVA with *post hoc* Newman-Keuls. *A Newman-Keuls *post-hoc* test was utilised in this study due to a previously commenced peer-review process of the data using this multiple comparisons test prior to publication.

Chapter 6: Behavioural and immunohistochemical data were analysed via three-way ANOVA with *post hoc* Bonferroni.

Chapter 3

Modelling the pathophysiology of inflammatory-based Parkinsonism in the rat

3.1 Introduction

Inflammation is a defensive immune reaction against tissue injury or infection comprised of diverse cellular & molecular processes equipped for the removal and/or inactivation of noxious agents in order to limit their detrimental effects (Wyss-Coray and Mucke, 2002). The inflammatory process is phased by checkpoints of control based on the integration of molecular cues to escalate or suppress the immune commitment in response to tissue injury in a time & context-dependent manner (Nathan, 2002). Chronic neuro-inflammation is widely considered to be a major contributor in the pathogenesis of neurodegenerative disease (Qin et al., 2007a; Glass et al., 2010; Tansey and Goldberg, 2010) with brain resident glial cells (microglia & astrocytes) acting as cellular effectors of inflammation-mediated neurodegeneration (Block and Hong, 2005; Maragakis and Rothstein, 2006). In multiple CNS disease states, including Parkinson's disease, neuro-immune interactions between damaged/dying neurons and dysregulated over-activated microglia shape a self-perpetuating cycle of uncontrolled chronic inflammation which propels neurodegenerative disease progression (Gao and Hong, 2008).

Microglia are classified as brain resident macrophages of haematopoietic origin, constituting about 12% of cells in the human brain (& 5-20% of total rodent glial cells), existing as benign surveillants with dynamic effector functions in the healthy and pathologic CNS (Lawson et al., 1990; Hanisch and Kettenmann, 2007). Microglia constantly screen CNS tissue (Davalos et al., 2005a), and upon activation of these brain resident macrophages, transition from a ''resting'' ramified morphology to an amoeboid state accompanied by an upregulated script of cell surface molecules is observed (Cho et al., 2006), including major histocompatibility (MHC) molecules, CR3, CD80 + CD86, DC SIGN, CD14 (Ponomarev et al., 2005), chemokine receptors and several others, as reviewed in (Galea et al., 2007). Within this activated state, immunocompetent microglia may serve to prevent toxic debris accumulation and maintain neuronal viability (Streit, 2002; Simard et al., 2006) and enhance their survival via release of trophic factors (Morgan et al., 2004). Furthermore, microglia within the mature CNS influence neural precursor cell fate, play a causative role in annulling excess glutamate-induced neurotoxicity via GLT-1 mediated glutamate uptake (Persson et al., 2005), are effective mediators of repair by virtue of guiding migratory stem cells to sites of injury/inflammation via the release of soluble factors (Aarum et al., 2003) and are

reportedly involved in maintaining hippocampal neurogenesis (Walton et al., 2006; Ziv et al., 2006).

Thus, within this state, activated microglia carry out diverse duties essential for neuronal survival. In spite of this, microglial over-activation may lead to copious production of cytotoxic factors including nitric oxide (NO) (Liu et al., 2002), superoxide (Giulian et al., 1993) and TNF-α (Lee et al., 1993) which in turn holds deleterious neurotoxic consequences for neuronal populations (Block et al., 2007a). Prolonged activation of these glial cells leads to the unwarranted rapport of reactive microgliosis and self-perpetuating neurotoxicity, a hallmark feature of pathological inflammatory states in neurodegenerative disease (Qin et al., 2004; Kettenmann et al., 2011). Thus, there is a wide body of literature stapling inflammation as a major contributor to the pathogenesis of neurodegenerative disease, a process in which microglia are at the crux of the issue (Hanisch and Kettenmann, 2007; Saijo et al., 2013).

Toll-like receptor 4 (TLR4) is a member of the toll-like family of receptors that constitute an integral partition of the first line of innate immune defence against infectious disease. TLR4 recognises LPS from gram-negative bacterium (Poltorak et al., 1998) and is expressed on brain-resident microglia and to a lesser extent on astrocytes (Jack et al., 2005). TLR4-mediated signalling can induce potent inflammatory responses and is thought to be implicated in the pathogenesis of Parkinson's disease (Lu et al., 2008; Panaro et al., 2008). TLR4 expression is increased in peripheral immune cells and in the caudate/putamen of PD patients (Drouin-Ouellet et al., 2015). TLR4 is required for α-synuclein-mediated microglial activation, phagocytic activity, pro-inflammatory cytokine secretion and ROS production (Fellner et al., 2013). Moreover, $TLR4^{-/-}$ animals are less vulnerable to MPTP-induced striatal dopamine depletion (Conte et al., 2017), and are partially protected against MPTP-induced loss of dopamine neurons in the SNpc, findings which were associated with reduced numbers of nigral $Iba1^{+}$ and MHC II^{+} activated microglia, thus highlighting inflammation-mediated neurodegeneration as a partially TLR4-dependent paradigm in the Parkinsonian brain. Taken together, promoting TLR4 signalling in nigral microglia is a useful method to recapitulate glial-derived pro-inflammatory mediator production and dopaminergic neuropathology in experimental Parkinson's disease.

Lipopolysaccharide (LPS) is an endotoxin and TLR4 agonist derived from Gram-negative bacteria that has been previously used in rodents to study how inflammatory processes contribute to the pathogenesis of PD (Liu and Bing, 2011). Unilateral intra-nigral LPS (10µg in 2µl sterile saline) injection induces a robust ionized calcium binding adaptor molecule 1-positive ($Iba1^{+}$) localised nigral microgliosis leading to nigrostriatal degeneration over a 20 day period culminating in pronounced, stable contralateral motor impairments (Hoban et al., 2013). Intra-nigral injection of LPS (5µg in 2µl) upregulates microglial iNOS expression and leads to dopaminergic neurodegeneration in the SNpc, possibly due to the release of NO (Le et al., 2001) and/or its metabolites (Arimoto and Bing, 2003). Persistent glutathione (GSH) impairments have previously been reported in the SNpc when assessed at 1, 3 and 7

days post bilateral intra-nigral injection of LPS (2µg in 1µl), accopmpanied by microglial activation and delayed deficits in striatal DA content (Ariza et al., 2010b). Consistent with the LPS-induced reduction of GSH in the SNpc of these animals, Parkinsonian patients classically exhibit an approximate 40% depletion in nigral GSH content (Perry et al., 1982; Perry and Yong, 1986), which, under the premise that glutathione is a powerful natural antioxidant that detoxifies free-radicals and combats reactive oxygen species production, thus implies that LPS is triggering oxidative stress-mediated cell death of midbrain dopaminergic neurons. Moreover, when injected into the striatum, LPS (2.5 µg/µl) leads to progressive TH^+ dopaminergic cell loss in the ipsilateral SN, whilst depleting 58% of striatal DA content when assessed 4 weeks later (Choi et al., 2009). The authors further show that LPS-induced S-nitrosylation/nitration of mitochondrial complex I and subsequent respiratory impairment was implicated in the progressive degeneration of the nigrostriatal system. The surviving nigral DA neurons in this study exhibited intracytoplasmic α-synuclein & ubiquitin accumulation 4 weeks after intra-striatal LPS injection. Thus, given that neuroinflammation (Hirsch et al., 2012), mitochondrial impairment (Exner et al., 2012), α-synucleinopathy (Lo Bianco et al., 2002) and the selective loss of dopaminergic neurons (Xu et al., 2002) are salient features of the substantia nigra in Parkinson's disease, lesioning the nigrostriatal system with LPS promotes itself as an attractive method to model the pathophysiology of PD in rodents.

Studies by (Herrera et al., 2000) have highlighted the effects of LPS-driven inflammatory reactions on nigrostriatal integrity and glial cell activation in the rat. The authors have demonstrated that when assessed at 15 days, 3 months, 6 months and 1 year post intranigral LPS (2µg) injection, dopamine levels in the SN were decreased to 63%, 82%, 73% and 76% of the contralateral hemisphere respectively. Moreover striatal DA levels were depleted to approximately 63%, 67%, 67% and 58% of the contralateral hemisphere at these time-points. The authors show that the deficit in DA content was preceeded by TH^+ dopaminergic neuronal loss occurring as early as 4 days post intranigral injection of LPS, and was underpinned by extensive OX-42 microglial reactivity and a paucity in nigral $GFAP^+$ astrocytes. Interestingly, the LPS-induced neuroinflammatory damage to the nigrostriatal dopaminergic system did not revert after 1 year post lesioning, highlighting the sensitivity of the midbrain to LPS and the permanent effects of a local inflammatory stimulus on dopaminergic neurons. These results are likely to be congruent with the cytoarchitecture of the midbrain, as it has been shown to contain the highest density of microglial cells in the rodent brain (Lawson et al., 1990). Interestingly, stereotaxic injection of LPS (5 or 10µg in 2µl) into the hippocampus, cortex or substantia nigra of adult rats leads to neurodegeneration 7 days later in the SN only, and treatment of neuron-glia mesencephalic cultures with LPS (1µg/ml) for 72 hours drastically reduces $MAP\text{-}2^+$ neuronal counts whilst hippocamapal and cortical cultures remain insensitive to LPS insult at concentrations as high as 10 µg/ml (Kim et al., 2000). Moreover, the authors further demonstrate that the mesencephalic cultures contain 4- to 8-fold more microglia than that of other regions, and that supplementation of LPS-insensitive cortical neuronal-glial cultures with enriched

microglia derived from the mesencephalon rendered the cortical cultures sensitive to LPS-induced neurotoxicity, highlighting the differential susceptibility of brain regions to LPS based on the regional density of brain resident microglia.

From a genetic perspective, missense mutations in the leucine-rich repeat kinase 2 (LRRK2) gene have been implicated in late onset Parkinson's disease (Zimprich et al., 2004; West et al., 2005). Studies by (Daher et al., 2014) involving LRRK2 wildtype (WT) rats have demonstrated that an intra-nigral LPS injection drives a change in the morphological phenotype of Iba1-positive microglia (ramified → amoeboid) whilst provoking CD68-positive myeloid cell recruitment to the midbrain which reportedly correlated strongly with TH-immunopositive dopaminergic cell loss in the SNpc 2 weeks post intra-nigral LPS exposure. Interestingly, the same authors show that the observed LPS-induced microglial activation, neuroinflammatory response and overt dopaminergic cell loss was abrogated in LRRK2-deficient rats. Studies by (Waak et al., 2009) have shown that LPS-treated astrocytes deficient in the Parkinson's disease associated gene *Dj-1* produce >10 times more NO than wildtype astrocytes, findings which were coincident with iNOS hyperactivation, MAPK phosphorylation and exacerbations in cyclooxygenase-2 (COX-2) and interleukin-6 (IL-6) expression. Moreover, the same authors demonstrate that primary neurons grown on *Dj-1*-deficient astrocytes become apoptotic in response to LPS in an iNOS-dependent fashion, thus also highlighting the capabilitiy of reactive astrocytes in propagating inflammatory processes leading to neuronal loss, and a role for DJ-1 in regulating astrocytic inflammatory responses to immune challenge.

Taken together, these studies implicate TLR4-mediated neuroinflammatory events in the pathogenesis of Parkinson's disease, and highlights the intra-nigral LPS model as a clinically relevant approach to characterise how immune-mediated events can influence PD-related neuropathology and motor dysfunction. Henceforth, the establishment & characterisation of an inflammatory-driven experimental model of PD sets the premise for future preclinical studies investigating the role of glial cells in PD-related neuropathology & motor deficits, and is therefore of great experimental value in subsequently assessing the therapeutic efficacy of pharmacologiaclly targeting these glial cells for anti-inflammatory and potentially neuroprotective effects *in vivo*. As of such, we hypothesize that a unilateral intra-nigral injection of bacterial LPS will activate microglia and dopaminergic neurodegeneration eill ensue along the nigrostriatal tract, leading to depletion of striatal dopamine reserves and lateralised motor deficits, thereby conceiving the establishment of a rodent model of experimental hemiparkinsonism.

3.2.1 Study aims & objectives

The aim of this study was to establish and characterise an LPS-mediated inflammatory-based animal model of Parkinson's disease. We aimed to examine the effects of an intra-nigral LPS (10µg/2µl) injection at a behavioural, biochemical & immunohistochemical level in *vivo*. Our main objective was to assess the impact of a unilateral intra-nigral LPS injection on nigral microglia, dopamine neurons within the SNpc and their affiliated nerve terminals in the striatum, nigrostriatal dopamine concentrations, and lateralised motor function in rats. Specifically, we aimed to investigate whether the potential impact of a single unilateral intra-nigral LPS injection on glial cell activation and nigrostriatal integrity was capable of inducing motor dysfunction and PD-related neuropathology in order to construct an *in vivo* model in which potentially disease-modifying therapies can be subsequently tested in future preclinical studies (see schematic below illustrating the effect(s) of an inflammatory lesion on the nigrostriatal tract).

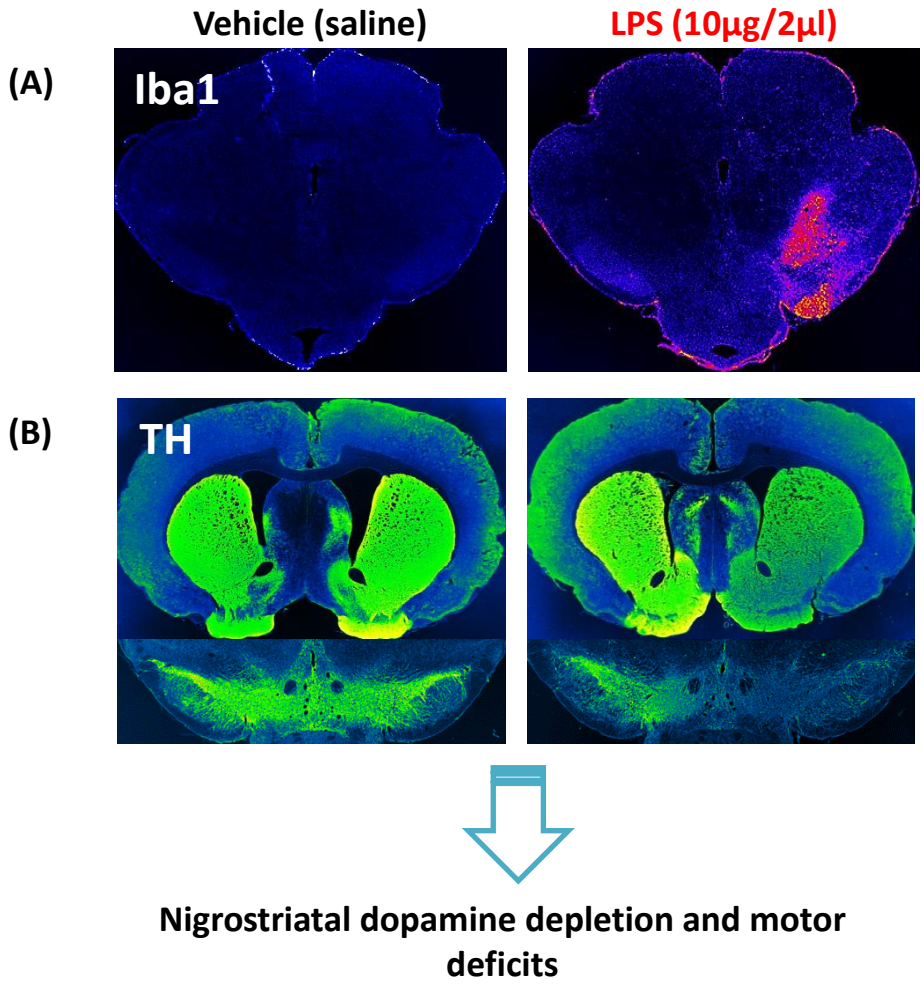


Figure 3.8 An LPS-induced microgliopathy within the substantia nigra sets the premise for nigrostriatal neurodegeneration, dopamine loss and motor dysfunction. Unilateral intra-nigral injection of bacterial LPS (10µg/2µl) induces robust microgliosis (A) dopaminergic neurodegeneration in the SNpc and affiliated nerve terminal loss in the striatum (B), culminating in nigrostriatal dopamine loss and associated motor deficits.

3.2.2 Experimental design

Adult male Wistar rats aged 6-8 weeks weighing 250-300g were habituated to the housing facilities for at least 1 week prior to commencing any experimental procedures. Rats received a minimum of 3 training sessions in the staircase test prior to recording baseline behaviour to establish competency. Rats received a stereotactic unilateral injection of LPS (10µg/2µl) into the substantia nigra a dose previously shown to induce microglial activation, dopaminergic neurodegeneration and stable motor deficits detectable at two-weeks (Hoban et al., 2013). Control animals received an intra-nigral injection of 2µl sterile saline (Vehicle). Behavioural testing in the staircase, stepping and cylinder tests was conducted 1 week prior to lesioning to assess baseline motor function and again thereafter at 7 and 14 days post-lesioning to assess the impact of an intra-nigral LPS injection on motor function. The following day rats were either euthanized by transcardial perfusion-fixation with 4% paraformaldehyde and their brains used for post mortem immunohistochemical analysis or hand dissected on dry ice in preparation for HPLC analysis of biogenic amine concentrations (see experimental timeline, below).

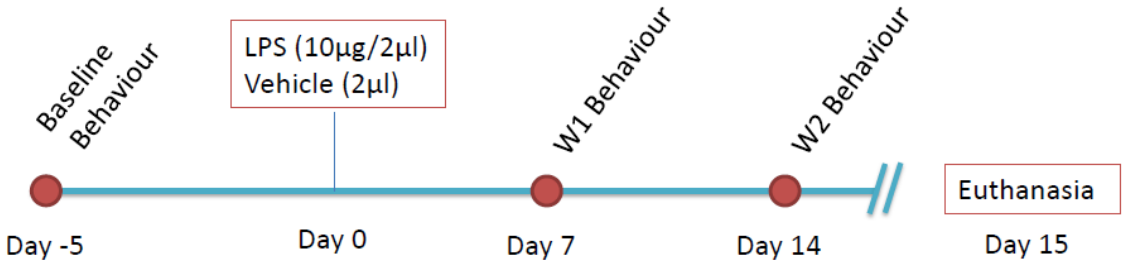


Figure 3.2 Experimental timeline of investigations. Rats underwent 3 rounds of behavioural testing in the staircase, stepping and cylinder tests at baseline & weeks 1 (day 7) and 2 (day 14) post-lesioning. Rats were euthanized 2 weeks post-surgical procedures (day 15) by transcardial perfusion fixation in preparation for immunohistochemistry (IHC) or by decapitation in preparation for high performance liquid chromatography with electrochemical detection (HPLC-ECD).

3.3 Results

3.3.1 Intra-nigral LPS administration induces lateralised deficits in skilled motor function in the staircase test.

The staircase test was used as a method to assess the impact of a unilateral intra-nigral LPS injection on skilled motor function. A two-way repeated measures ANOVA demonstrated an effect of an intra-nigral LPS lesion ($F_{(1,28)}$ = 42.02, P<0.0001), an effect of time ($F_{(2,28)}$ = 37.26, P<0.0001) and an interaction between LPS and time ($F_{(2,28)}$ = 20.74, P<0.0001) on the success rate of contralateral grasping ability in the staircase test. Bonferroni *post hoc* analysis revealed that LPS induced contralateral impairments in skilled motor function at both 7 (P < 0.001) and 13 (P < 0.001) days post lesioning by 31% and 36% on average respectively relative to baseline behavioural testing. LPS had no effect on the total number of food pellets eaten using the contralateral forelimb at any time-point tested.

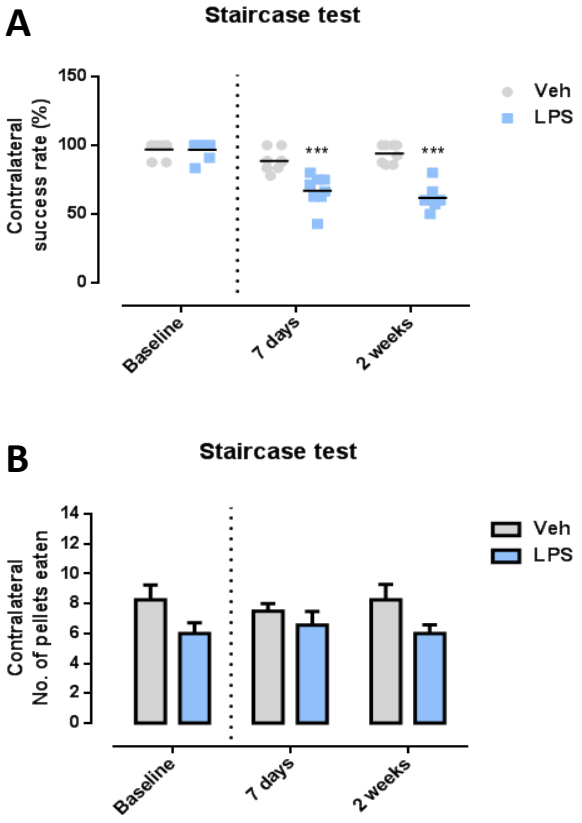


Figure 3.3. Intra-nigral LPS induces lateralised deficits in skilled motor function in the staircase test. The effect of an intra-nigral unilateral LPS injection on skilled motor function was assessed at 7 (W1) and 13 days (W2) post lesioning using the staircase test. Unilateral injection of LPS (10µg/2µl) into the substantia nigra induced stable deficits in contralateral skilled motor function at both 7 & 13 days post lesioning (A). LPS had no effect on the total number of contralateral food pellets eaten by any rat tested at either time-point (B). Data are expressed as mean ± S.E.M. (n=7-8) *** P<0.001 vs. vehicle by 2-way repeated measures ANOVA with *post hoc* Bonferroni.

3.3.2 Intra-nigral LPS induces forelimb akinesia in the stepping test.

The stepping test was performed to assess the impact of a unilateral intra-nigral LPS injection on forelimb kinesis. A two-way repeated measures ANOVA demonstrated an effect of LPS ($F_{(1,28)}$ = 7.565, P<0.0165) and an effect of time ($F_{(2,26)}$ = 5.384, P=0.0111) on forelimb akinesia. A Bonferroni *post hoc* test revealed that LPS reduced the number of contralateral adjusting steps made in the forehand direction by 29% on average at both 7 (P < 0.05) and 13 (P < 0.05) days post lesioning. Similarly, a two-way repeated measures ANOVA demonstrated an effect of LPS ($F_{(1.26)}$ = 5.934, P<0.0300) and time ($F_{(2,26)}$ = 7.370, P<0.0029) on forelimb kinetic behaviour in the backhand direction. A Bonferroni *post hoc* test revealed a reduction in contralateral adjusting steps was apparent by 26% on average in the backhand direction at 7 (P<0.05), but not significantly 13 days post lesioning with LPS.

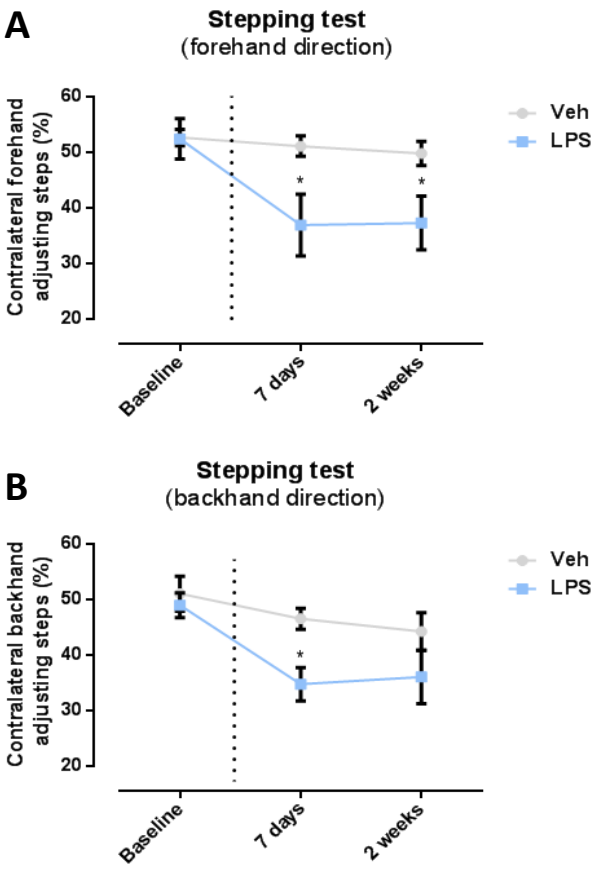


Figure 3.4 Intra-nigral LPS induces contralateral forelimb akinesia in the stepping test. The effect of a unilateral intra-nigral LPS injection on forelimb kinesis (the ability to initiate forelimb movement) was assessed in the forehand (A) & backhand (B) direction at 7 (W1) and 13 days (W2) post lesioning using the stepping test. Unilateral injection of LPS (10µg/2µl) into the substantia nigra induced akinetic behaviour in the forehand direction at both 7 and 13 days post lesioning. LPS also induced contralateral motor impairments in the backhand direction at 7, but not 13 days post lesioning. Data are expressed as mean ± S.E.M. (n=7-8) * P<0.05 vs. vehicle by 2-way repeated measures ANOVA with *post hoc* Bonferroni.

3.3.3 Intra-nigral LPS induces asymmetric limb use in the cylinder test.

The cylinder test was used to assess the impact of a unilateral intra-nigral LPS injection on forelimb use asymmetry. Two-way repeated measures ANOVA revealed an effect of lesioning with LPS ($F_{(1,26)}$ = 5.376, P=0.0374) and an interaction between LPS and time ($F_{(2,26)}$ = 4.038, P=0.0297) on forelimb use asymmetry in the cylinder test. Time alone had no effect on forelimb use preference in the cylinder test ($F_{(2,26)}$ = 3.301, P=0.0528). Bonferroni *post hoc* analysis revealed that LPS significantly reduced the number of contralateral forelimb wall placements at 7 (P<0.05), and at 13 days post lesioning by 43% and 44% on average respectively. LPS had no effect on forelimb use preference for floor placements at either time-point tested in the cylinder test.

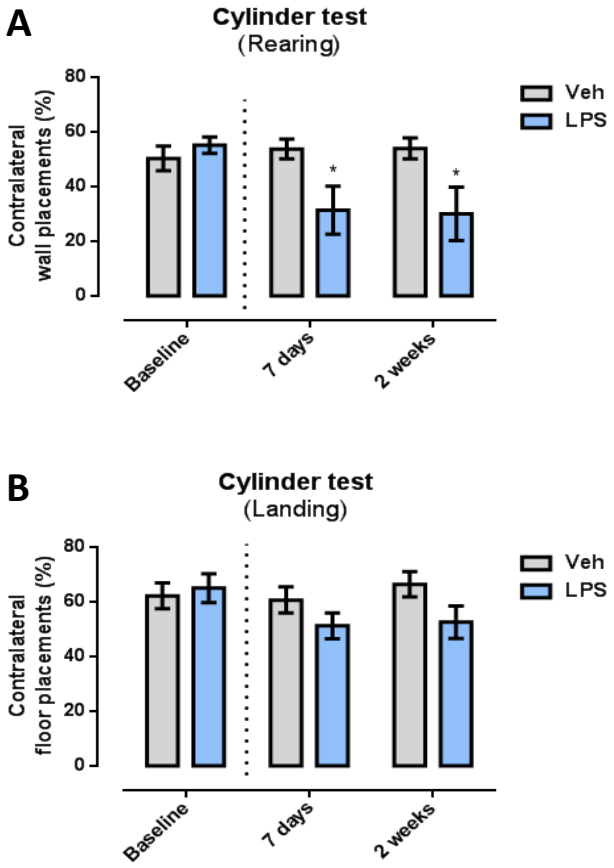
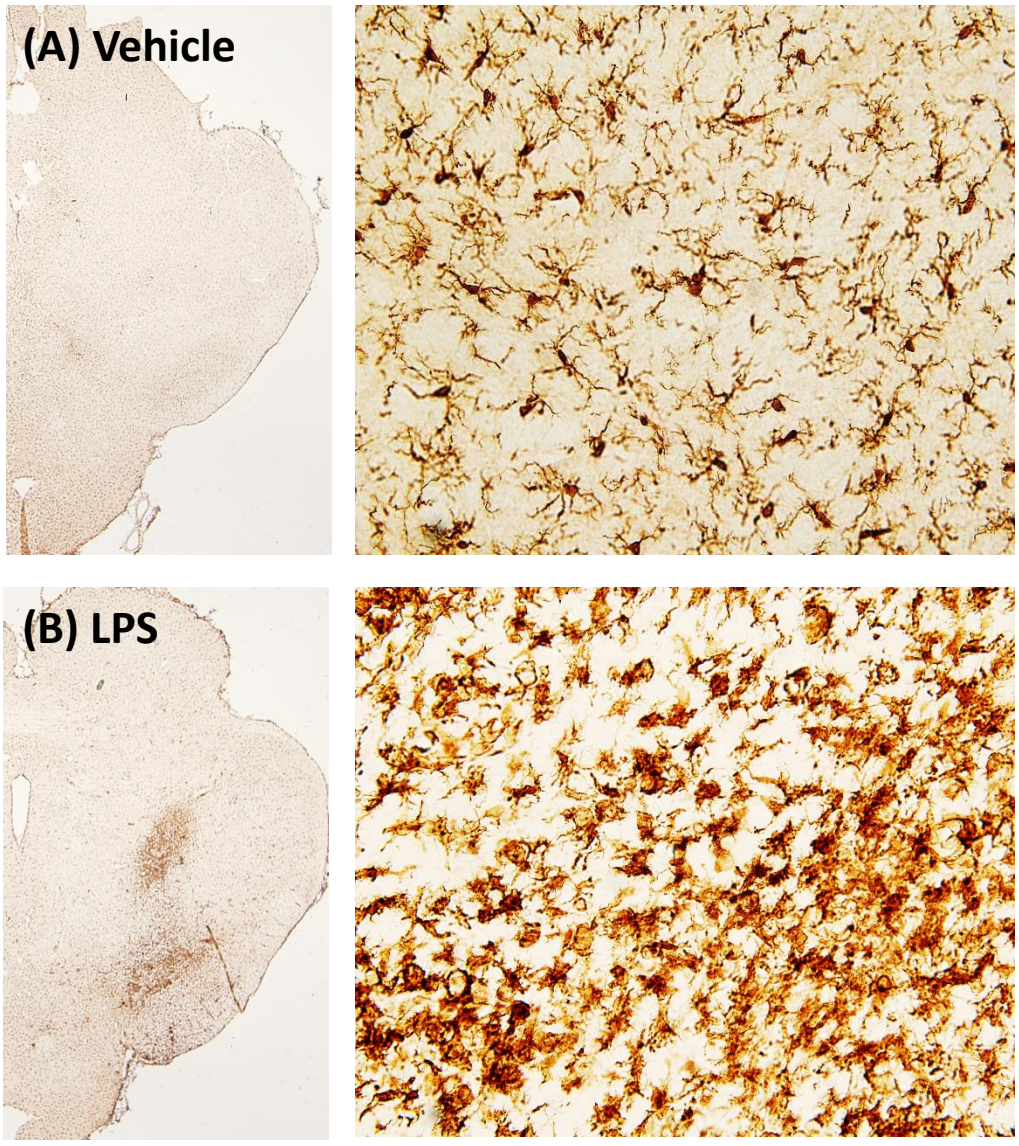


Figure 3.5 Intra-nigral LPS induces asymmetric forelimb use in the cylinder test. The effect of unilateral intra-nigral LPS injection on limb use asymmetry was assessed for wall (A) and subsequent floor (B) placements at 7 (W1) and 13 days (W2) post lesioning using the cylinder test. Unilateral injection of LPS (10µg/2µl) into the substantia nigra induced deficits in the number of contralateral wall placements at 7, but not 13 days post lesioning. Data are expressed as mean ± S.E.M. (n=7-8) * P<0.05 vs. vehicle by 2-way repeated measures ANOVA with *post hoc* Bonferroni.

3.3.4 Intra-nigral LPS administration induces a robust localised microgliosis within the ipsilateral substantia nigra.

Anti-Iba1 immunohistochemistry was performed to assess the impact of an intra-nigral LPS injection on microglial activation. A student's unpaired t-test revealed that at 2 weeks post lesioning with LPS there was a marked upregulation in Iba1^{+} reactive microglia within the SNpc relative to vehicle injected controls ($t_{(14)}$ = 9.086, P<0.0001). Iba1-immunopositive cell perimeter analysis was used as a morphometric parameter to assess the effect of a unilateral intra-nigral LPS injection on microglial morphology. A student's unpaired t-test revealed that the microglial cells present within the lesioned nigral hemisphere of LPS-injected animals exhibited an observed increase in cell soma size and an apparent retraction of processes, culminating in an overall decrease in cell perimeter length (P<0.05).



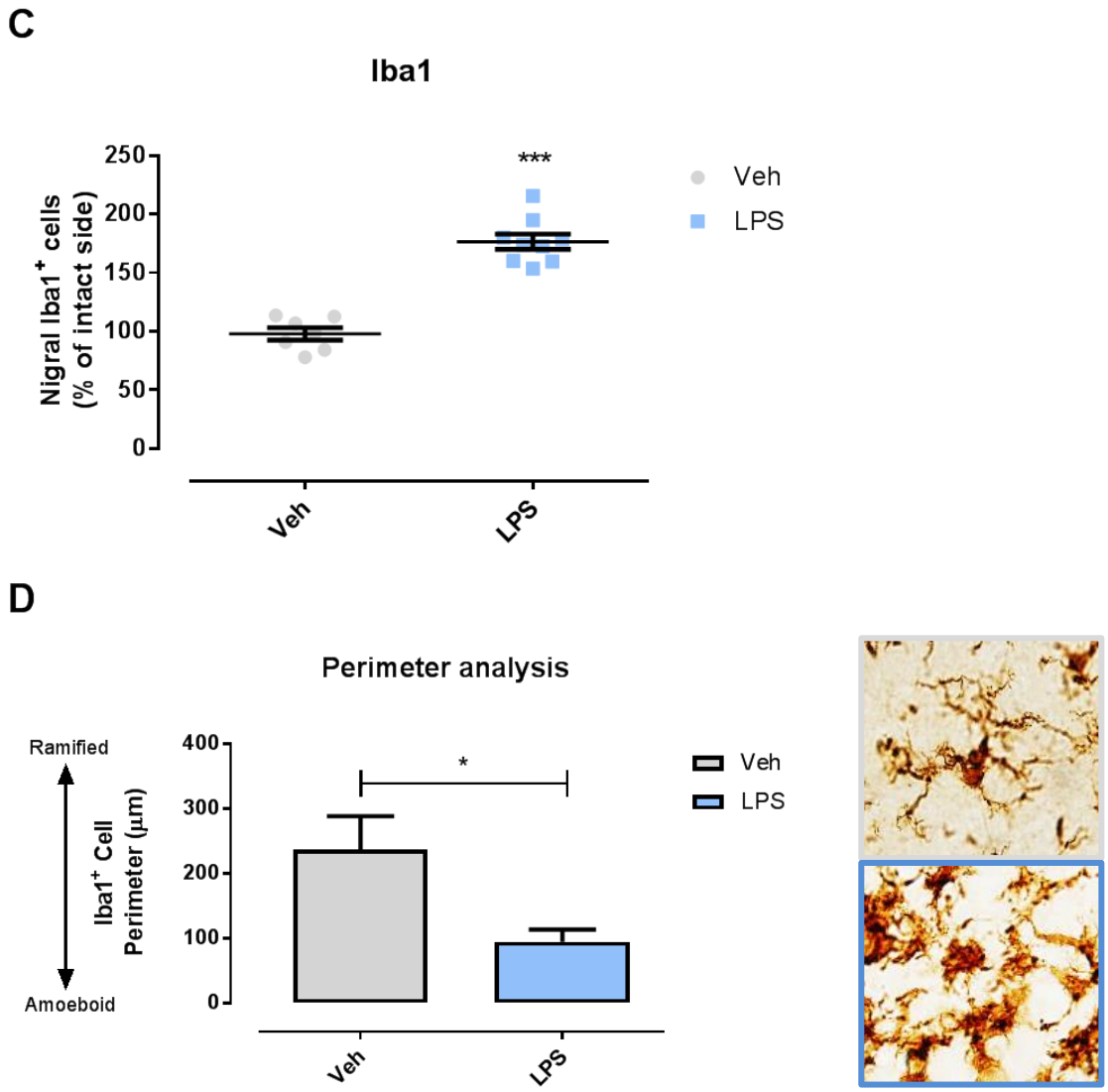
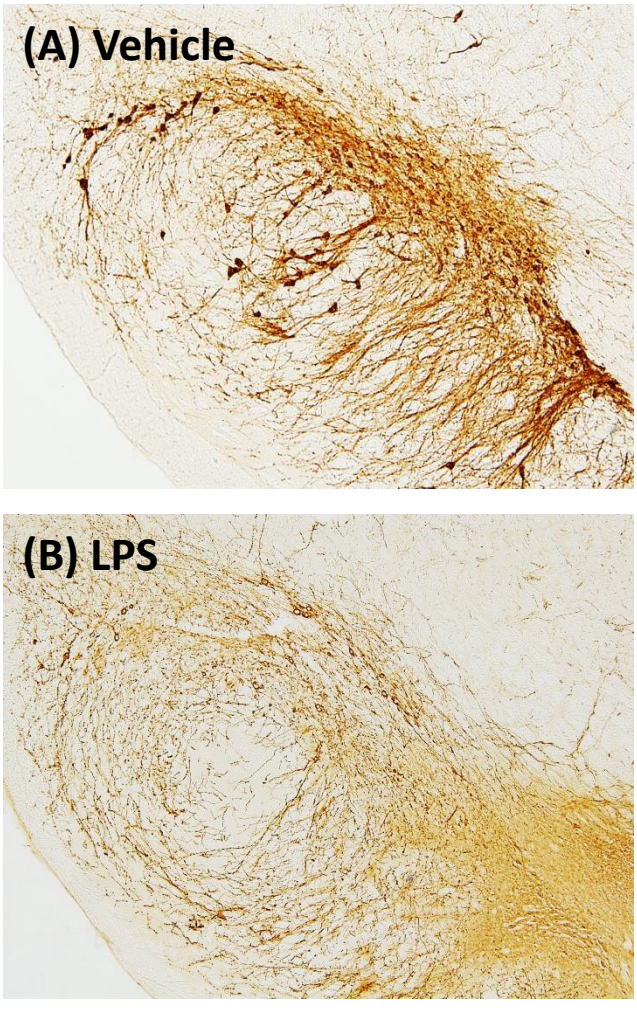


Figure 3.6 Intra-nigral LPS injection induces a robust microgliosis ipsilateral to the lesioned hemisphere. The effect of a unilateral LPS injection on nigral microgliotic reactivity was assessed via anti-Iba1 immunohistochemistry. Unilateral injection of LPS (10µg/2µl) into the substantia nigra induced a robust increase in Iba1-immunopositive microglial cells ipsilateral to the injection site (A-C). Intra-nigral LPS injection induces morphological alterations in nigral microglia (D). Data are expressed as mean ± S.E.M. (n=7-9) * P<0.05, *** P<0.001 vs. vehicle by Student's unpaired *t*-test SNpc: *substantia nigra pars compacta*, SNL: *substantia nigra, lateral*.

3.3.5 Intra-nigral LPS injection induces dopaminergic cell death within the substantia nigra.

Anti-tyrosine hydroxylase (TH) immunohistochemistry was conducted to assess the impact of an intra-nigral LPS injection on dopaminergic neurons within the substantia nigra. An unpaired student's t-test revealed that at 2 weeks post lesioning with LPS there was severe dopaminergic neuronal loss within the ipsilateral compact region of the substantia nigra relative to vehicle-treated controls ($t_{(14)}$ = 7.817, P<0.0001). On average, there was an estimated 52% deficit in TH^+-immunoreactivity in the ipsilateral nigral hemisphere of LPS-lesioned animals relative to vehicle-injected controls. To assess the link between dopaminergic neurodegeneration and microglial activation, the correlation between TH-immunopositive deficits with the extent of LPS-induced microgliotic reactivity was established via linear regression analysis. Unilateral injection of LPS (10µg/2µl) into the substantia nigra produced severe TH^+ neuronal degeneration that was strongly correlated with the extent of $Iba1^+$ microgliosis ipsilateral to the lesioned hemisphere (r = -0.7932, P = 0.0002).



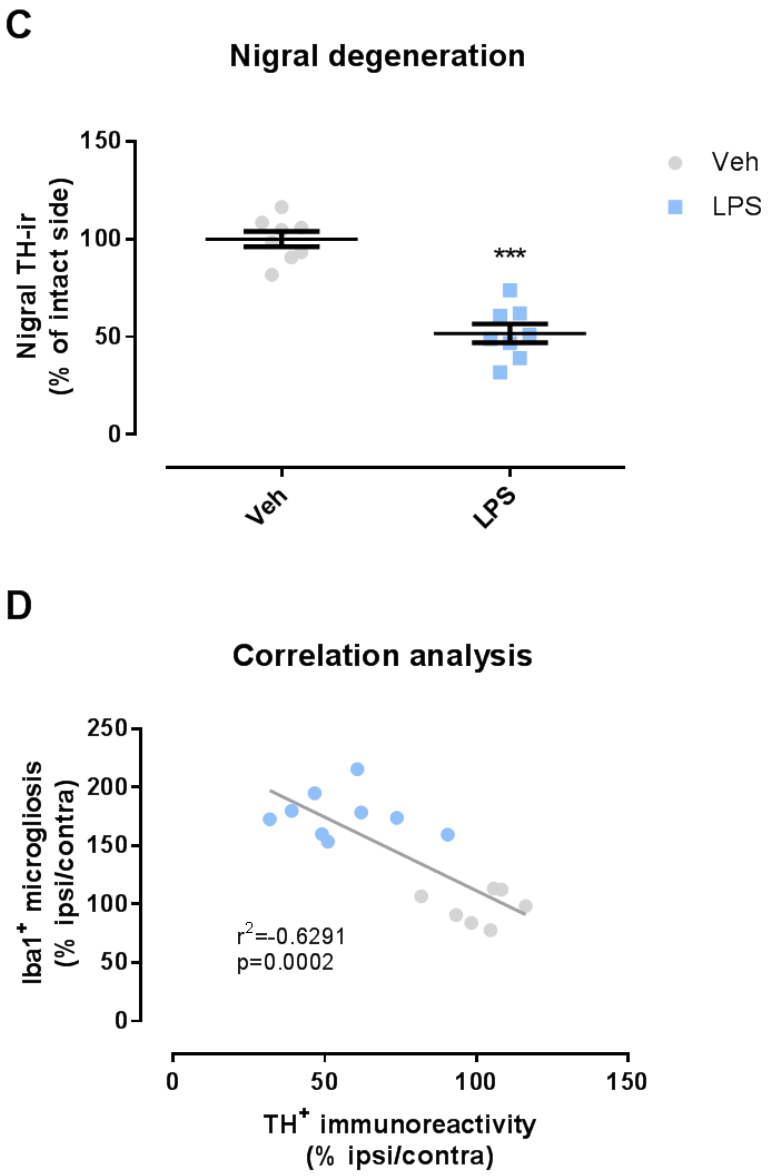
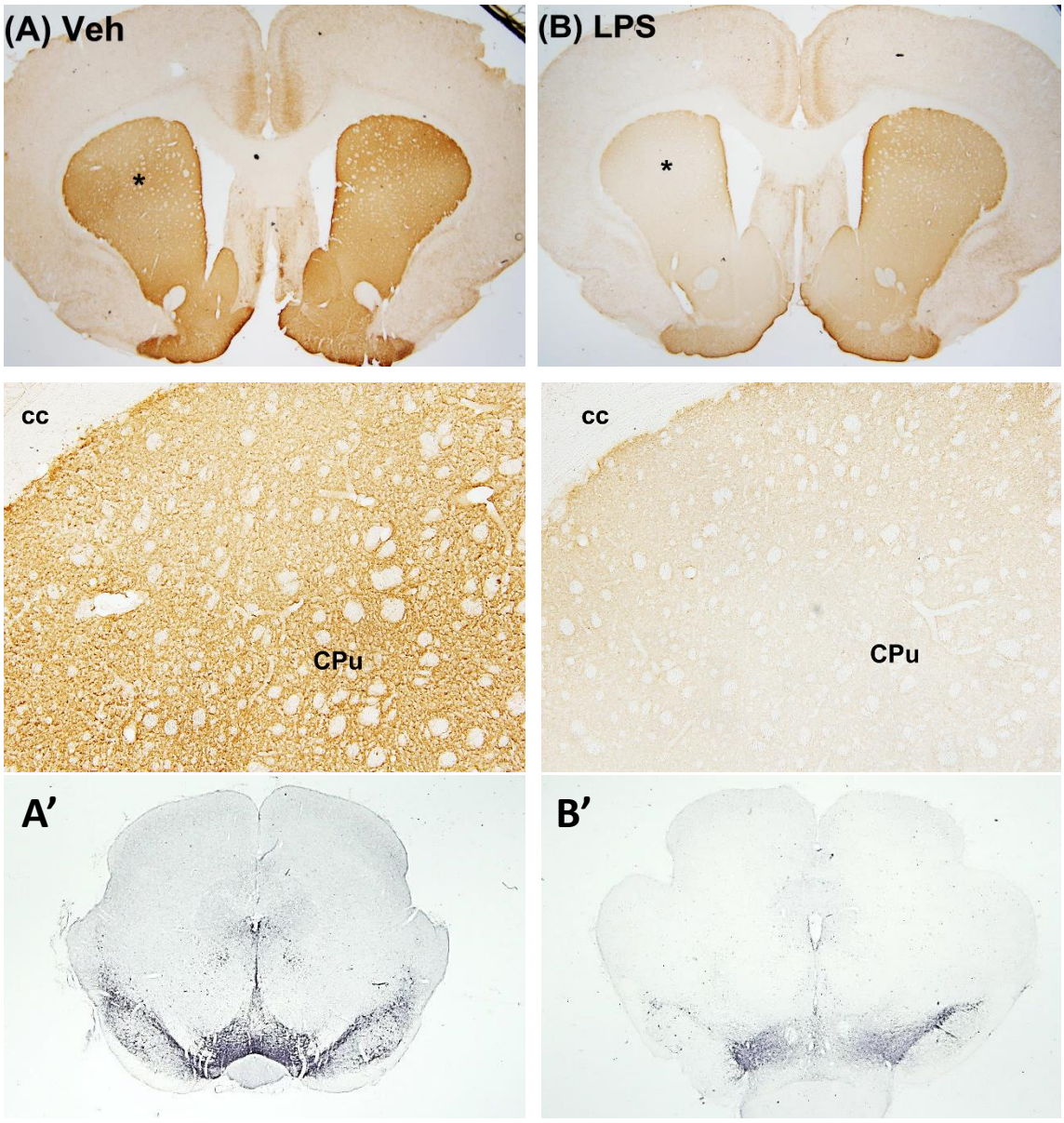


Figure 3.7 Intra-nigral LPS degenerates dopaminergic cell bodies and fibers in the substantia nigra. The effect of a unilateral intra-nigral LPS injection on dopaminergic neuronal integrity was assessed via anti-tyrosine hydroxylase (TH) immunohistochemistry. Unilateral injection of LPS (10µg/2µl) into the substantia nigra induced a severe deficit in TH-immunoreactivity (TH-ir) ipsilateral to the injection site (A-C). Dopaminergic neurodegeneration within the SNpc correlates strongly with microglial reactivity in response to LPS (D). Data are expressed as mean ± S.E.M. (n=8) *** P<0.001 vs. vehicle by Student's unpaired *t*-test. SNpc: *substantia nigra pars compacta.*

3.3.6 Intra-nigral LPS injection induces dopaminergic nerve terminal degeneration in the ipsilateral striatum.

The impact of a unilateral intra-nigral LPS (10µg/2µl) injection on striatal nerve terminal integrity was assessed 2 weeks post lesioning via anti-tyrosine hydroxylase immunostaining. A student's unpaired t-test revealed that intra-nigral injection of LPS induced nerve terminal denervation in the ipsilateral striatum relative to vehicle injected controls ($t_{(14)}$ = 4.091, = P=0.0011). On average, the area of TH-immunoreactivity (TH-ir) within the striatum was reduced by approximately 36% in LPS-lesioned rats compared to vehicle injected controls. The extent of LPS-induced microgliosis within the substantia nigra correlated with ipsilateral striatal denervation.



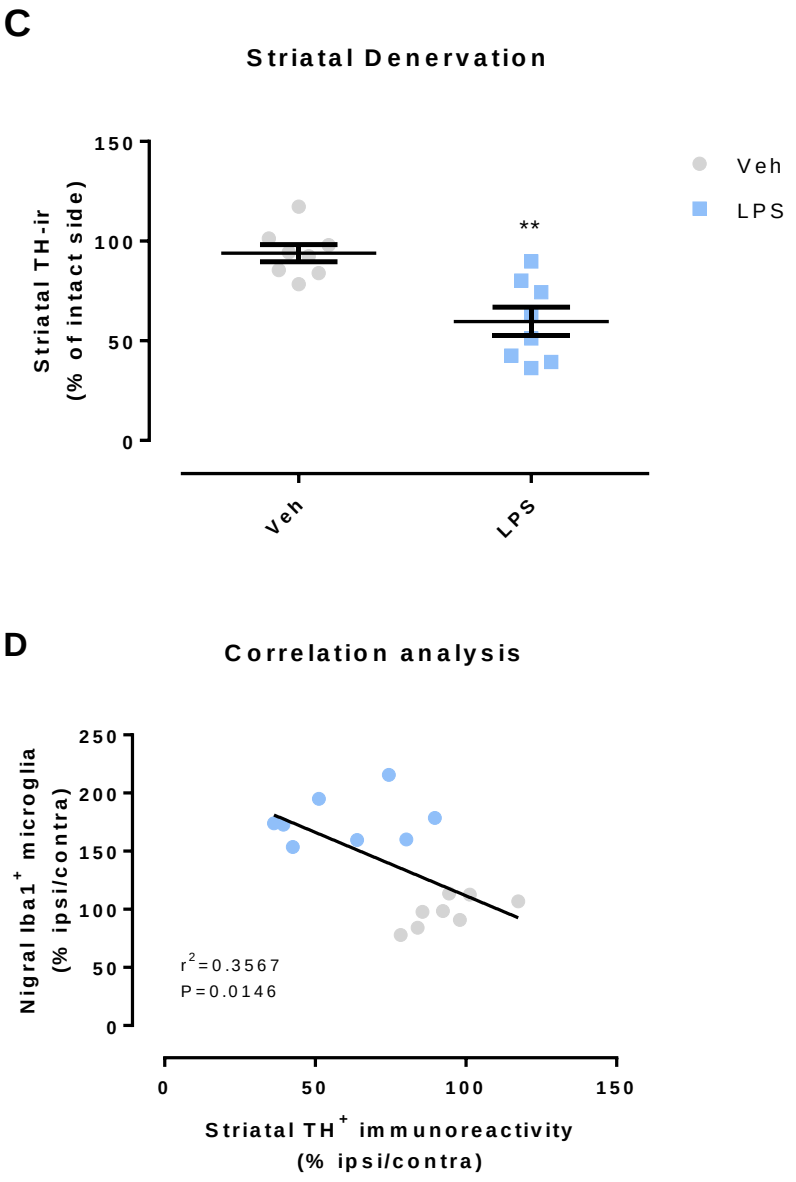
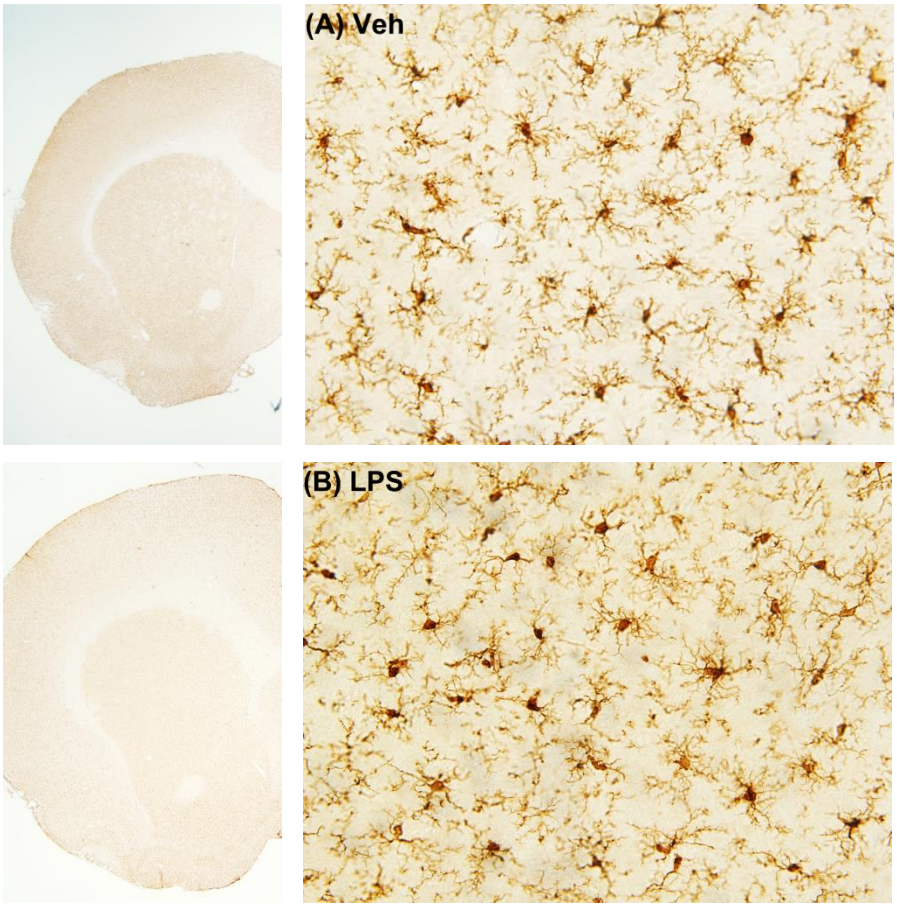


Figure 3.8 Intra-nigral LPS decreases TH-immunoreactivity (TH-ir) in the ipsilateral striatum. The effect of a unilateral intranigral LPS injection on striatal nerve-terminal integrity was assessed via anti-tyrosine hydroxylase (TH) immunohistochemistry. Unilateral injection of LPS (10µg/2µl) into the substantia nigra induced severe TH-immunopositive terminal denervation in the ipsilateral striatum (A-C, affiliated nigral sections are shown in A' & B'). The extent of LPS-induced nigral microglial activation correlates with nerve terminal loss in the ipsilateral striatum (D). Data are expressed as mean ± S.E.M. (n=8) ** P<0.01 vs. vehicle by Student's unpaired *t*-test. CPu: *caudate putamen* (striatum), cc: *corpus callosum*.

3.3.7 Intra-nigral LPS has no effect on microglial cell numbers in the ipsilateral striatum.

Anti-Iba1 immunohistochemistry was performed to assess microglial activation in the striatum at 2 weeks post lesioning with LPS. A student's unpaired t-test revealed that at 2 weeks post lesioning there was no difference in the number of $Iba1^+$ cell counts in the ipsilateral striatal hemisphere of LPS-injected rats relative to vehicle-injected controls ($t_{(14)}$ = 0.8025, P=0.4357). Striatal denervation did not correlate with striatal microglial reactivity.



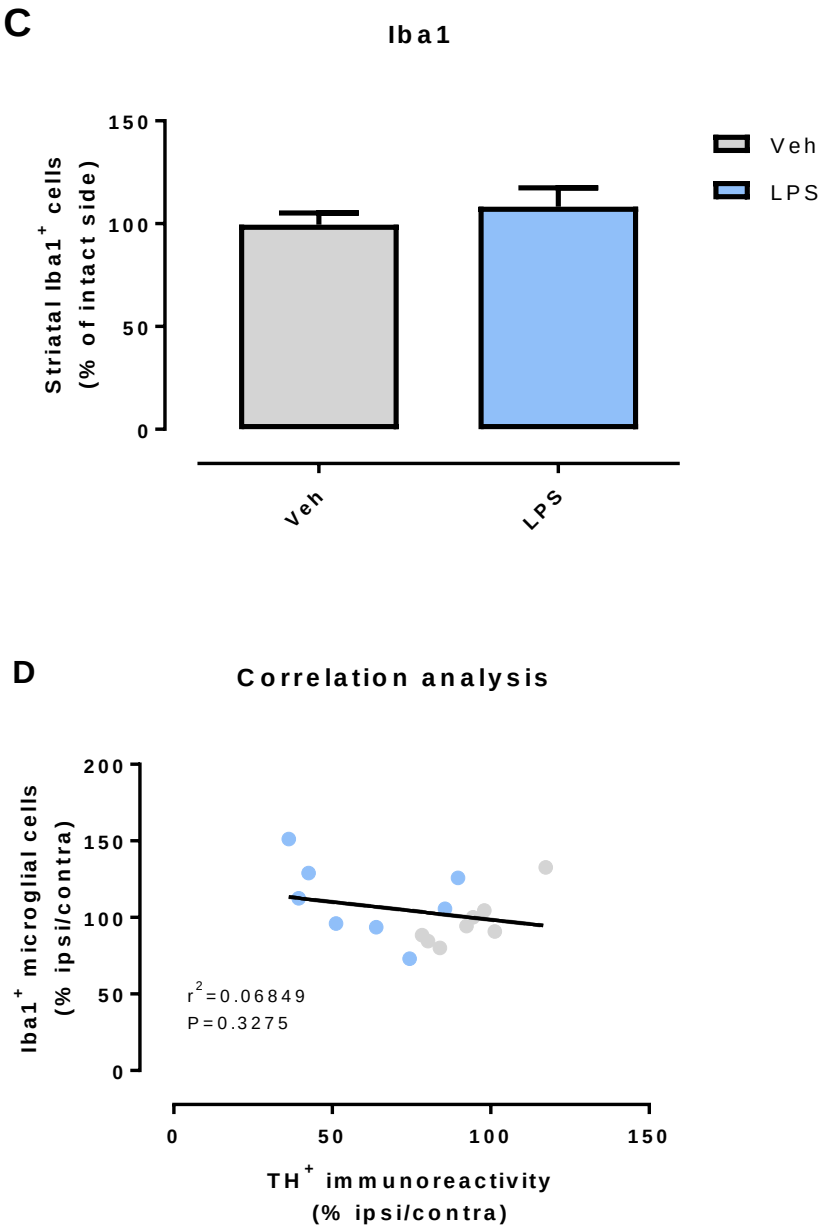
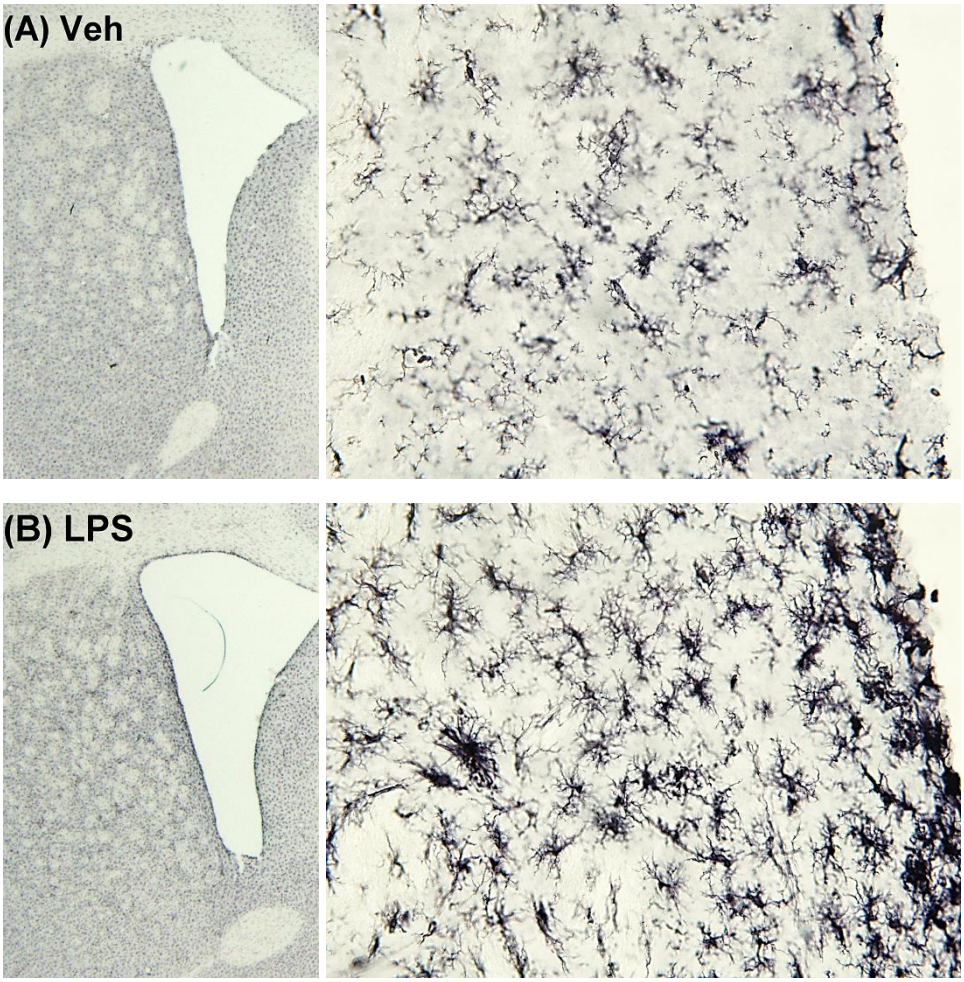


Figure 3.9 Intra-nigral LPS injection does not affect microglial cell numbers in the ipsilateral striatum. The effect of a unilateral LPS injection on striatal microgliotic reactivity was assessed via anti-Iba1 immunohistochemistry. Unilateral injection of LPS (10µg/2µl) into the substantia nigra had no effect on the quantity of Iba1-immunopositive reactive microglia in the ipsilateral striatum (A-C). The extent of LPS driven nerve terminal loss in the ipsilateral striatum was not correlated with striatal microglial reactivity (D). Data are expressed as mean ± S.E.M. (n=6) * P<0.05 vs. vehicle by Student's unpaired *t*-test.

3.3.8 Intra-nigral LPS increases the number of peri-ventricular microglia at the level of the ipsilateral striatum.

Anti-Iba1 immunohistochemistry was performed to assess microglial activation in the peri-ventricular space at the level of the striatum at 2 weeks post lesioning with LPS. A student's unpaired t-test revealed that at 2 weeks post lesioning there was a significant increase in the number of microglia relative to vehicle injected controls ($t_{(8)}$=4.329, P=0.0025). On average there was a 57% increase in the number of Iba1^{+} microglia in LPS-lesioned rats compared to vehicle injected controls.



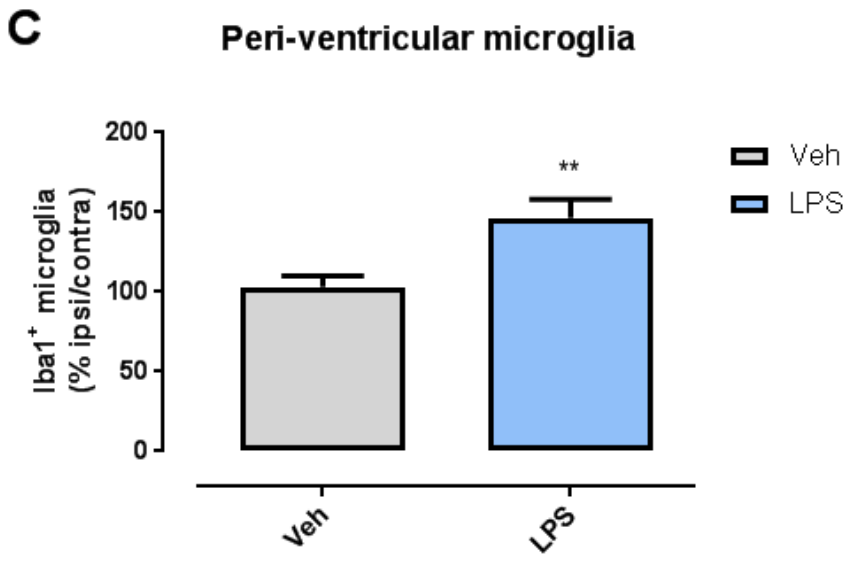


Figure 3.10 Intra-nigral LPS injection lead to an increase in the number of peri-ventricular microglia at the level of the striatum. The effect of a unilateral LPS injection on striatal microgliotic reactivity was assessed via anti-Iba1 immunohistochemistry. Unilateral injection of LPS (10µg/2µl) into the substantia nigra induced an increase in the number of Iba1-immunopositive reactive microglia at the level of the ipsilateral striatum (A-C), (n=6) ** P<0.01 vs. vehicle by Student's unpaired t-test.

3.3.9 Intra-nigral LPS injection reduces nigrostriatal dopamine content

High performance liquid chromatography with electrochemical detection (HPLC-ECD) was performed to assess the effect of a unilateral intra-nigral LPS injection on nigrostriatal dopamine content 2 weeks post lesioning with LPS. A student's unpaired t-test revealed that LPS reduced DA levels by approximately 21% in the midbrain ($t_{(12)}$ = 2.899, P=0.0134) and by approximately 28% in the ipsilateral striatum ($t_{(10)}$ = 2.662, P=0.0238) relative to vehicle injected controls.

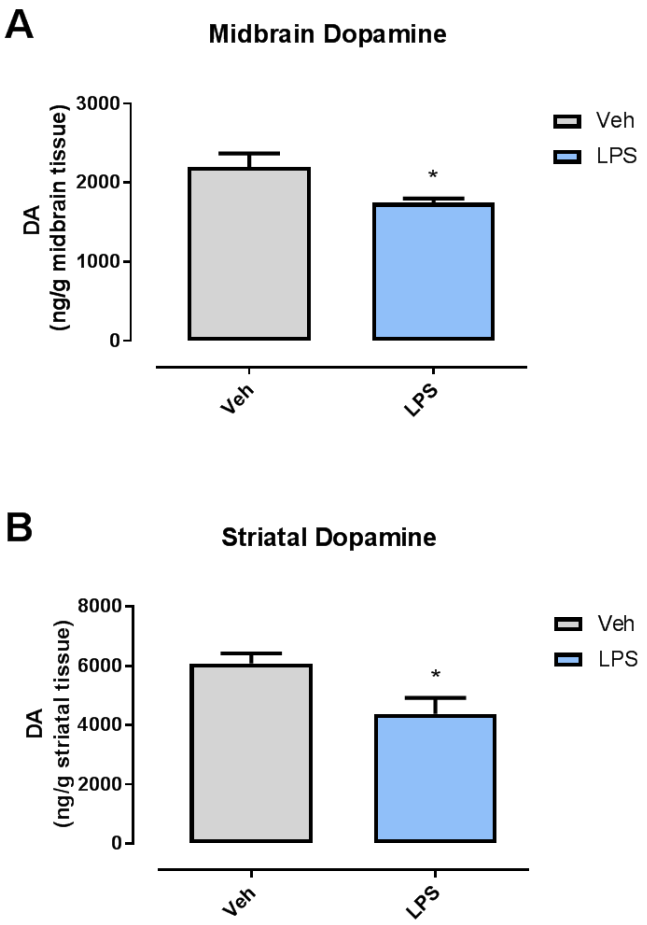
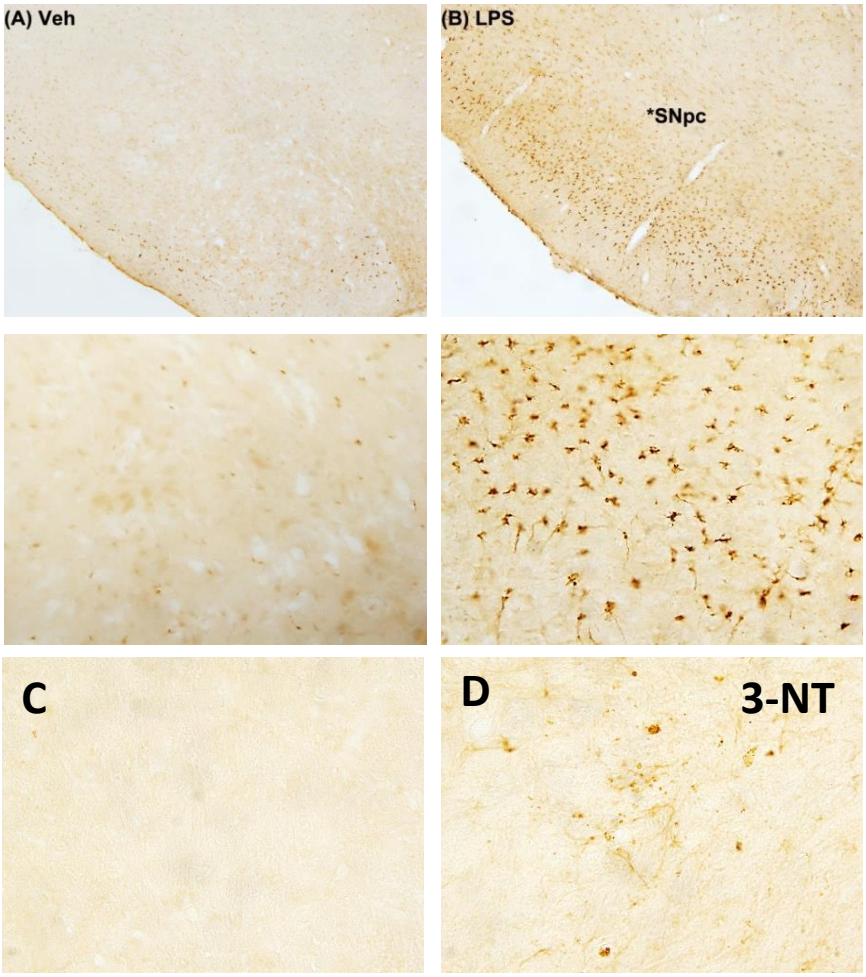


Figure 3.11 Intra-nigral LPS injection reduces nigrostriatal dopamine (DA) content. LPS was stereotactically injected into the substantia nigra, and at 15 days post lesioning neurochemical analysis of biogenic amines was conducted via HPLC-ECD. Unilateral LPS (10µg/2µl) injection depleted ipsilateral midbrain dopamine content by approximately 21% (A) and by approximately 28% in the ipsilateral striatum (B). Data are expressed as mean ± S.E.M. (n=6-8) * P<0.05 vs. vehicle by Student's unpaired *t*-test.

3.3.10 Intra-nigral LPS injection upregulates iNOS expression within the lesioned nigral hemisphere

Anti-iNOS immunohistochemistry was conducted 2 weeks post lesioning to assess the impact of an intra-nigral LPS injection on inducible nitric oxide synthase expression within the substantia nigra. A student's unpaired t-test revealed that iNOS expression was upregulated ipsilateral to the injection site of LPS-lesioned animals compared to vehicle-injected controls. ($t_{(14)}$ = 9.782, P<0.0001). There was an approximate 95% increase in the number of $iNOS^+$ cell counts within the lesioned nigral hemisphere of LPS-injected rats relative to vehicle-injected controls. Moreover, evidence of 3-nitrotyrosine (3-NT) formation was apparent within the SNpc of LPS-lesioned rats but absent in vehicle injected controls.



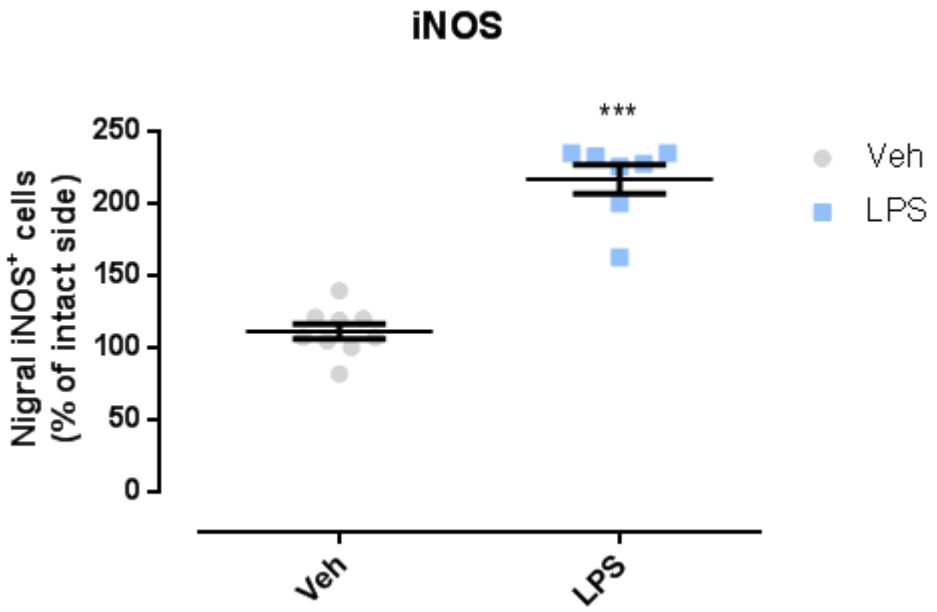


Figure 3.12 Intra-nigral LPS injection upregulates iNOS expression and induces 3-NT formation ipsilateral to the lesioned hemisphere. The effect of a unilateral intra-nigral LPS injection on inducible nitric oxide synthase expression and 3-nitrotyrosine formation was assessed via anti-iNOS and anti-3-NT immunohistochemistry respectively. Unilateral injection of LPS (10µg/2µl) into the substantia nigra stimulated a robust increase in the number of iNOS-immunopositive cell bodies ipsilateral to the injection site (A+B). Intra-nigral LPS also induced an observed increase in 3-NT formation within the SNpc (C+D). Data are expressed as mean ± S.E.M. (n=7-9) *** P<0.001 vs. vehicle by Student's unpaired *t*-test

3.4 Discussion

Here we show that a unilateral injection of LPS into the substantia nigra stimulates a neuroinflammatory response within the nigrostriatal dopaminergic system, leading to neurodegeneration and a Parkinsonian phenotype in male Wistar rats. LPS induced severe dopaminergic cell loss within the compact region of the substantia nigra and concurrent nerve terminal degeneration in the ipsilateral striatum, findings which were accompanied by reductions in nigrostriatal dopamine content and lateralised motor deficits. The degeneration of the dopaminergic system was underpinned by a robust LPS-driven microgliosis and elevations in iNOS$^+$ cell bodies ipsilateral to the lesioned hemisphere. Here we report that an LPS-induced microgliopathy within the substantia nigra sets the premise for dopaminergic neurodegeneration, nigrostriatal dopamine loss and spontaneous motor deficits. These findings compliment previous studies demonstrating that an LPS-induced microgliosis within the substantia nigra leads to degeneration of the nigrostriatal dopaminergic system, dopamine loss and motor dysfunction (Herrera et al., 2000; Zhou et al., 2005; Ariza et al., 2010a; Hoban et al., 2013).

The present study demonstrates that a unilateral injection of LPS (10µg/2µl) into the left hemisphere of the substantia nigra induces robust microglial activation and dopaminergic neuronal loss, culminating in a reduction in nigrostriatal dopamine levels and contralateral deficits in motor function. Intra-nigral injection of LPS lead to a robust 81% elevation in $Iba1^+$ activated microglial cells and decreased TH^+ dopaminergic cell bodies and fibers by approximately 52% within the ipsilateral substantia nigra. The extent of LPS-induced DAergic neurodegeneration correlated strongly with the level of LPS-driven microgliotic reactivity. Moreover, morphometric analysis of nigral microglia revealed a shift in morphology towards an amoeboid phenotype in LPS-injected animals, indicative of a microglial activation state. Concurrently, unilateral intra-nigral LPS administration lead to a 36% decrease in TH^+ nerve terminals in the ipsilateral striatal hemisphere, where $Iba1^+$ microglia were also apparent. Our finding that the extent of striatal denervation did not correlate with striatal microglial reactivity highlights that the dopaminergic nerve terminal loss is likely linked to the LPS-induced nigral cell loss as opposed to a secondary inflammatory reaction in the ipsilateral striatum. Thus, activated nigral microglia are likely to be the main cellular culprits in degeneration of the nigrostriatal dopaminergic system in the LPS model of Parkinson's disease.

Profuse numbers of activated microglia are present in the vicinity of damaged/dying dopaminergic neurons within the SNpc of patients with Parkinson's disease (McGeer et al., 1988). Degenerating dopamine neurons release multiple factors capable of activating microglia such as α-synuclein, neuromelanin and matrix metalloproteinase 3 (MMP3) leading to microglial-derived ROS production and a self-perpetuating cycle of reactive microgliosis and DAergic neurodegeneration (Block et al., 2007a). Our results showing that the level of LPS-induced microgliosis within the substantia nigra correlates strongly with the extent of dopaminergic neurodegeneration are corroborated by previous studies demonstrating that the amount of $CD68^+$ cells recruited to the midbrain of LPS-injected rats correlates strongly with the level of dopaminergic neuronal loss within the SNpc (Daher et al., 2014), and that the amount of nigral $Iba1^+$ microglial cells correlates strongly with the loss of TH^+ cells in the SN when assessed at 7 days post lesioning with MPTP (Noelker et al., 2013). Thus, robust microglial activation is likely to be a major cellular process leading to the demise of dopaminergic neurons within the substantia nigra in response to an inflammatory stimulus such as LPS. Moreover, the midbrain reportedly contains the highest density of microglial cells in the entire rodent brain (Lawson et al., 1990), highlighting its susceptibility to the cytotoxic effects of an inflammogen. Similar to our findings, LPS-mediated dopaminergic neurodegeneration within the substantia nigra has previously been reported to be accompanied by an increase in $OX\text{-}42^+$ macrophages at the focal point of the lesion (Castano et al., 2002a). Thus, the nigrostriatal dopaminergic system may be selectively vulnerable to an inflammatory stimulus such as LPS, due to the glial cyto-architecture of the midbrain, which promotes this brain region as a hotspot for neurodegeneration, particularly in circumstances where inflammation sets the precedence for neuropathology.

An experimental model of Parkinson's disease should successfully replicate to some extent, the motoric dysfunction typical of a Parkinsonian state. Following a unilateral injection of LPS into the substantia nigra, the resulting microglial activation, neuroinflammation, and neurodegeneration lead to contralateral behavioural impairments when assessed at 1, and 2 weeks post lesioning. We found that unilateral lesioning of the substantia nigra with LPS induced pronounced, yet stable deficits in contralateral skilled motor function (staircase test), and to a lesser extent forelimb akinesia (stepping test) and forelimb use asymmetry (cylinder test). These lateralised motor disturbances in response to lesioning of the nigrostriatal tract with LPS are similar to those reported in studies by (Hoban et al., 2013), and are highly relevant to the human condition (Sabatini et al., 2000; Djaldetti et al., 2006; Jankovic, 2008). A unilateral intra-nigral injection of 10µg LPS has previously been shown to induce nigrostriatal neurodegeneration of sufficient magnitude to stimulate ipsiversive turning in response to amphetamine (5 mg/kg i.p.) challenge (Iravani et al., 2005). Moreover, unilateral intra-striatal administration of LPS (2.5 µg/µl) induces progressive degeneration of the nigrostriatal dopaminergic system and leads to sustained asymmetric forelimb use in the cylinder test and D-amphetamine (2.5 mg/kg i.p.)-induced ipsilateral rotational behaviour when assessed at 4 weeks post lesioning (Choi et al., 2009). As described in the studies above, the amphetamine rotation challenge is usually conducted to confirm the presence of a lesion in the nigrostriatal dopaminergic system but is essentially an artificial measurement of motor asymmetry (Hoban et al., 2013), relying on the release of dopamine from intact DAergic terminals to enforce drug-induced rotation, without necessarily illuminating any distinct patterns of motor dysfunction as seen in the human condition. Moreover, in the present study, given the possibility for D-amphetamine administration to infringe on the accurate measurement of nigrostriatal DA levels as assessed by HPLC, we did not include this test in our assessment of the impact of an intra-nigral LPS injection on motor behaviour, choosing solely to focus on clinically relevant assessments of lateralised motor performance instead.

LPS has previously been shown to induce sickness behaviour in rodents characterised by deficits in locomotor activity, social interaction and food intake when administered centrally (Huang et al., 2008; Lawson et al., 2013) or systemically (Henry et al., 2008; Hines et al., 2013), and are exacerbated in aged mice (Godbout et al., 2005). Bearing these reports in mind, we aimed to clarify whether the motor dysfunction observed in response to lesioning the substantia nigra with LPS was due to direct injury to the nigrostriatal dopaminergic system as opposed to an LPS driven sickness response. Our observation that injecting the SNpc with LPS did not alter the total amount of food pellets eaten using the contralateral limb at 7 or 13 days post lesioning highlights that direct injury to the nigrostriatal dopaminergic system is culpable for the pronounced deficits in skilled motor function observed, and that these deficits in skilled motor function are unlikely to be attributable to a generalized LPS-induced sickness behaviour.

A salient pathological feature of Parkinson's disease is extensive loss of the neurotransmitter dopamine which is linked to the preceding degeneration of the nigrostriatal dopaminergic system in the brains of PD patients (Christopher et al., 2013; Kordower et al., 2013). In the present study, 2 weeks following an intra-nigral injection of LPS dopamine content was reduced on average by about 21% in the midbrain, and by approximately 28% in the ipsilateral striatum relative to vehicle-injected controls. Dopamine content in the striatum has previously been reported to be reduced to 63.4% of control values after 7 days, extending to 60.1% after 14 days post a single intra-nigral LPS injection (2µg; from *Escherichia coli*, serotype 026:B6) (Castano et al., 2002b). Previously, it has been shown that an intra-striatal injection of LPS depletes dopamine content and increases the turnover rates of DOPAC & HVA to DA in the striatum when assessed as early as 3 days post-surgical procedures (Hunter et al., 2007). Contrary to these findings, intra-nigral injection of LPS of the same serotype used in our present investigation (2µg/1µl; from *Escherichia coli*, serotype 0111:B4) did not alter striatal DA, DOPAC or HVA levels when measured via HPLC at 21 days post lesioning (Santiago et al., 2010). Studies by (Chien et al., 2016) have also demonstrated that an intra-nigral LPS injection of an alternate serotype (1µg/1µl) does not significantly alter striatal DA content when assessed 5 days post lesioning, but LPS-induced dopamine loss is promoted in DJ-1 (PARK7) KO mice, a genetic deletion linked to the development of early-onset Parkinson's disease. Thus, the impact of lesioning the nigrostriatal tract with LPS on cerebral dopamine content is dependent on the dose and serotype of LPS injected, its site of administration, the timing of monoamine analysis post lesioning and the genetic makeup of subjects under investigation.

In the present study, 2 weeks following an intra-nigral LPS injection, $iNOS^+$ cells with a non-neuronal, punctate morphology were visible within the ventral midbrain. The cluster of $iNOS^+$ cell bodies within the ventral midbrain of LPS-injected animals were in close proximity to TH^+ dopaminergic neurons within the SNpc, and therefore, the amount of iNOS-derived NO potentially produced that could reach nigral dopamine neurons is conceivably substantial. This is of particular clinical relevance as iNOS expression is reportedly upregulated in glial cells of the mesencephalon of patients with idiopathic Parkinson's disease (Hunot et al., 1996). In the present study, it's likely that the LPS-induced iNOS-derived NO production within the ventral midbrain could flood surrounding dopaminergic structures at the peak of inflammation and contribute to neuronal loss within the substantia nigra. Nitric oxide may react with superoxide (O_{2^-}) to form the cogent oxidant peroxynitrite ($ONOO^-$) which may cause direct oxidative stress mediated injury to dopamine neurons (Oh-hashi et al., 1999; Park et al., 2002). Moreover, tyrosine residues are a target for nitration following exposure to peroxynitrite (Ara et al., 1998), a process previously shown to be implicated in the MPTP (Ara et al., 1998), 6-OHDA (Mihm et al., 2001) and LPS (Tomás-Camardiel et al., 2004) models of Parkinson's disease. In the current study, 3-NT formation was evident within the SNpc of LPS-lesioned rats. Hence, LPS-induced elevations in iNOS expression may contribute to the death of DAergic cells within the SNpc via an oxidative

stress mechanism and/or a process dependant on nitration of tyrosine residues. Thus, in combination with peroxynitrite-derived neurotoxicity, tyrosine nitration-induced TH enzymatic inactivation and consequent DA synthesis impairments may be a major biochemical progressor of Parkinsonian severity in the LPS model of PD.

In summary, we have established and characterised an inflammatory-based experimental rodent model of Parkinson's disease in which the mechanisms underlying how immune-mediated events can contribute to the pathophysiology of PD can be investigated. Intra-nigral injection of LPS lead to a localised microgliosis within the ventral midbrain and dopamine cell loss within the SNpc, findings which were accompanied by ipsilateral dopaminergic striatal denervation, nigrostriatal dopamine loss and lateralised motor deficits. We conclude that the intra-nigral LPS model may be a highly relevant model for investigating how inflammatory events influence PD-related neuropathology and for research into assessing the efficacy of potential disease modifying anti-inflammatory therapies to slow or halt disease progression.

Chapter 4

Glial crosstalk at the interface of nigrostriatal neurodegeneration;

implications for Parkinson's disease

4.1 Introduction

4.1.1 Role of astrocytes in the CNS

The term glia originates from the Greek word gliok, meaning glue, but is also commonly translated as slime (Nedergaard et al., 2003). Astrocytes are however, highly complex, fibrous, multifunctional housekeeping cells of the CNS whose importance have long been overlooked by histologists due to the impressive complexity and beauty of their neuronal neighbours. Indeed, astrocytes are stellate glial cells functionally linked by gap junctions along astrocytic processes proximately interspersed with capillaries and neurons, forming a gliovascular syncytium in which they perform diverse duties integral to the structural and functional architecture of the CNS.

Mediators of calcium signalling: Astrocytes communicate with other glia & modulate the synaptic activity of adjacent cells (neurons and oligodendrocytes) via a calcium-mediated mode of intercellular communication (Verderio and Matteoli, 2001). ATP released from astrocytes via connexin hemichannels has been identified as a diffusible messenger responsible for mediating glial calcium wave signalling (Guthrie et al., 1999; Stout et al., 2002). Astrocytes express the purinoreceptors P2X7, P2Y1, P2Y2 & P2Y4 and at least two of these are involved in ATP-evoked intercellular Ca^{2+} wave signalling (James and Butt, 2001; Suadicani et al., 2006). Gap junction proteins (particularly connexins) are also involved in this process however, as ectopic expression of connexins increases the radius of Ca^{2+} wave propagation and cell lines that express Cx26, Cx32, or Cx43 release 5-20 fold more ATP than connexin-deficient controls (Cotrina et al., 1998; Cotrina et al., 2000). Thus, astrocytes are excitable glial cells that respond to Ca^{2+} fluctuations in order to integrate and convey information, form neuroglial interactions and influence synaptic activity.

Guardians of glutamate: Glutamate is the main excitatory neurotransmitter in the brain and is the most prevalent neurotransmitter in the vertebrate CNS, being excitatory at over 90% of synaptic connections in the human brain (Meldrum, 2000). Astrocytes are cellular gatekeepers of glutamate; they recycle glutamate from the extracellular space back into presynaptic neuronal terminals (in the form of glutamine) via the glutamate-glutamine

shuttle (Steele and Robinson, 2012). It is estimated that >80% of glutamate is metabolised within astrocytes to glutamine and the rapid recovery of this glutamate from the synaptic cleft occurs via astrocytic GLAST and GLT-1 glutamate transporters (also known as EAAT1 & EAAT2 respectively) (Anderson and Swanson, 2000). Astrocytic uptake, catabolism & recycling of glutamate is of particular importance in maintaining neuronal viability due to the excitotoxic nature of glutamate at high extracellular concentrations (Pitt et al., 2000).

Spatial buffering of potassium: Astrocytes play a major role in extracellular potassium homeostasis by buffering K^+ ions with potassium channels located at synapses & end-foot processes (Kofuji and Newman, 2004). Neurons are bathed in an extracellular fluid rich in Na^+ and low in K^+ but these concentrations are reversed inside the cell. Any flux in potassium concentrations in the extracellular space (ECS), due to an influx of sodium and an efflux of potassium, has an impact on neuronal processes, such as the maintenance of membrane potential, voltage gated ion channel activation and synaptic transmission. Astrocytic gap junctional coupling has been shown to be important for the spatial buffering of potassium ions in the brain (Wallraff et al., 2006). Moreover, inwardly rectifying K^+ (Kir) channels on astrocytic end-foot processes play an integral role in mopping up extracellular K^+ from synaptic sites of excited neurons (Kofuji et al., 2002). The dense expression of strongly rectifying Kir channels on astrocytic end-foot processes thus contributes greatly to preventing the overt outward leak of K^+ from neuronal sources which would undoubtedly disturb neuronal transmission.

Trophic support: Glial cell line-derived neurotrophic factor (GDNF) is a widely studied trophic factor in PD research. Recombinant human GDNF promoted survival & differentiation of embryonic midbrain dopaminergic neurons, and increased their high affinity uptake of dopamine (Lin et al., 1993b). *Ex vivo* intra-nigral transplantation of lentiviral-mediated GDNF-transduced primary astrocytes 1 week prior to an intra-striatal 6-OHDA lesion protected against dopaminergic neurodegeneration within the SN, highlighting the efficacy astrocyte derived GDNF production against dopaminergic neurodegeneration (Ericson et al., 2005). Recently it has been shown that intranasal delivery of chitosan (CS)-coated nanostructured lipid carriers with trans-activating of transcription (TAT) peptide encapsulating GDNF modulates MPTP-induced microglial activation within the SN, alleviates nigrostriatal neurodegeneration and promotes functional recovery from motor impairments in the rotarod test (Hernando et al., 2018). Thus, pharmacologically targeting astrocytes for increased growth factor expression may be a valuable therapeutic prospect for conferring neuroprotection in Parkinson's disease.

4.1.2 Astrocytes in neurodegenerative disease

It has become increasingly apparent in recent decades from post mortem histological studies on brains of Parkinson's (PD), Alzheimer's (AD), Huntington's (HD) and Amyotrophic lateral sclerosis (ALS) disease patients (to name a few) that astrocytes play a role in the pathophysiology of multiple neurodegenerative disease states. Increasing experimental evidence indicates that astrocytes are also dysfunctional in multiple neurodegenerative disorders and in some cases this early glial dysfunction precedes the onset of neurodegeneration. Up until the 1990's the instigation, and indeed progression of neurological disease states has been attributed to primary neuronal dysfunction, with the role of neuroglia in this process, although speculative and intriguing, denoted to playing second fiddle to their neuronal counterparts. Re-investing research efforts into the active role of astrocytes in primary neurodegenerative disorders of the human CNS may prove that these glial cells are more pertinent to fitting the pieces of this puzzle together than what we originally thought.

Huntington's disease: Huntington's disease (HD) is an autosomal dominant, progressive, fatal neurodegenerative disorder characterised by the loss of medium spiny neurons (MSN's) within the striatum (Tang et al., 2005). The disease is underpinned by a polyglutamine (CAG) repeat expansion of the N-terminal domain of the huntington gene (htt), leading to a toxic gain of function mutation (mutant htt) associated with dystonia, chorea, incoordination and cognitive dysfunction (Walker, 2007). Nuclear mutant htt aggregates were found in the striatum of R6/2 mouse model of Huntington's disease, accompanied by a decrease in GLT-1 expression and a reduction in glutamate uptake (Shin et al., 2005). The same authors show in a neuron-glia co-culture system that wild-type astrocytes protect neurons from glutamate toxicity, whereas astrocytes expressing mutant htt increased the vulnerability of neurons to glutamate-induced neurotoxicity. Given the seminal role of astrocytes in mopping up extracellular glutamate, this particular finding may highlight major astrocytic relevance in the pathogenesis of HD, as MSN's receive substantial glutamatergic axonal input and thus, may be inherently sensitive to glutamate-induced excitotoxicity.

Amyotrophic lateral sclerosis: Transplantation of glial restricted precursor cells (GRP's) harbouring the human ALS-linked $SOD1^{G93A}$ mutation into the cervical spinal column of WT rats induces focal motor neuron degeneration, forelimb motor dysfunction, respiratory impairments, astrocytosis and reductions in GLT-1 expression (Papadeas et al., 2011). Using human $SOD1^{G93A}$ transgenic mice, studies by (Rossi et al., 2008) have shown that astrocytic degeneration is spatially confined to the microenvironment of spinal cord motor neuron degeneration, and that astrocytes expressing mutant human SOD^{G93A} are highly vulnerable to glutamate toxicity downstream of mGluR5 activation. Moreover, the authors demonstrate that blockade of mGluR5 *in vivo* slows down astrocytic degeneration and delays the onset of clinical symptoms in $hSOD^{G93A}$ transgenic mice. Conversely,

overexpressing GLT-1 in astrocytes in mSOD1 mice enhances the survival of motor neurons and delays disease onset (Guo et al., 2003). Moreover, delivery of the β-lactam antibiotic ceftriaxone (200 mg/kg i.p.) daily to $SOD1^{G93A}$ transgenic mice increases cerebral GLT-1 expression and activity, and delays loss of motor neurons and associated muscle strength (Rothstein et al., 2005). Taken together, astrocytes are involved in the pathogenesis of ALS, contextualised by a mechanistic link that exists between the focal losses of astrocytic GLT-1 expression, astrocytosis and the development of motor neuron degeneration.

Alzheimer's disease: In the Alzheimer's field, studies by (Olabarria et al., 2010) have demonstrated that early astroglial atrophy (detected at 6, 12 & 18 months) within the hippocampus of a triple transgenic mouse model of Alzheimer's disease (3xTg-AD) may contribute to the synaptic loss and cognitive decline observed in AD patients, whereas in the later stages of the disease hypertrophic reactive astrocytes localise exclusively around neuritic Aβ plaques in both the CA1 and dentate gyrus hippocampal brain regions. Treatment of cultured rat hippocampal neurons with conditioned media from β-amyloid treated astrocytes increases the number of $TUNEL^{+}$ apoptotic cells and markedly increases S100B expression, thus implying that astrocytes contribute to Aβ-neurotoxicity in Alzheimer's disease (Malchiodi-Albedi et al., 2001). Ultrastructural studies on post mortem brain tissue of Alzheimer's disease patients have also shown that cortical $A\beta_{1\text{-}42}$ deposits are found within astrocytes and that activated astrocytes are abundant within areas of AD pathology (Wisniewski and Wegiel, 1991). Moreover, astrocytes internalise Aβ42 material and undergo cell lysis when overburdened with amyloid-β, leading to $GFAP^{+}$ astrocytic amyloid plaque deposition (Nagele et al., 2004). Thus, astrocytes contribute towards the clearance of Aβ, albeit the cellular internalisation of Aβ has shown to be deleterious for astrocytes, leading to mitochondrial dysfunction, Ca^{2+} signalling impairments and depletions in intracellular glutathione (Swerdlow, 2011). Astrocytes reportedly clear Aβ in an *ApoE*-dependent fashion as $ApoE^{-/-}$ mice are reportedly incapable of degrading Aβ (Gupta et al., 2013). In any case, the contribution of astrocytes in the pathogenesis of Alzheimer's disease is relatively unknown and remains a topic of ongoing clinical research.

Parkinson's disease: Administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a Parkinsonian symptomology in humans and mice that is almost indistinguishable from the human condition. MPTP-induced toxicity to the nigrostriatal dopaminergic system requires conversion of MPTP to MPP^{+} via the astrocytic enzyme monoamine oxidase B (Mallajosyula et al., 2008). Multiple studies have also highlighted astrocytes as glial gatekeepers towards alpha-synuclein mediated dopaminergic neuropathology; Astrocytic uptake of aberrant human α-synuclein from differentiated SH-SY5Y cells leads to the consequent formation of perinuclear inclusion bodies and a shift towards a pro-inflammatory gene expression profile (Lee et al., 2010). The same authors demonstrate neuron-derived α-synuclein accumulation within astrocytes in transgenic mice over-expressing human α-synuclein, a finding previously reported to initiate non-cell autonomous neurotoxicity to dopaminergic neurons (Rappold and Tieu, 2010). Interestingly, targeted

disruption of Nrf2 (a transcription factor involved in the induction of antioxidant & phase 2 detoxification enzymes) sensitizes mice to 6-OHDA & MPTP toxicity, and astrocyte-restricted Nrf2 activation protects against MPTP-induced dopaminergic neurodegeneration in vivo (Burton et al., 2006; Jakel et al., 2007; Chen et al., 2009). Studies by (Lazzarini et al., 2013) have shown that 6-OHDA-induced motor dysfunction and dopaminergic neuropathology in the SNpc & striatum is associated with increases in $GFAP^+$ reactive astrocytes when assessed 25 days post unilateral striatal lesioning, whereas the neuroprotection conferred on foot of treatment with doxycycline is associated with a reduction in the nigral & striatal astroglial response to 6-OHDA. Other groups have demonstrated that treatment with the naturally derived antibiotic rapamycin (7.5 mg/kg) elevates astrocytic glutamate transporter (GLT-1) expression and function, raises IL-6 production and downregulates pro-inflammatory cytokine expression, ultimately providing protection against the MPTP-induced loss of nigral dopaminergic neurons (Zhang et al., 2017). Taken together, in a context-dependent manner, reactive astrocytes may be polarised to play a protective or neurotoxic role towards midbrain dopamine neurons in classical toxin-based animal models of PD, yet the role of midbrain astrocytes in inflammatory derived Parkinsonism has been relatively unexplored to date.

4.1.3 L-alpha-aminoadipic acid (L-AAA)

L-alpha-aminoadipic acid (L-AAA) is an astroglial-specific toxin that induces a transient dysfunction of astrocytes *in vivo* by entering astroglial cells via Na+-dependent glutamate transporters and inhibiting cellular functions including protein synthesis and metabolic processes (Brown and Kretzschma, 1998; Lima et al., 2014). Bilateral injection of L-AAA into the medial prefrontal cortex (mPFC) induces site-directed astrocytic pathology, progressive neuronal loss and dendritic atrophy of surviving neurons, findings which set the premise for impairments in attentional set shifting & working memory deficits in the water maze (Lima et al., 2014). The authors proclaim that infusion of the astrocyte-specific toxin L-AAA into the PFC is a useful method for better understanding the mechanisms underlying the pathophysiology of specific illnesses involving astrocytic loss/dysfunction within the PFC, such as depression, schizophrenia or bipolar disorder. Similar studies have also shown that L-AAA-induced astroglial ablation within the PFC impairs the glutamate-glutamine cycle and provokes depressive like behaviours in mice and rats (Banasr and Duman, 2008; Lee et al., 2013). Previous studies by (Khurgel et al., 1996) on the characterisation of this astrotoxin has demonstrated that L-AAA exerts a depletion of astrocytes as early as 4 hours post injection, and induces a most prominent depletion of $GFAP^+$ and $S100B^+$ astrocytes at 48 hours post injection. The authors demonstrate that the astrocyte ''free-zone'' was still apparent at 7 days post injection, albeit much reduced in comparison to the earlier time-points tested. Interestingly, at 48 hours post injection, the same authors show vimentin-positive structures (most likely astrocytic processes & debris) at the core of the injection site

but no Vim^+ astrocytes in that area, there were however Vim^+ astrocytes surrounding the focal point of the lesion, and thus, when taken together with the loss of GFAP & S100B-immunopositive astrocytes at the lesion site, indicates astrocytic dysfunction in that area.

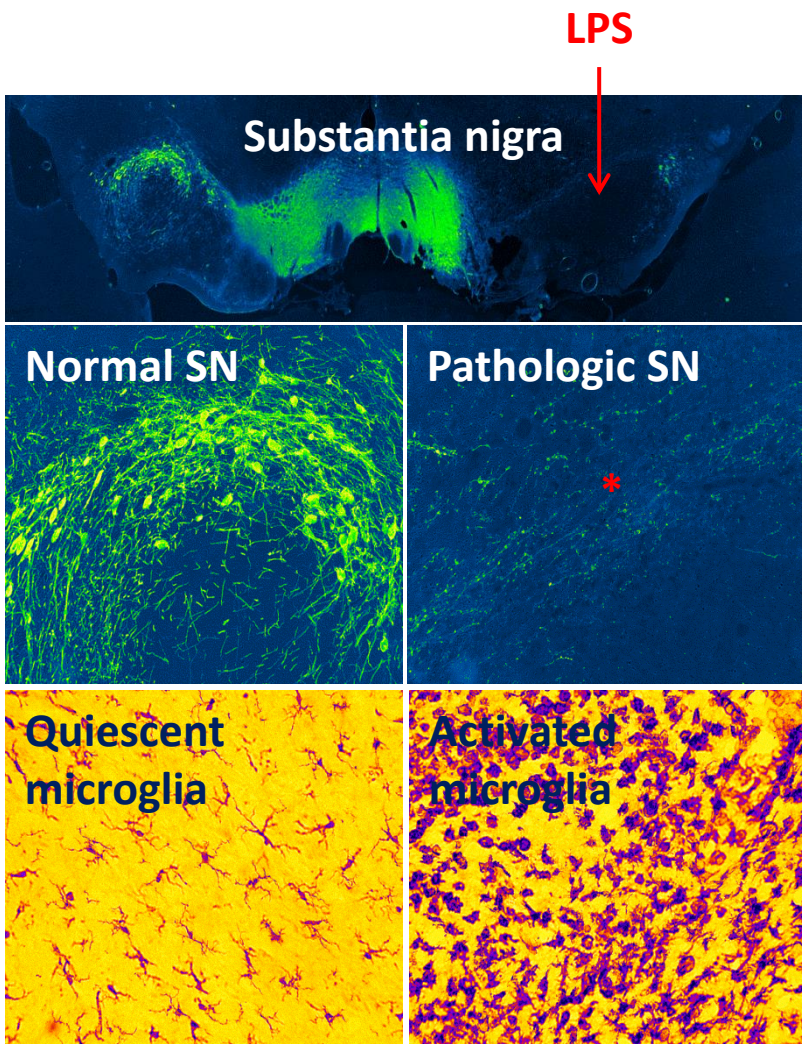
4.2.1 Study aims & objectives

The main aim of this study was to investigate the role of midbrain astrocytes in the pathogenesis of Parkinson's disease. Using the selective astrocytic gliotoxin L-alpha-aminoadipic acid (L-AAA) we aimed to establish whether concurrent astroglial dysfunction affects LPS-induced PD-related neuropathology and motor dysfunction. The three major objectives of the study were as follows:

(1) Behavioural tests of motor function (staircase test of skilled motor function, stepping test of forelimb akinesia and the cylinder test of forelimb use asymmetry) to assess motor function.
(2) Immunohistochemical analysis of midbrain and striatal TH^+ dopaminergic neurons & nerve terminals, $Iba1^+$ microglia and $GFAP^+$ & $S100B^+$ astrocytes to assess nigrostriatal integrity & glial cell activation respectively.
(3) HPLC-ECD analysis of midbrain & striatal DA, DOPAC and HVA concentrations to determine nigrostriatal dopamine content.

Previously, we have demonstrated that a single unilateral intra-nigral injection of LPS (10µg/2µl) induces a robust nigral microgliosis, dopaminergic neurodegeneration, nigrostriatal dopamine loss, and associated motor deficits. Now we aim to focus primarily on the role of astrocytes in this process, i.e. is there evidence to suggest that functional astrocytes play a beneficial role to dopaminergic structures under circumstances of LPS-mediated neuroinflammation? Or do immunocompetent astroglia actively sustain this pro-inflammatory state and contribute to the neurodegenerative process in this rat model of Parkinson's disease? See below for a schematic overview of the possible beneficial/harmful role of reactive astrocytes in Parkinson's disease.

Assessing the role of astrocytes in the inflammatory component of Parkinson's disease



Beneficial

- **α-synuclein uptake & degradation** (major component of Lewy body pathology)
- **Trophic support**
  -GDNF
  -BDNF
  -CDNF
  -MANF
- **Astrogliosis** glial scar formation (prevents overt damage to CNS tissue)
- **GSH** production & release (powerful antioxidant properties)
- **Iron homeostasis** (excess nigral iron = more oxidative stress)

Harmful

- **MAO-B** substrate catabolism induces H_20_2 (ROS) production
- **S100B** (DAMP) secretable cytokine & induces apoptosis at high concentrations
- **Astrogliosis** changes in gene expression (overt IL-6 production)
- **Glial crosstalk** with microglia (activated microglia induce A1 neurotoxic astrocytes)
- **Extracellular purines** (purines bind A_{2A} receptors which opposes DAergic signalling)

Figure 4.1 Assessing the beneficial/harmful role of astrocytes in LPS-mediated neurotoxicity. The present study sought to investigate the role of nigral astrocytes in the pathophysiology of LPS-induced Parkinsonism in the rat. Here we aimed to assess whether reactive astrocytes play a beneficial or harmful role in the degenerating nigrostriatal dopaminergic system in response to an intra-nigral injection of the inflammagen LPS. Currently, we have provided data demonstrating that intra-nigral LPS induces a robust microgliosis, dopaminergic neuronal loss and motor deficits albeit whether or not astrocytes contribute towards or protect against this LPS-mediated Parkinsonian state remains to be established and is indeed, the focus of this study.

4.2.2 Experimental design

Initially, adult male Wistar rats were obtained to characterise the effects of L-AAA, LPS and a combination of LPS+L-AAA on nigral astrocytes, microglia and dopamine neurons at 48 hours (n=16) & at 7 days (n=16) post lesioning, time-points previously shown to prominently and selectively ablate cerebral astrocyte populations surrounding the injection site, particularly at 48 hours post injection (p.i.), with a substantial recovery of $GFAP^+$ astrocytes at 7 days p.i. (Khurgel et al., 1996). For the main study, adult male Wistar rats (n=32) aged 7-8 weeks (220-250g) were obtained from the comparative medicines unit (CMU, TCD) and were group housed in cages of 3 in climate controlled rooms set to 21°C on a 12:12 hour light-dark cycle. Animals were allowed food and water *ad libitum* and were habituated to the animal housing facilities for 1 week prior to commencing any experimental procedures. All behavioural testing was conducted between 09:00 and 18:00. The experimental protocols involved were in compliance with the European directive 2010/63/EU on the protection of animals used for scientific purposes.

Rats were sorted into four treatment groups: (1) Vehicle + Saline, (2) LPS + Saline, L-AAA + Saline, (3) LPS + L-AAA, *n* = 8 per group. Baseline behavioural testing was conducted 5 days prior to stereotactic surgery. Rats received a unilateral intra-nigral injection of LPS (10µg/2µl), vehicle (2µl 0.89% sterile saline), L-AAA (50µg/µl; 2µl) or a combination of LPS + L-AAA (dose as above). The coordinates for SNpc infusion were as follows: anteroposterior (AP) -5.3 mm, mediolateral (ML) ± 2.0 mm and dorsoventral (DV) -8.5 mm. Behavioural testing in the staircase, stepping and cylinder tests was conducted 7 and 14 days post-surgical procedures between the hours of 10-3pm. The following day, rats were either euthanized by transcardial perfusion fixation in preparation for immunohistochemical analysis of nigrostriatal integrity and glial cell activation or decapitated and their brains were free-hand dissected on dry ice in preparation for HPLC analysis of biogenic amine concentrations in the midbrain and striatum.

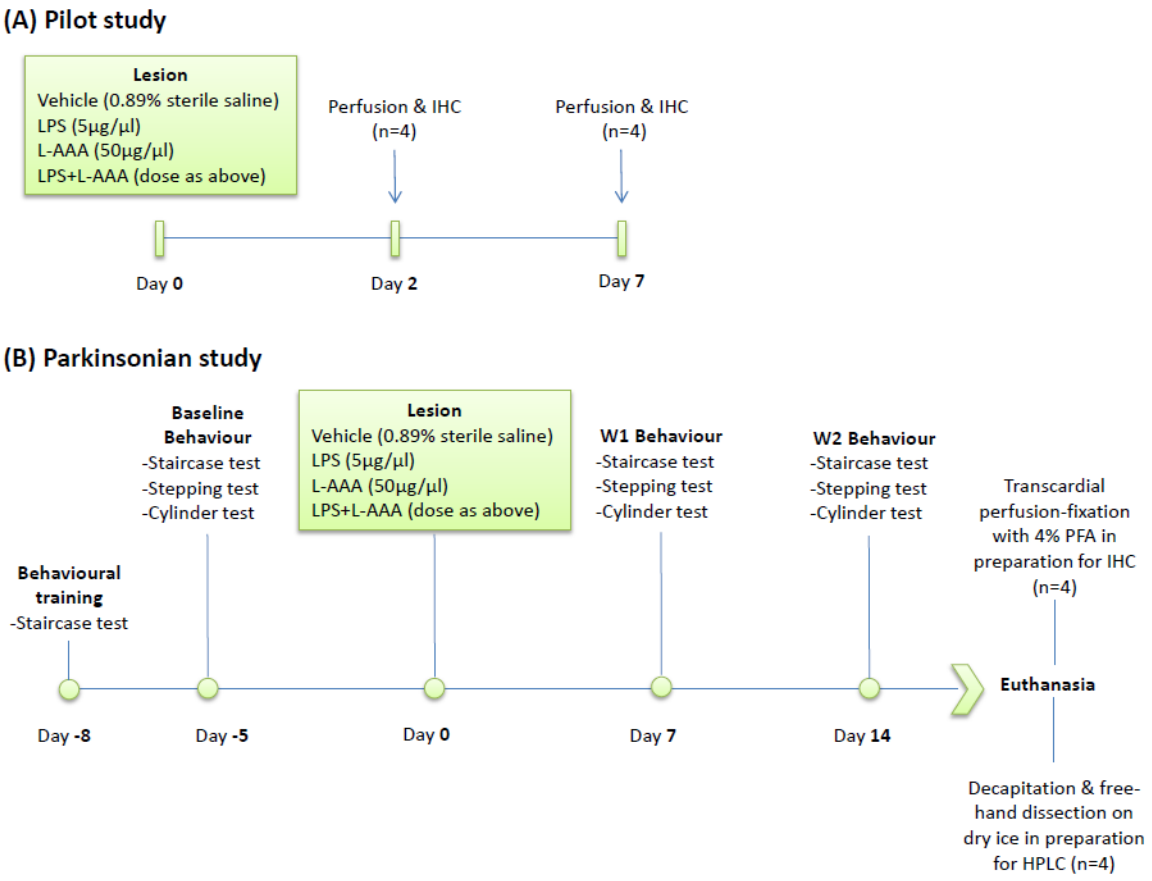


Figure 4.2 Experimental timeline of investigations. In the pilot study (A) rats received an intra-nigral injection of vehicle, LPS, L-AAA or a combination of LPS+L-AAA and were euthanized at either 48 hours or 7 days post lesioning for histopathological assessment. For the main Parkinsonian study (B) the impact of L-AAA-induced astrocytic dysfunction was assessed at a behavioural level in the LPS model of Parkinson's disease. DA concentrations were determined via HPLC analysis and DAergic neuronal loss was assessed by post mortem IHC as previously described.

4.3 Results

4.3.1 Acute astrocytic ablation represses LPS-driven deficits in skilled motor function.

Three-way ANOVA demonstrated an effect of time ($F_{(2, 72)}$ = 30.36, P<0.0001), LPS ($F_{(1, 72)}$ = 47.02, P<0.0001), L-AAA ($F_{(1, 72)}$ = 8.383, P=0.0050), a time x LPS interaction ($F_{(2, 72)}$ = 8.166, P=0.0006), a time x L-AAA interaction ($F_{(2, 72)}$ = 7.889, P=0.0008) an LPS x L-AAA interaction ($F_{(1, 72)}$ = 8.675, P=0.0043) and a time x LPS x L-AAA interaction ($F_{(2, 72)}$ = 5.496, P=0.0060) on contralateral success rate in the staircase test. Bonferroni *post hoc* test revealed that intra-nigral LPS injection reduced the contralateral success rate at both 7 (P<0.01) & 14 (P<0.001) days post lesioning by 22% and 33% respectively relative to vehicle-injected controls. Intra-nigral injection of L-AAA alone had no effect on skilled motor function at 7 or 14 days post lesioning. Rats injected with LPS in combination with L-AAA had a higher contralateral success rate than animals injected with LPS alone at both 7 (P<0.001) and 14 days (P<0.05) post lesioning; co-injection of L-AAA completely attenuated the LPS-induced deficit in skilled motor function at 7 days post-lesioning. At 14 days post-lesioning, the contralateral success rate of animals co-injected with LPS in combination with L-AAA was reduced by a mere 16% There were no significant differences in the number of pellets eaten using the contralateral forelimb between the 4 treatment groups at any time-point tested.

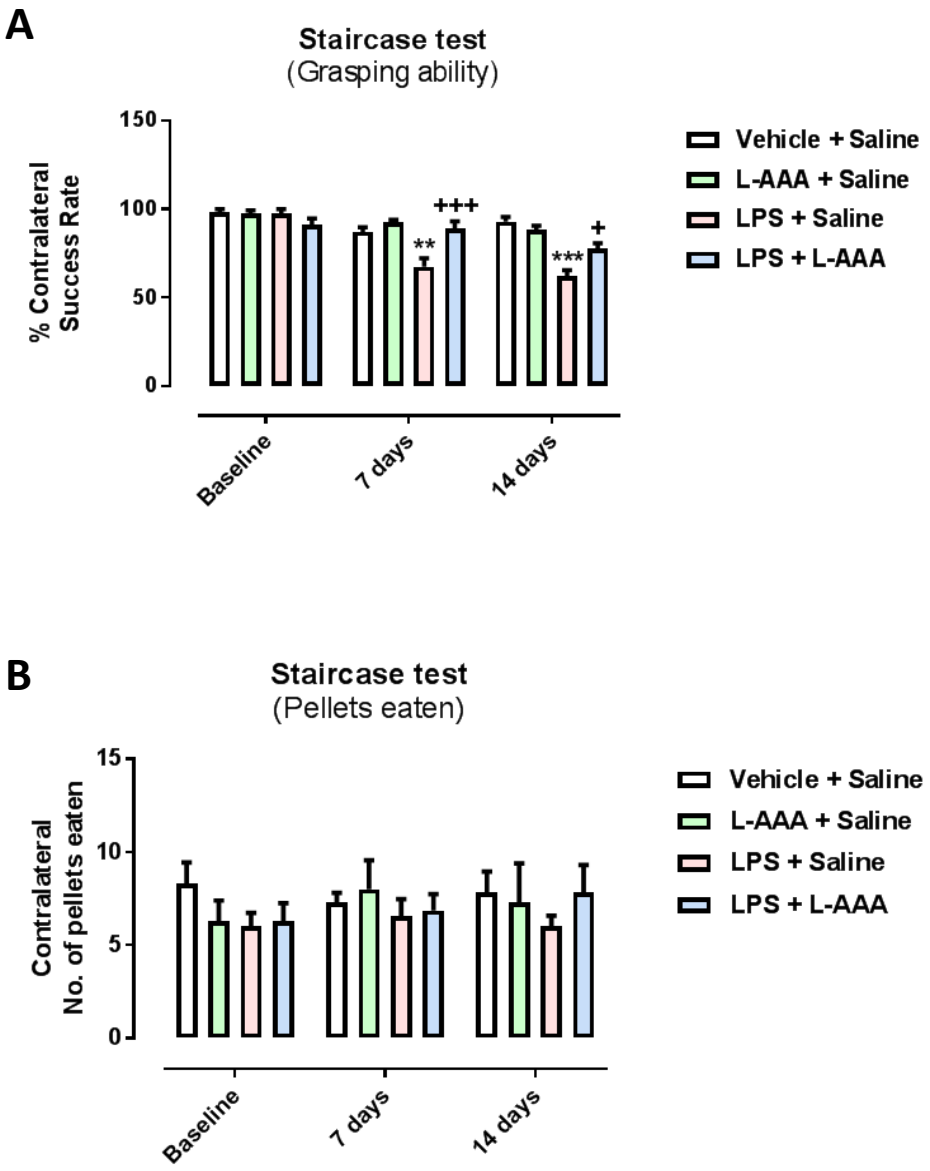


Figure 4.3 Intra-nigral injection of L-AAA represses LPS-induced deficits in skilled motor function in the staircase test. The effect of a unilateral intra-nigral L-AAA injection alone and in combination with LPS on forelimb skilled motor function was assessed using the staircase test. Intra-nigral injection of LPS induced contralateral forelimb deficits in skilled motor function at 7 & 14 days post lesioning. Co-injection of L-AAA impeded LPS-driven impairments in skilled motor function at 7, and to a lesser extent at 14 days post lesioning (A). Neither LPS nor L-AAA had any effect on the total number of food pellets eaten using the contralateral limb at either time-point tested (B). Data are expressed as mean ± S.E.M. (n=8) **P<0.01, ***P<0.001 vs. Vehicle-injected controls, +P<0.05, +++P<0.001 vs. LPS-injected controls by 3-way ANOVA with *post hoc* Bonferroni.

4.3.2 Acute astrocytic ablation inhibits LPS-induced forelimb akinesia.

A three-way ANOVA demonstrated an effect of time ($F_{(2, 84)}$ = 8.767, P=0.0003), LPS ($F_{(1, 84)}$ = 11.71, P=0.0010), an interaction between time x LPS ($F_{(2, 84)}$ = 5.878, P=0.0041) and between LPS x L-AAA ($F_{(1, 84)}$ = 8.731, P=0.0041) on the number of contralateral forehand adjusting steps made in the stepping test of forelimb akinesia. Bonferroni *post hoc* analysis revealed that intra-nigral LPS reduced the no. of adjusting steps made at both 7 and 14 days post lesioning by 27% relative to vehicle-injected controls (P<0.05). Intra-nigral injection of L-AAA alone had no effect on forelimb kinesis in the forehand direction. Intra-nigral co-injection of L-AAA completely attenuated LPS-mediated forelimb akinesia in the forehand direction at 7 days (P<0.05), but not at 14 days post-lesioning whereby a 20% deficit in forelimb kinesis was observed in animals co-injected with LPS in combination with L-AAA.

A three-way ANOVA demonstrated an effect of time ($F_{(2, 84)}$ = 7.612, P=0.0009), LPS ($F_{(1, 84)}$ = 13.06, P=0.0005), and an interaction between time x LPS ($F_{(2, 84)}$ = 3.341, P=0.0401) and between LPS x L-AAA ($F_{(1, 84)}$ = 5.208, P=0.0250) on the number of contralateral backhand adjusting steps made in the stepping test of forelimb akinesia. Bonferroni *post hoc* analysis revealed that intra-nigral LPS reduced the no. of adjusting steps made at both 7 and 14 days post lesioning by approximately 27% on average relative to vehicle-injected controls (P<0.05). Intra-nigral injection of L-AAA alone had no effect on forelimb kinesis in the backhand direction. Animals co-injected with intra-nigral LPS in combination with L-AAA performed a higher no. of contralateral adjusting steps in the backhand direction relative to rats lesioned with intra-nigral LPS alone at both 7 & 14 days post-lesioning, albeit these results were not deemed statistically significant.

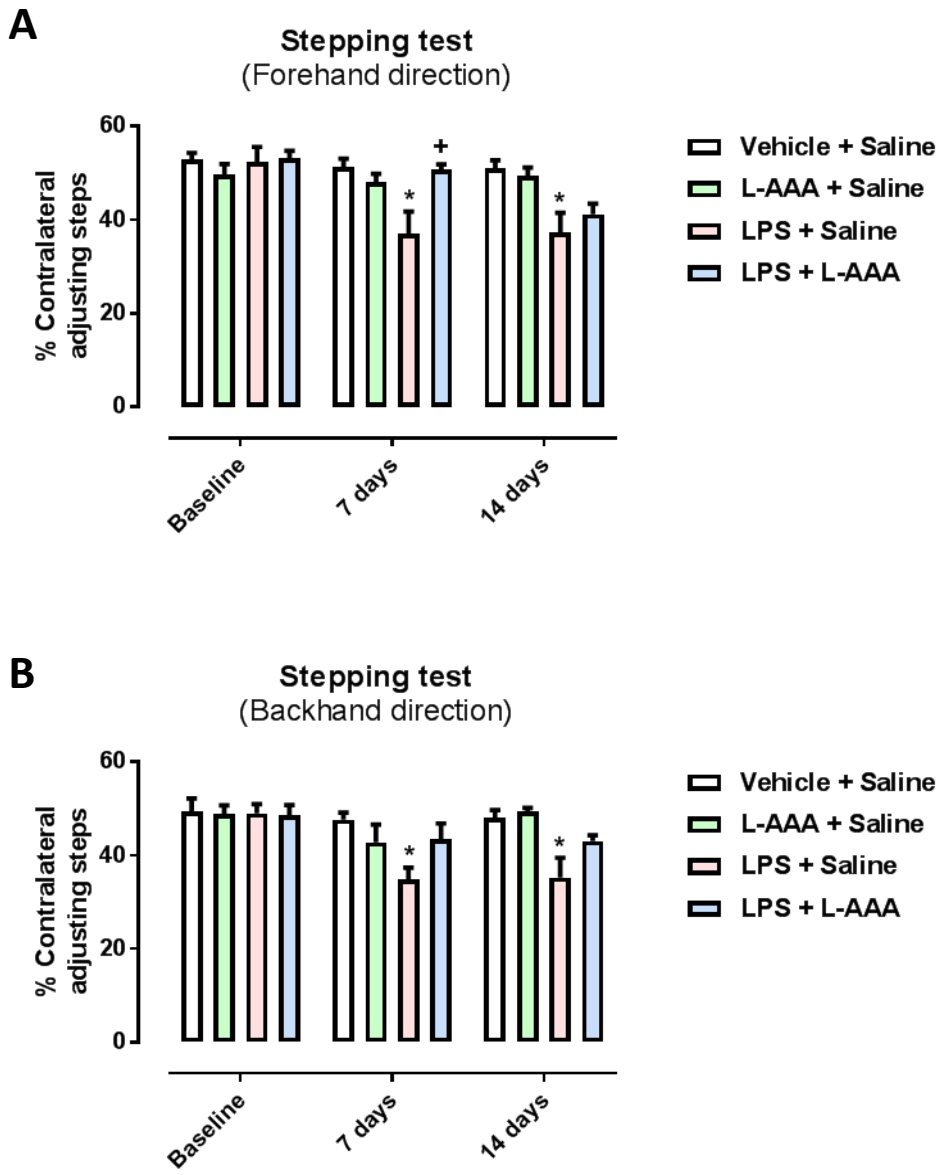


Figure 4.4 Intra-nigral L-AAA injection inhibits LPS-induced forelimb akinesia in the stepping test. The effect of a unilateral intra-nigral L-AAA injection alone and in combination with LPS on forelimb kinesis was assessed using the stepping test. Intra-nigral injection of LPS induced forelimb akinesia in the forehand and backhand direction at 7 & 14 days post lesioning. Co-injection of L-AAA prevented LPS-induced akinetic behaviour in the forehand direction at 7 days post lesioning. Data are expressed as mean ± S.E.M. (n=8) *P<0.05, vs. Vehicle-injected controls, +P<0.05 vs. LPS-injected controls by 3-way ANOVA with *post hoc* Bonferroni.

4.3.3 Acute astrocytic ablation trends towards a suppression of LPS-driven forelimb use asymmetry.

A three-way ANOVA demonstrated an effect of LPS ($F_{(1, 84)}$ = 12.82, P=0.0006) on contralateral wall placements made upon rearing in the cylinder test of asymmetric limb use. Intra-nigral LPS injection induced an observed reduction in contralateral wall placements relative to vehicle-injected controls at both 7 & 14 days post-lesioning, albeit this result was not deemed statistically significant. Intra-nigral injection of L-AAA had no effect on rearing behaviour in the cylinder test. The no. of contralateral wall placements made upon rearing was observably higher in LPS + L-AAA-injected animals at both 7 & 14 days post-lesioning relative to rats receiving an intra-nigral injection of LPS alone, albeit this difference was not deemed statistically significant. Neither intra-nigral LPS or L-AAA alone or in combination had any effect on the number of contralateral floor placements made in the cylinder test at either time-point tested.

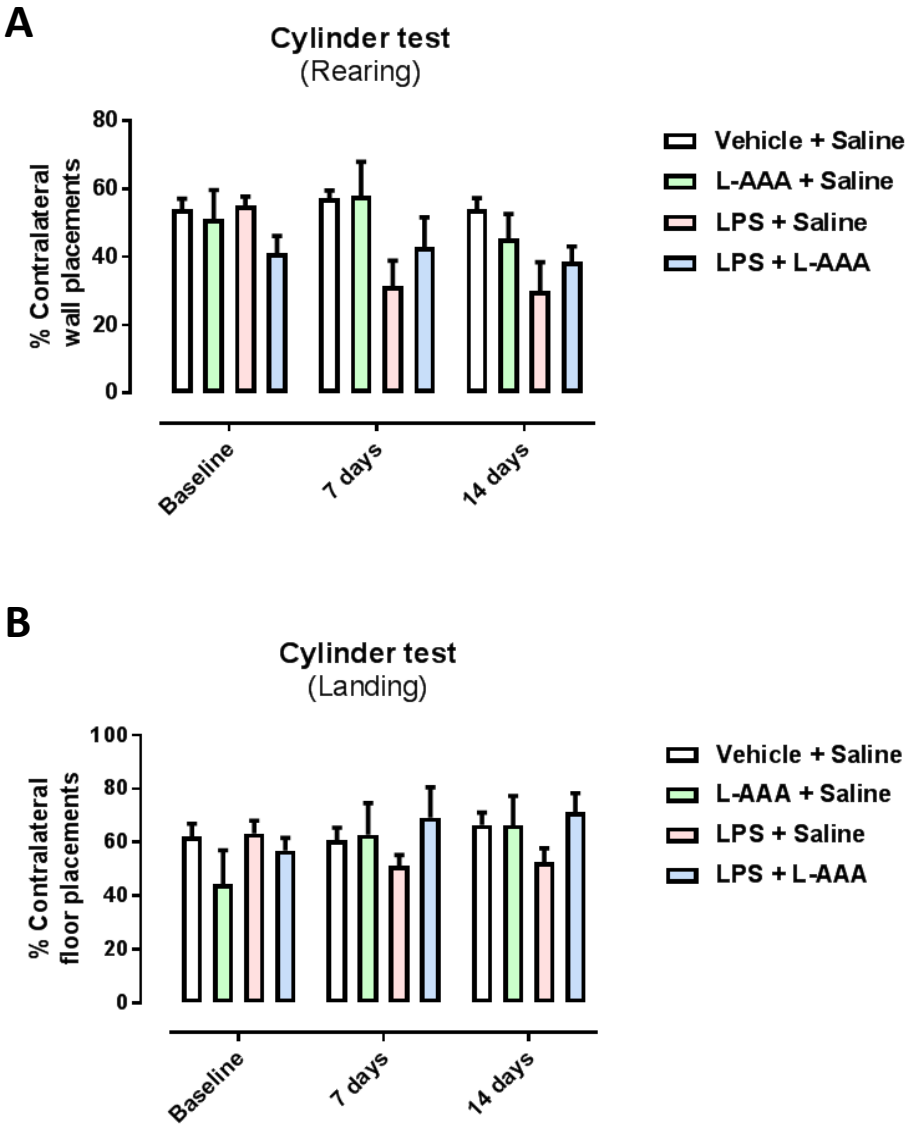


Figure 4.5 Intra-nigral L-AAA injection leads to an observed suppression in forelimb use asymmetry in the cylinder test. The effect of a unilateral intra-nigral L-AAA injection alone and in combination with LPS on forelimb use asymmetry was assessed using the cylinder test. Unilateral injection of LPS lead to an observed reduction in the number of wall placements made using the contralateral limb at 7 & 14 days post lesioning. Co-injection of L-AAA lead to an observed suppression of LPS-induced reductions in wall placements made using the contralateral forelimb at 7 & 14 days post lesioning (A). Neither intra-nigral injection of LPS or L-AAA alone or in combination had any effect on floor placements upon landing in the cylinder test (B). Data are expressed as mean ± S.E.M. (n=8) by 3-way ANOVA with *post hoc* Bonferroni.

4.3.4 Acute astrocytic ablation represses LPS-induced nigrostriatal dopamine loss.

A two-way ANOVA demonstrated an effect of LPS ($F_{(1, 12)}$ = 8.760, P=0.0119) on midbrain dopamine concentration. Bonferroni *post hoc* analysis revealed that unilateral intra-nigral LPS injection induced on average, a 44% reduction in midbrain dopamine content relative to vehicle-injected controls (P<0.05) at 14 days post-lesioning. Intra-nigral injection of L-AAA had no effect on midbrain DA concentrations. Intra-nigral injection of LPS failed to significantly reduce dopamine levels (decreased on average by 28%) in the midbrain when injected in combination with L-AAA relative to vehicle-injected controls. A two-way ANOVA demonstrated an effect of LPS ($F_{(1, 12)}$ = 15.42, P=0.0020) on striatal dopamine concentration. Bonferroni *post hoc* analysis revealed that unilateral intra-nigral LPS injection induced on average, a 31% reduction in striatal dopamine content relative to vehicle-injected controls (P<0.05) at 14 days post-lesioning. Intra-nigral injection of L-AAA had no effect on striatal DA concentrations. Intra-nigral injection of LPS failed to significantly reduce dopamine levels (decreased on average by 15%) in the striatum when injected in combination with L-AAA relative to vehicle-injected controls.

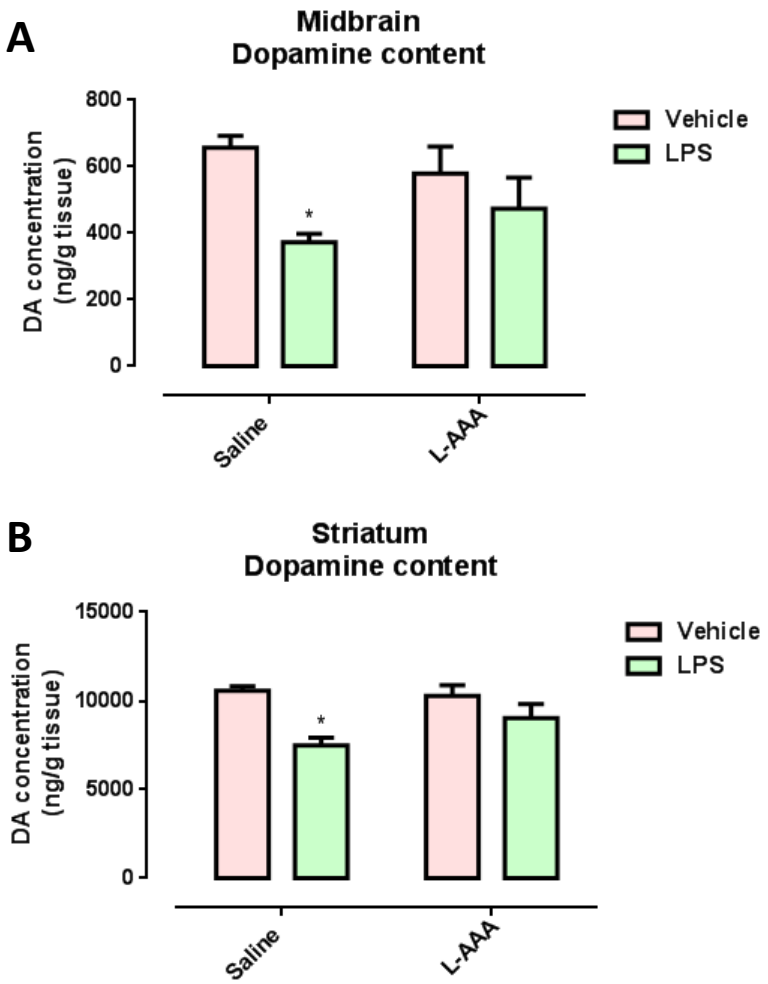
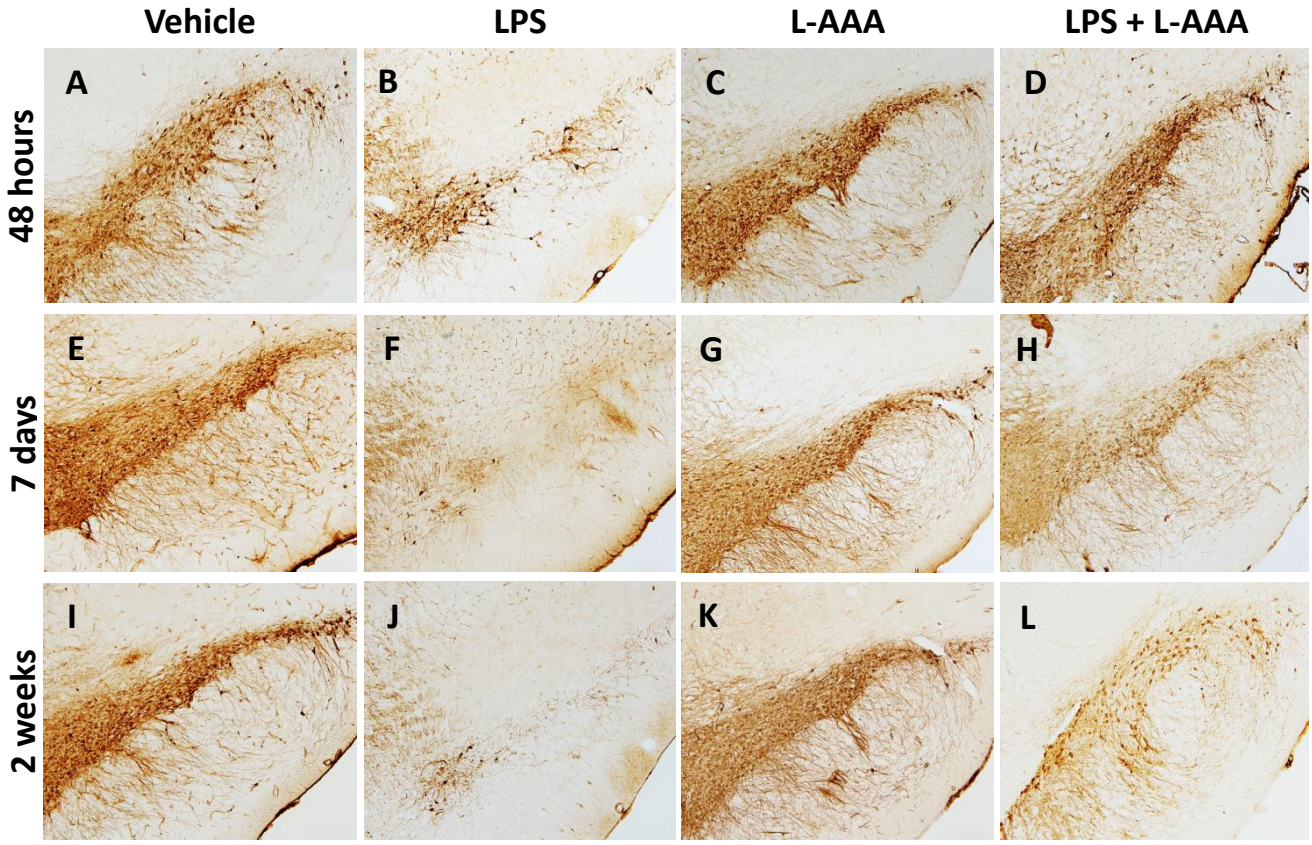


Figure 4.6 Intra-nigral injection of L-AAA represses LPS-driven dopamine loss in the midbrain and striatum. The effect of a unilateral intra-nigral injection of L-AAA alone and in combination with LPS on nigrostriatal dopamine levels was assessed via HPLC-ECD. Unilateral intra-nigral injection of LPS reduced midbrain and striatal DA content. A reduction in DA concentrations was not evident in the midbrain or striatum when LPS was co-injected with L-AAA (A+B). Data are expressed as mean ± S.E.M. (n=4) *P<0.05 vs. control by 2-way ANOVA with *post hoc* Bonferroni.

4.3.5 Acute Astrocytic ablation limits LPS-induced dopaminergic neurodegeneration within the substantia nigra.

A three-way ANOVA demonstrated an effect of LPS ($F_{(1, 36)}$ = 61.74 P<0.0001), an effect of L-AAA ($F_{(1, 36)}$ = 6.364, P=0.0162) and an interaction effect between LPS x L-AAA ($F_{(1, 36)}$ = 6.646, P=0.0142) on nigral TH-immunoreactivity. Bonferroni *post hoc* analysis revealed a reduction in TH^+ dopamine neurons in the SNpc at 7 & 14 days post-lesioning by 48% and 58% on average respectively relative to vehicle-injected controls. Intra-nigral injection of L-AAA had no effect on nigral TH-immunoreactivity when assessed at any time-point. Despite inducing observed reductions in nigral TH-immunoreactivity, intra-nigral LPS failed to induce a significant loss of dopamine cells in the SNpc when simultaneously co-injected with L-AAA at 48 hours, 7 days, and 2 weeks post-lesioning.



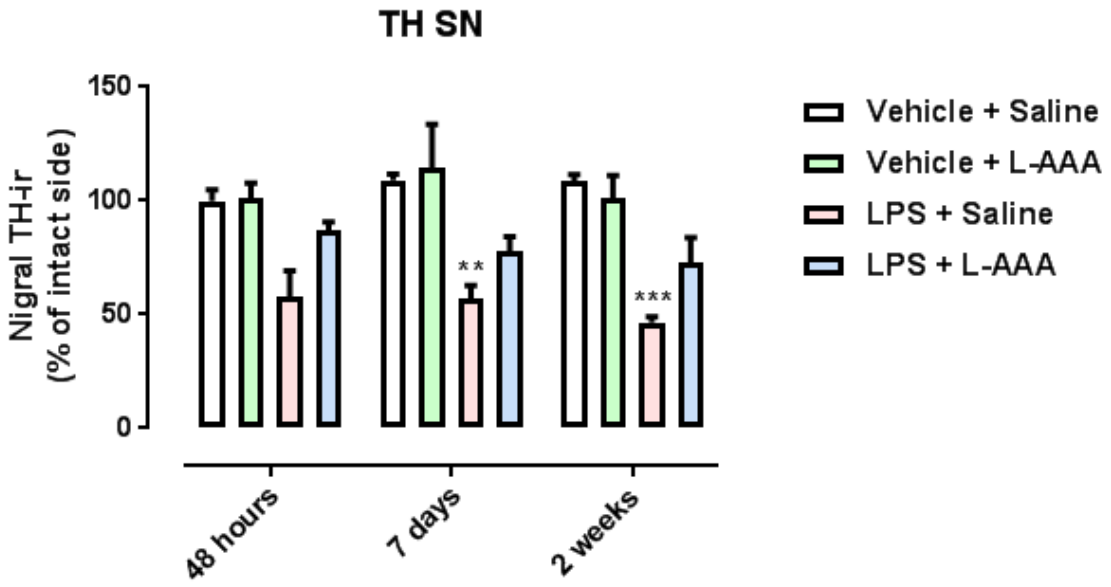
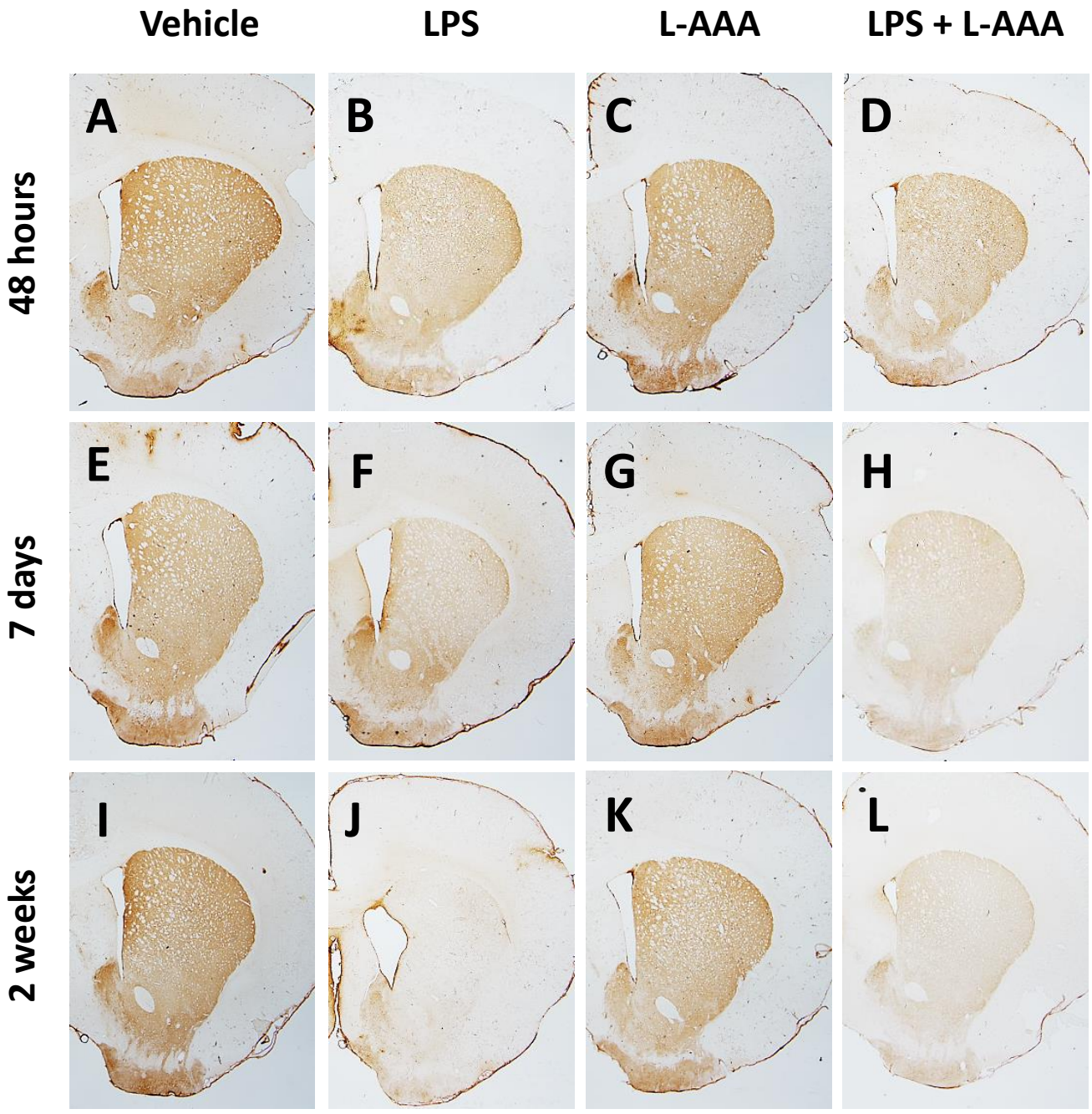


Figure 4.7 Intra-nigral injection of L-AAA supressed LPS-induced dopaminergic neurodegeneration within the substantia nigra. The effect of a unilateral intra-nigral injection of L-AAA alone and in combination with LPS on nigral dopamine neurons was assessed via anti-TH immunohistochemistry. Unilateral intra-nigral LPS injection induced dopaminergic neurodegeneration within the SNpc at 48 hours, 7 days & 2 weeks post lesioning. Unilateral co-injection of L-AAA limited the extent of LPS-induced dopaminergic cell loss within the substantia nigra (A-L). Data are expressed as mean ± S.E.M. (n=4) **P<0.01, ***P<0.001 vs. Vehicle-injected controls by 3-way ANOVA with *post hoc* Bonferroni.

4.3.6 Acute astrocytic ablation delays the progression of LPS-induced nerve terminal degeneration in the ipsilateral striatum.

A three-way ANOVA demonstrated an effect of time ($F_{(2, 36)} = 3.914$, P=0.0290) an effect of LPS ($F_{(1, 36)} = 45.22$, P<0.0001) and an interaction effect between time x LPS ($F_{(2, 36)} = 5.566$, P=0.0078) on striatal TH-immunoreactivity. Bonferroni *post hoc* analysis revealed that intra-nigral LPS injection induced a robust loss of TH^+ dopaminergic nerve fibres in the ipsilateral striatum by approximately 59% on average when compared to vehicle-injected controls at 14 days post-lesioning. Intra-nigral injection of L-AAA alone had no effect on striatal TH-immunoreactivity at any time-point tested. Intra-nigral injection of LPS in combination with L-AAA reduced striatal TH-immunoreactivity by approximately 42% on average at 14 days post-lesioning relative to vehicle-injected controls (P<0.05), albeit not to the same extent as when intra-nigral LPS was injected alone.


Vehicle
LPS
L-AAA
LPS + L-AAA
48 hours
7 days
2 weeks
A
B
C
D
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H
I
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K
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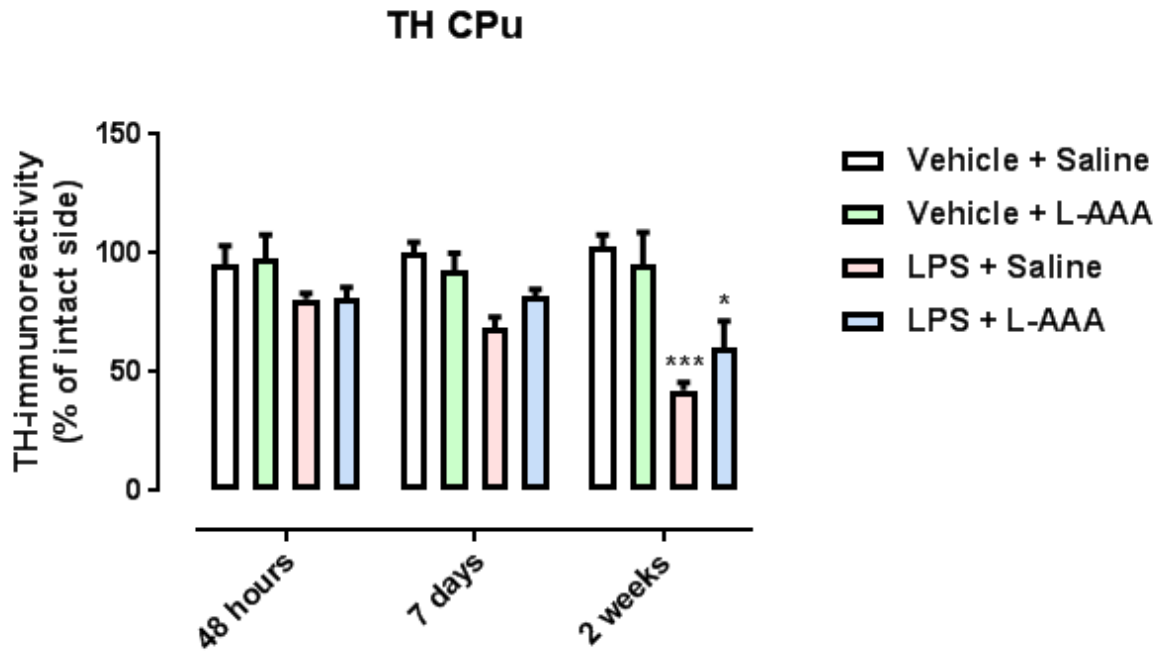
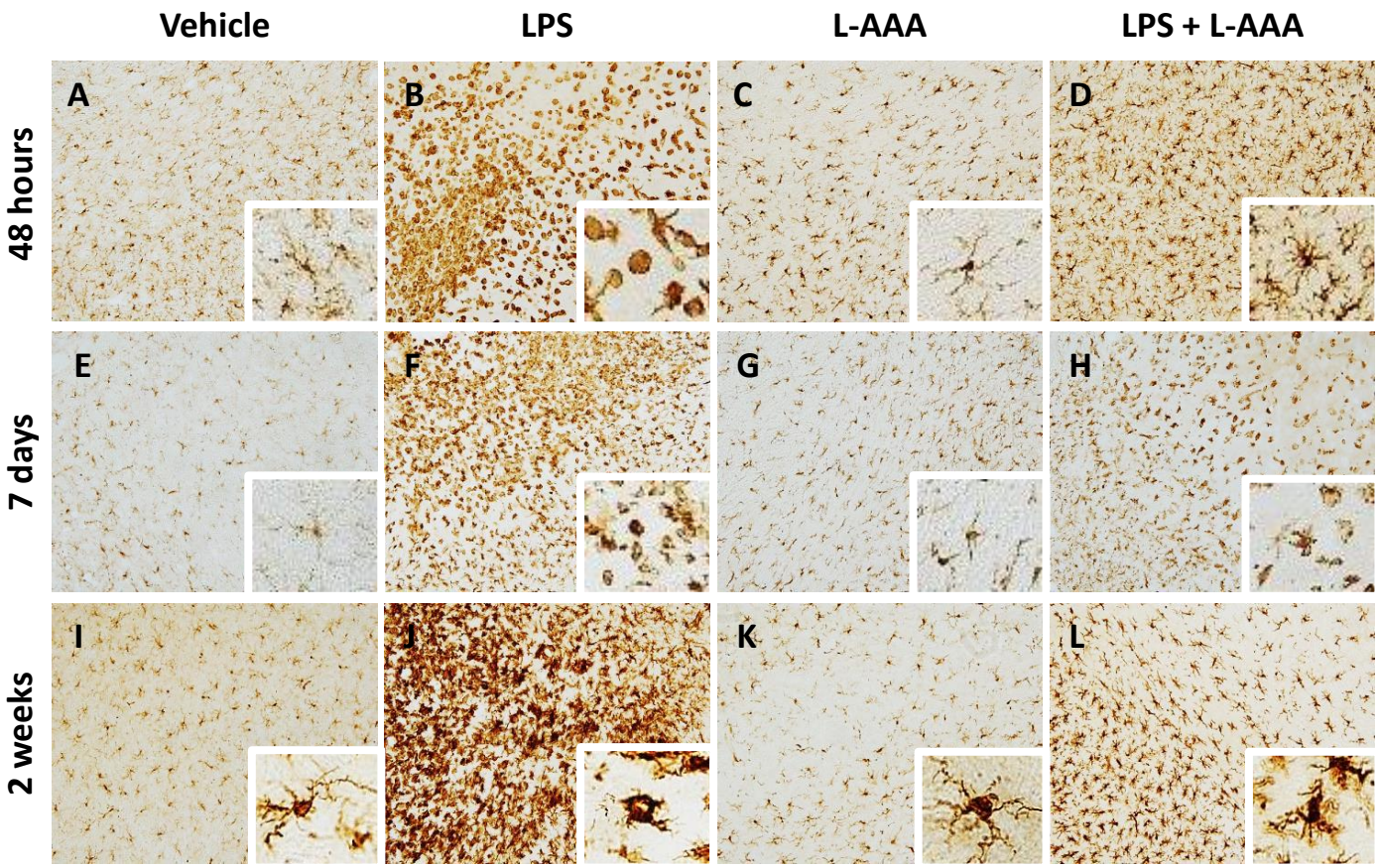


Figure 4.8 Intra-nigral injection of L-AAA restrains LPS-induced dopaminergic nerve terminal degeneration in the striatum. The effect of a unilateral intra-nigral injection of L-AAA alone and in combination with LPS on striatal nerve terminal integrity was assessed via anti-TH immunohistochemistry. Unilateral intra-nigral injection of LPS induced dopaminergic nerve terminal degeneration in the ipsilateral striatum. Co-injection of L-AAA repressed LPS-induced dopaminergic nerve terminal loss in the ipsilateral striatum (A-L). Data are expressed as mean ± S.E.M. (n=4) *P<0.05, *** P<0.001 vs. Vehicle-injected controls by 3-way ANOVA with *post hoc* Bonferroni.

4.3.7 Acute astrocytic ablation inhibits LPS-induced microglial activation within the substantia nigra.

A three-way ANOVA demonstrated an effect of time ($F_{(2, 36)}$ = 7.084, P=0.0025), an effect of LPS ($F_{(1, 36)}$ = 106, P<0.0001), an effect of L-AAA (F(1, 36) = 29.73, P<0.0001), an interaction effect of time x LPS ($F_{(2, 36)}$ = 6.3, P=0.0045), between LPS x L-AAA ($F_{(2, 36)}$ = 11.71, P=0.0001) and between time x LPS x L-AAA ($F_{(2, 36)}$ = 10.34, P=0.0003) on nigral Iba1-immunoreactivity. Bonferroni *post hoc* analysis revealed that intra-nigral LPS injection increased the no. of $Iba1^+$ cells in the SN at 48 hours (P<0.01), 7 days (P<0.001) & 2 weeks (P<0.001) post-lesioning relative to vehicle-injected controls. Intra-nigral injection of L-AAA alone had no effect on Iba1-immunoreactivity at any time-point tested. Co-injection of L-AAA suppressed the LPS-induced microgliosis at 7 days post lesioning (P<0.001).

A three-way ANOVA demonstrated an effect of LPS ($F_{(1, 36)}$ = 52.07, P<0.0001) and an interaction effect of LPS x L-AAA ($F_{(1, 36)}$ = 16.22, P=0.0003) on nigral Iba1-immunoreactivity. Bonferroni *post hoc* analysis revealed that intra-nigral LPS decreased $Iba1^+$ cell perimeter length at 48 hours (P<0.01), 7 days (P<0.05) & 2 weeks (P<0.001) post lesioning relative to vehicle-injected controls. Intra-nigral injection of L-AAA alone had no effect on $Iba1^+$ cell perimeter length. Co-injection of L-AAA lead to an observed mitigation in LPS-induced decreases in $Iba1^+$ cell perimeter length at all time-points tested, albeit these differences were not deemed statistically significant.



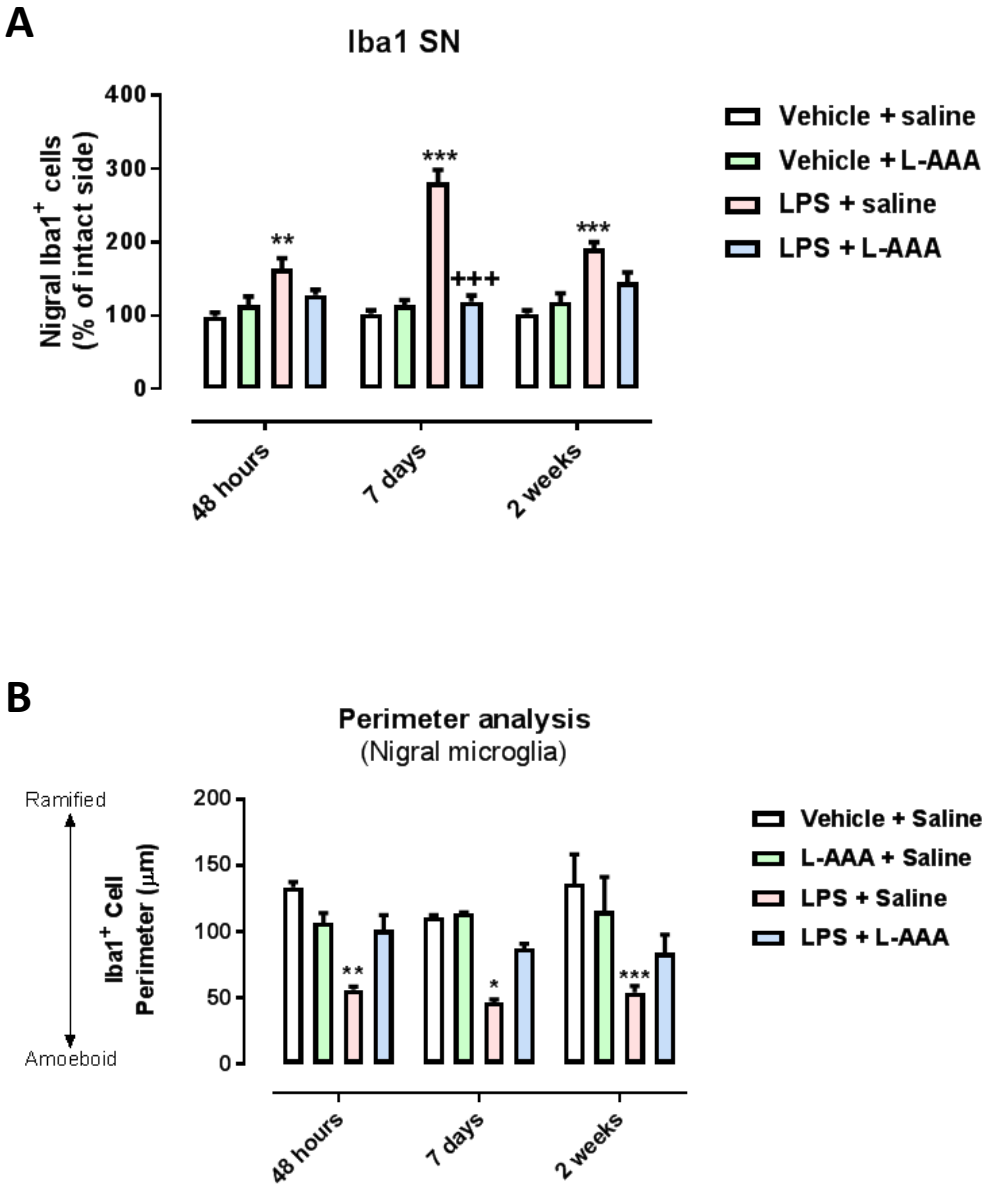


Figure 4.9 Intra-nigral L-AAA administration restrains LPS-induced microglial activation within the substantia nigra. The effect of a unilateral intra-nigral injection of L-AAA alone and in combination with LPS on nigral microglial activation was assessed via anti-Iba1 immunohistochemistry. Unilateral intra-nigral injection of LPS induced a robust microgliosis ipsilateral to the injection site. Unilateral injection of L-AAA in combination with LPS curtailed the LPS-induced increase in microglial cell numbers (A) and suppressed microglial activation (B) within the substantia nigra. Data are expressed as mean ± S.E.M. (n=4) *P<0.05, **P<0.01, *** P<0.001 vs. Vehicle-injected controls, +++P<0.001 vs. LPS-injected controls by 3-way ANOVA with *post hoc* Bonferroni.

4.3.8 Intra-nigral injection of L-AAA in combination with LPS suppresses astrocyte activation within the substantia nigra.

A three-way ANOVA demonstrated an effect of time ($F_{(2, 36)}$ = 7.19, P=0.0024), an effect of LPS ($F_{(1, 36)}$ = 129.1, P<0.0001), an effect of L-AAA ($F_{(1, 36)}$ = 104, P<0.0001) and an interaction effect between LPS x L-AAA ($F_{(1, 36)}$ = 72.19, P<0.0001), and between time x LPS x L-AAA ($F_{(2, 36)}$ = 7.997, P=0.0013) on nigral GFAP-immunoreactivity. Bonferroni *post hoc* analysis revealed an increase in the number of $GFAP^+$ astrocytes in response to intra-nigral lesioning with LPS at 48 hours (P<0.001), 7 days (P<0.001) & 2 weeks (P<0.001) relative to vehicle-injected controls. Intra-nigral injection of L-AAA reduced GFAP-immunoreactivity by 78% on average relative to vehicle-injected controls at 48 hours post-lesioning, albeit this result was not deemed statistically significant. The L-AAA-mediated deficits in nigral GFAP-immunoreactivity were largely diminished by 7 days post-lesioning and were completely absent by 2 weeks post-lesioning. Simultaneous co-injection of L-AAA abrogated the intra-nigral LPS-induced increases in nigral GFAP immunoreactivity at 48 hours (P<0.01), 7 days (P<0.001), & 2 weeks (P<0.001) post-lesioning.

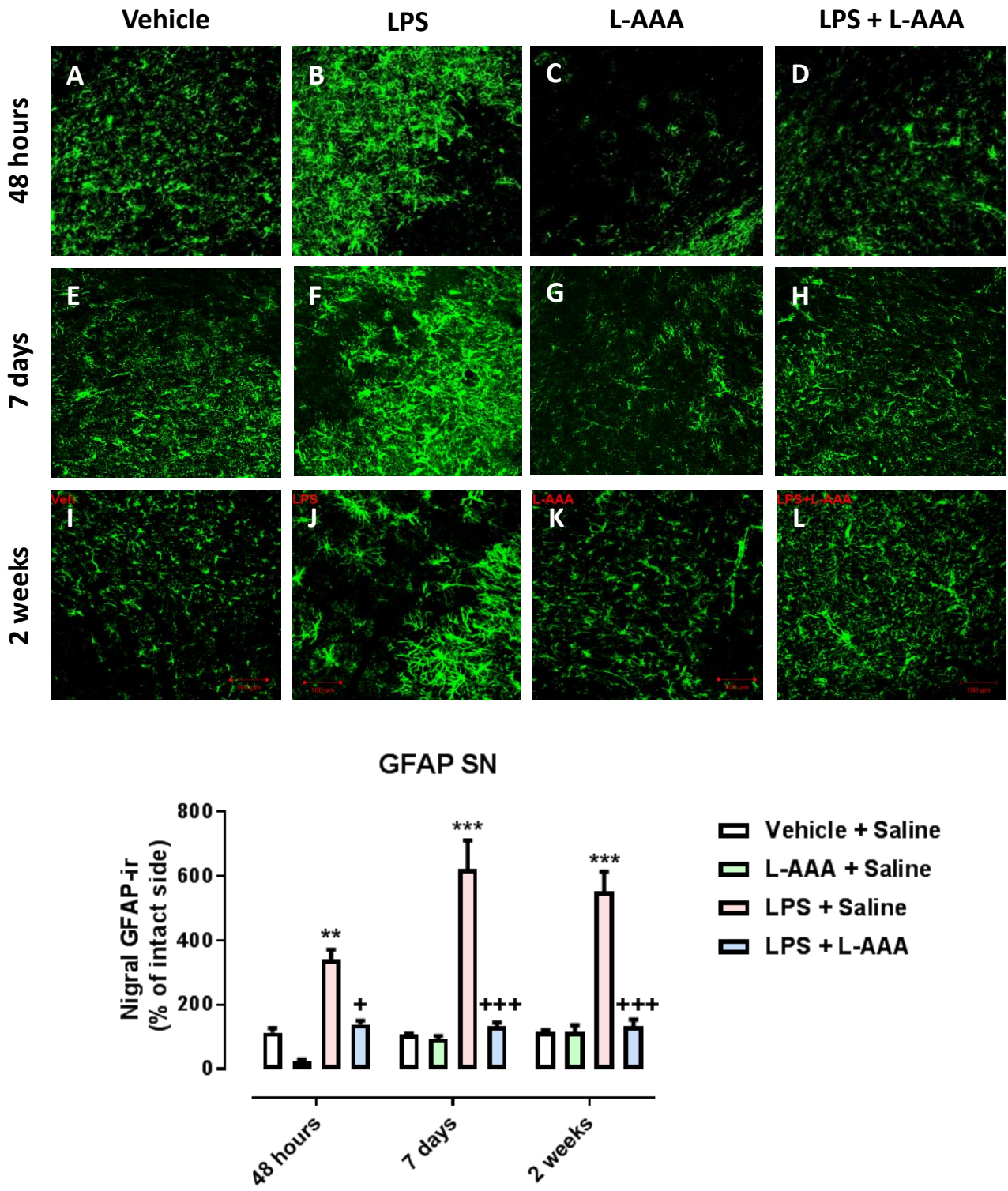


Figure 4.10 Intra-nigral L-AAA injection restrains LPS-induced astrocytic activation. The effect of a unilateral intra-nigral injection of L-AAA alone and in combination with LPS on nigral astrocyte populations was assessed via anti-GFAP immunohistochemistry. Unilateral intra-nigral injection of LPS induced a robust increase in GFAP-enriched astrocytes. Co-injection of L-AAA suppressed LPS-induced astrocytic activation (A-L). Data are expressed as mean ± S.E.M. (n=4) ** P<0.01, *** P<0.001 vs. vehicle-injected controls, + P<0.05, +++ P<0.001 vs. LPS-injected controls by 3-way ANOVA with *post hoc* Bonferroni.

4.3.9 Intra-nigral injection of L-AAA inhibits LPS-mediated increases in nigral S100β expression

A three-way ANOVA demonstrated an effect of LPS ($F_{(1, 36)}$ = 28.69, P<0.0001), an effect of L-AAA ($F_{(1, 36)}$ = 37.84, P<0.0001), an interaction effect between time x L-AAA ($F_{(2, 36)}$ = 6.603, P=0.0036) and between LPS x L-AAA ($F_{(1, 36)}$ = 14.1, P=0.0006) on nigral S100β expression. Bonferroni *post hoc* analysis revealed that intra-nigral LPS injection increased S100β expression at all time-points tested, but most prominently at 7 days post-lesioning relative to vehicle-injected controls (P<0.001). Intra-nigral injection of L-AAA decreased S100β expression on average by 74% at 48 hours post-lesioning relative to vehicle-injected controls albeit this result was not deemed statistically significant. The effect of L-AAA on nigral S100β expression was largely diminished by 7 days post-lesioning, and was completely absent when assessed at 2 weeks post-lesioning. Simultaneous intra-nigral co-injection of L-AAA largely suppressed the LPS-induced increases in S100β expression at 48 hours (P<0.001) and 7 days (P<0.001) post-lesioning, but not after 2 weeks.

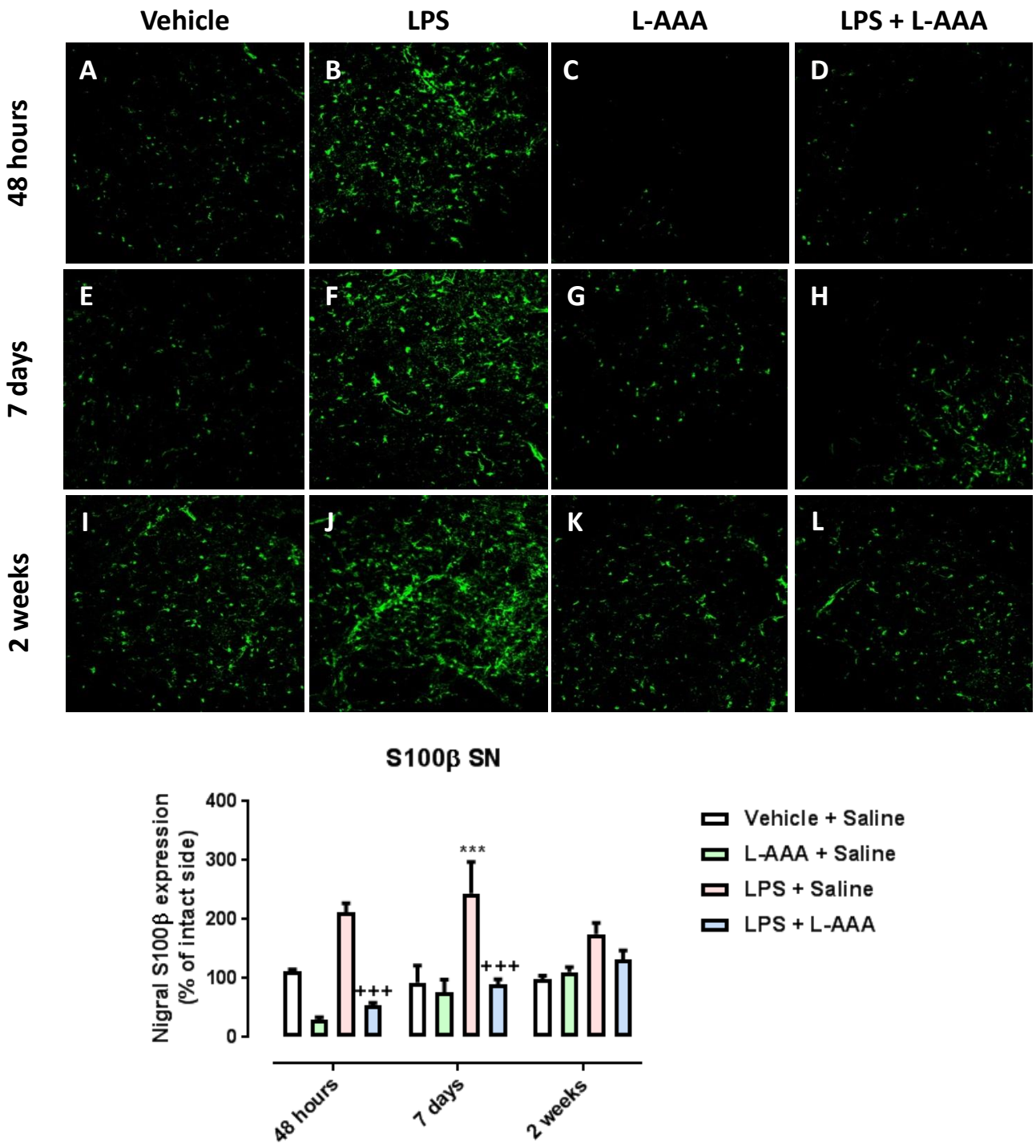


Figure 4.11 Intra-nigral L-AAA injection mitigates LPS-induced increases in S100B expression in the SNpc. The effect of a unilateral intra-nigral injection of L-AAA alone and in combination with LPS on nigral astrocyte activity was assessed via anti-S100β immunohistochemistry. Unilateral intra-nigral injection of LPS increased S100β expression. Co-injection of L-AAA suppressed LPS-induced S100β expression (A-L). Data are expressed as mean ± S.E.M. (n=4) ***P<0.001 vs. vehicle-injected controls, +++P<0.001 vs. LPS-injected controls by 3-way ANOVA with *post hoc* Bonferroni.

4.4 Discussion

Reactive astrogliosis, characterised by progressive changes in cellular gene expression profiles and glial scar formation (Sofroniew, 2009), is a graduated process that varies with insult severity in multiple neurodegenerative disease pathologies (Pluchino et al., 2003; Olabarria et al., 2010; Tong et al., 2014). It remains unclear however, whether or not reactive astrocytes play a beneficial or harmful role in an inflammatory context in Parkinson's disease, and indeed how prominent a role these cells play in contributing towards or protecting against a Parkinsonian state. The present study investigated the role of midbrain astrocytes in the pathophysiology of LPS-induced Parkinsonism in rats.

Unilateral intra-nigral LPS injection resulted in a robust gliosis within the substantia nigra, dopamine neuron loss, reductions in nigrostriatal dopamine levels and spontaneous motor deficits. Concurrent L-AAA mediated nigral astrocytic dysfunction suppressed the LPS-induced gliopathy within the substantia nigra, mitigated the nigrostriatal dopamine loss and partially abrogated the associated motor dysfunction. Specifically, concurrent intra-nigral L-AAA injection limited the LPS-mediated dopaminergic neurodegeneration within the substantia nigra, and repressed nerve terminal loss in the ipsilateral striatum. These findings were underpinned by attenuated glial cell activation within the SN in response to lesioning with LPS in the presence of L-AAA. Co-injection of L-AAA also suppressed the LPS-induced reductions in midbrain & striatal dopamine content and partially attenuated the ensuing impairments in forelimb kinesis and skilled motor function. Here, we have identified a causative role of reactive astrocytes in contributing towards the pathogenesis of a Parkinsonian phenotype in circumstances where inflammation sets the precedence for neuropathology and motor dysfunction.

A recent study by (Liddelow et al., 2017) has shown that LPS-activated microglia induce the formation of neurotoxic astrocytes (termed A1 astrocytes) via the secretion of IL-1α, TNF, and C1q, and that not only do A1 astrocytes kill oligodendrocytes and axotomized CNS neurons, but they also lose the ability to promote neuronal survival & outgrowth, to promote synapse formation and to phagocytose myelin debris *in vivo*. The author's further show through co-immunofluorescence staining for C3 & GFAP in post mortem Parkinson's disease brain tissue that 30-60% of nigral astrocytes exhibit an A1 neurotoxic phenotype, and that treatment of human embryonic stem cell differentiated dopamine neurons with A1 astrocyte conditioned media (50µg ml^{-1}) leads to a 25% loss in neuronal viability due to A1-mediated neurotoxicity. These findings are in line with our current data demonstrating that L-AAA-mediated nigral astrocytic dysfunction prevents overt damage to the nigrostriatal dopaminergic system when it is concurrently mediated by LPS-driven neurotoxicity, and thus highlights a role for neurotoxic astrocytes in the pathophysiology of Parkinson's disease. Given the emerging role that neurotoxic astrocytes play in neuroinflammatory conditions and neurodegenerative diseases (Lobsiger and Cleveland, 2007; Capani et al., 2016), one explanation for the results gathered herein is that L-AAA-induced astroglial

dysfunction prevented LPS-activated microglia from inducing the formation of A1 neurotoxic astrocytes, and thus mitigated the astrocytic contribution towards LPS lesioning of the nigrostriatal tract. In essence, L-AAA-induced astrocytic dysfunction suppressed the neurotoxic effects of astrocytes induced by LPS-activated microglia, leading to reduced gliosis, attenuated dopamine loss and overall improvements in motor function. Indeed, it has been shown recently that pharmacological blockade of microglial-mediated A1 neurotoxic astrocyte conversion with the long-acting, lipophilic glucagon-like peptide-1 receptor (GLP1R) agonist NLY01 (3 mg/kg s.c twice weekly for 5 months) attenuated dopaminergic neuronal loss in the SNpc, dopamine depletion in the striatum and motor deficits in the α-synuclein preformed fibril (α-syn PFF) mouse model of sporadic Parkinson's disease (Yun et al., 2018). NLY01 suppressed α-syn PFF-mediated increases in microglial IL-1α, TNF-α & C1q secretion, attenuated increases in nigral $C3^+$/$GFAP^+$ cells and associated increases in A1-specific transcripts, essentially blocking microglial-derived A1 reactive astrocyte formation. The authors further demonstrate that the therapeutic effects of NLY01 also extended to the human A53T α-synuclein (hA53T) transgenic mouse model of α-synucleinopathy-induced familial Parkinsonism, prolonging the lifespan and abrogating the development of neuropathological features & behavioural deficits in these animals. These findings, in conjunction with that of our own, provide strong evidence for the role of reactive astrocytes in contributing towards the pathogenesis of a Parkinson's disease state in response to secretable factors released from either LPS- or α-synuclein-induced microglial activation, and bear particular clinical relevance as Parkinson's disease itself is an age related neurodegenerative disease and the normal aging process alone has been demonstrated to induce an A1-like reactive phenotype in striatal astrocytes (Clarke et al., 2018).

At a behavioural level, similar to studies by (Hoban et al., 2013) demonstrating the impact of an intracerebral LPS injection on lateralised motor function in rats, intra-nigral LPS injection induced bi-directional forelimb akinesia and impairments in skilled motor function at 7 and 14 days post lesioning. Concurrent intra-nigral injection of L-AAA inhibited LPS-driven deficits in forelimb kinesis in the forehand & in the backhand direction of the stepping test at 7 and 14 days post lesioning and also protected against skilled motor dysfunction at 7 days, and to a lesser extent at 14 days post lesioning in the staircase test. These behavioural improvements are likely to be ascribed to the repression of LPS-induced nigrostriatal neurodegeneration & dopamine loss in rodents co-injected with L-AAA. At a biochemical level, as previously reported in studies by (Zhou et al., 2005), intra-nigral LPS injection reduced nigrostriatal dopamine content. Here, intra-nigral injection of LPS depleted midbrain & striatal dopamine content relative to vehicle-injected controls when administered alone, which was partially attenuated when injected in combination with L-AAA. Taken together, the suppression of LPS-mediated motor impairments in animals co-injected with L-AAA is afforded by increases in nigrostriatal dopamine concentrations by

virtue of restraining neurotoxic astrocytes from contributing to the detrimental effects of an LPS lesion on the nigrostriatal dopaminergic system.

These behavioural and biochemical findings are bolstered by an array of immunohistochemical data demonstrating a protective effect of L-AAA-mediated astrocytic dysfunction on glial cell activation and nigrostriatal degeneration in response to lesioning the SNpc with LPS. Our data demonstrate that intra-nigral LPS administration induced a reduction in nigral TH-immunoreactivity (dopamine neurons & associated neuronal fibers) and a loss of TH^+ dopaminergic nerve terminals in the ipsilateral striatum relative to vehicle-injected controls. When LPS was simultaneously co-injected with L-AAA however, dopaminergic neurodegeneration was limited in the SNpc, and nerve terminal loss in the ipsilateral striatum was also repressed when compared to vehicle-injected controls. These findings are similar to that of (Takada et al., 1990) demonstrating that concurrent intra-nigral L-AAA-induced astroglial ablation abolishes MPTP-mediated neurotoxicity of the nigrostriatal dopaminergic system. Intra-nigral LPS injection lead to a robust $Iba1^+$ microgliosis and a $GFAP^+$ astrogliosis in the lesioned SNpc. Perimeter analysis revealed that $Iba1^+$ microglia in the LPS-lesioned SN exhibited a retraction of processes and a slightly enlarged cell soma, ultimately culminating in a decrease in overall cell perimeter length, a classical morphological phenotype indicative of activated microglia. Interestingly, when LPS was simultaneously co-injected with L-AAA the microgliosis was attenuated, and there was not the same extent of morphological alterations observed in nigral $Iba1^+$ cells, the microglia in this instance were evidently exhibiting an intermediate activation state particularly at 14 days post-lesioning. Studies by (Yu et al., 2018) have shown that the oxidative stress-sensitive transcription factor early growth response-1 (Egr-1) is promptly, albeit transiently upregulated in protoplasmic $AldoC^+$ nigral astrocytes and promotes neuroinflammation and dopaminergic neurodegeneration in the MPTP model of PD. The same authors demonstrate that MPTP-induced upregulation of astrocytic Egr-1 expression precedes morphological microglial activation and that pharmacological inhibition of Egr-1 transcriptional activity with Mithramycin A, or genetic ablation of *Egr-1* suppresses both astrogliosis and microgliosis, decreases midbrain pro-inflammatory cytokine expression and attenuates the loss of dopamine neurons in the SNpc in response to MPTP administration. Taken together, these results would imply that reactive astrocytes promote microglial activation within the nigra in response to oxidative stress and contribute to dopaminergic neurotoxicity in a comparable manner as to what we have shown in response to lesioning with LPS in our present investigation.

Thus, we propose that bidirectional glial crosstalk between LPS-activated microglia and neighbouring midbrain astrocytes at the interface of dopaminergic neurodegeneration plays a prominent role in the pathogenesis of inflammatory-derived Parkinsonism. Glial-crosstalk has also been reported to be implicated in the pathogenesis of Alzheimer's disease, as Aβ-induced astroglial NfκB hyper-activation and subsequent C3a release and microglial C3a-C3aR signalling compromises microglial phagocytosis of Aβ *in vitro*, and heightens Aβ plaque

load in APP transgenic mice *in vivo,* whereas treatment with a C3aR antagonist suppresses microgliosis & amyloid pathology, thus corroborating the astrocytic C3a-microglial C3aR axis to Aβ dynamics & AD neuropathology (Lian et al., 2016). More recent studies in this field by (Xu et al., 2017) have shown that astrocyte-derived CCL2 production promotes microglial activation and M1 polarisation via the CCL2-CCR2 axis, leading to elevations in hippocampal TNF-α, IL-1β, Tau protein expression, neuronal apoptosis & learning memory deficits in rats, findings which were inhibited by a CCR2 antagonist. These findings support our data indicating that reactive astrocytes actively sustain microglial activation in the inflamed brain, as in our current study, intra-nigral injection of LPS failed to induce the same extent of $Iba1^+$ microglial activation in the presence of dysfunctional astrocytes. Thus, glial crosstalk is likely to be a bidirectional process between astrocytes and microglia, proceeding at the interface of LPS-mediated neurotoxicity of dopamine neurons.

It is important to note however, that despite the evident crosstalk between microglia & astrocytes under pro-inflammatory conditions, astrocytes themselves are responsive to TLR-4 stimulation as well, and are also solely capable of signalling through NfκB, MAPK & Jak1/Stat1 pathways leading to the production of TNF-α, IL-15, IL-27, VCAM-1, IL-10 & MMP-9 which may contribute directly to an LPS lesion (Gorina et al., 2011). Indeed, cytokine stimulation (TNF & IL-1) of human astrocytes reduces their neuroprotective properties and switches their gene profile to a neurodegenerative one, triggering the synthesis & release of soluble, transferrable factors that actively kill human neurons *in vitro* (Efremova et al., 2017). Astrocytes are an inductive cellular source of Interleukin-6 (IL-6) production in the inflamed brain (Van Wagoner et al., 1999), a key soluble mediator linked to pro-inflammatory signalling cascades leading to neurodegeneration (Goetzl et al.; Bellaver et al., 2018; Neal et al., 2018). Elevations in nigral IL-6 expression has previously been reported in the MPTP (Kohutnicka et al., 1998), intra-striatal 6-OHDA (Goes et al., 2018), rotenone (Sharma et al.) and intra-nigral LPS (Yssel et al., 2018) animal models of PD, and is upregulated in the CSF of Parkinson's disease patients (Blum-Degena et al., 1995) in a manner which is inversely correlated to disease severity (Müller et al., 1998).

Keeping in line with the documented evidence that reactive astrocytes can secrete soluble factors that activate microglia and promote neurodegeneration, studies by (Kim et al., 2016) have demonstrated a pathogenic role for glial-derived Lipocalin-2 (LCN2) in the nigrostriatal dopaminergic system of Parkinson's disease patients and in the MPTP & 6-OHDA animal models of PD. The authors show that LCN2 expression is upregulated in the SN of PD patients and in MPTP & 6-OHDA lesioned animals, primarily within reactive astrocytes of the nigra and the striatum. The MPTP-induced glial cell activation (increases in nigral $Iba1^+$ microglia & $GFAP^+$ astrocytes), pro-inflammatory mediator production (raised nigral IL-1β & TNF-α expression), dopaminergic neurodegeneration and abnormal locomotor activity were ameliorated in LCN2-deficient mice. Moreover, data from the same study demonstrates that the MPP^+-induced neurotoxicity of co-cultured mesencephalic neurons and WT astrocytes was abrogated in mesencephalic neuron-enriched co-cultures containing astrocytes

harbouring an LCN2 gene deficiency. Hence, reactive astrocytes are likely to be immense inflammatory effectors in the process of cytokine-mediated neurodegeneration. This is further exemplified in studies by (Barcia et al., 2011) demonstrating that TNF-α is more highly expressed in astroglia than microglia within the SNpc of chronic Parkinsonian monkeys in response to lesioning the DAergic system with MPTP, which is an important finding considering that mice deficient in TNF receptors ($TNFR1^{-/-}$ & $TNFR2^{-/-}$) are protected against MPTP (12.5 mg/kg s.c.)-induced dopaminergic neurotoxicity and astrogliosis (SRIRAM et al., 2002). Data from the same Barcia, Ros et al. study has also shown that MPTP-induced elevations in IFN-γ & TNF-α are crucial pro-inflammatory cytokines for microglial & astroglial activation prior to the loss of dopamine neurons and that the levels of IFN-γ & TNF-α in the SNpc of Parkinsonian monkeys correlates with the degree of dopaminergic neurodegeneration and motor impairment. The authors show in experiments involving MPTP-injected IFN-γ & TNF-α KO mice that both cytokines play a synergistic role in perpetuating surrounding glial cell activation states in a reciprocal manner and highlight that intercellular crosstalk between astrocytes and microglia via IFN-γ & TNF-α signalling is essential to fully activate both types of glial cells.

Moreover, numerous astrocytes within the LPS-lesioned SNpc were full-bodied and exhibited GFAP-enriched fibrous projections entangled in a glial scar, indicative of astrogliosis. When LPS was simultaneously co-injected with L-AAA however, there was limited evidence of astrogliosis, and few full-bodied astrocytes were discernible in the lesioned SNpc relative to that of rats lesioned with LPS alone. It is likely therefore, that L-AAA-induced astrocytic dysfunction in this instance, prevented LPS from inducing an astrogliosis in response to the inflammagen. The L-AAA-mediated impediment in glial scar formation in response to lesioning the nigra with LPS underscores the level of astrocytic impairment, which may contribute at least partially, to the suppressive effect of astroglial dysfunction on the neuro-inflammatory profile & toxicity of dopamine neurons within the SNpc. This may indeed facilitate the influx of soluble factors capable of slowing the propagation of the inflammatory lesion which may have otherwise been incapable of doing so due to the densely hypertrophied filamentous meshwork of $GFAP^{+}$ astrocytes blocking their entrance, as evident in LPS-lesioned animals with fully immunocompetent astroglia present.

Studies by (Sathe et al., 2012) have revealed that S100B protein expression is increased in the substantia nigra of post mortem Parkinson's disease brain tissue and that S100B CSF levels are higher in these patients compared with controls. The same authors demonstrate that genetic ablation of S100B reduces dopaminergic neurodegeneration, nigral microgliosis and TNF-α expression in MPTP-injected mice, demonstrating a role for astrocytic S100B in the pathophysiology of Parkinson's disease. Given that S100B acts as a damage associated molecular pattern (DAMP) protein in Parkinson's disease via the RAGE/TNF-α pathway (Sathe et al., 2012), causes neuronal death by apoptosis at high concentrations (Sorci et al., 2010) and is linked to striatal DA loss and impairments in motor coordination on the rotarod

test (Liu et al., 2011), we decided in the present investigation, to assess whether increases in S100B protein expression could be implicated in the LPS-mediated neurotoxicity of dopaminergic neurons. Here we show that intra-nigral LPS injection upregulates S100B protein expression in the post mortem SNpc. Simultaneous L-AAA-induced astrocytic dysfunction protects against LPS-induced increases in nigral S100B expression however, findings which were associated with a mitigated nigral microgliosis, death of dopaminergic neurons, nigrostriatal dopamine loss and associated motor deficits in these animals. Studies by (Xu et al., 2016) have shown that treatment of primary microglial cultures with recombinant S100β activates microglia and upregulates TNF-α, IL-1β & iNOS gene expression to a greater extent than LPS, and promotes the release of NO and matrix metalloproteinase 9 (MMP9), findings which were replicated when cultured microglia were exposed to lysed astrocytes but blocked in the presence of a PARP-1 (regulator of microglial activation) inhibitor. Likewise, the same authors demonstrate that intra-striatal delivery of S100β (0.5µg) induces robust microglial activation, as indexed morphologically by a retraction in $Iba1^{+}$ cell processes & an enlargement of their cell soma, accompanied by increases in TNF-α, IL-1β & iNOS mRNA expression and MMP9 immunoreactivity when assessed 24 hours later in the mouse striatum, findings which were again attenuated by the inhibition of PARP-1. Ligation of S100B to receptor for advanced glycation end products (RAGE) induces the formation of hypertrophic stellate astrocytes, promotes local astroglial mitosis, polarises astrocytes into a pro-inflammatory phenotype (increased TLR2, iNOS & IL-1β expression), facilitates cell migration towards sites of CNS injury and upregulates RAGE expression in astrocytes, which can further potentiate S100B-mediated feed-forward autocrine loops (Villarreal et al., 2014). But what is perhaps more concerting than the autocrine effects of astrocytic S100B production are the paracrine effects of S100B on microglia; it has been reported that S100B activates microglia via engaging with RAGE, stimulating NfκB and AP-1 transcriptional activity and the ensuing production of IL-1β, TNF-α & COX-2 expression and that S100B synergises with IL-1β & TNF-α to upregulate COX-2 expression in microglia (Bianchi et al., 2010).

Given that both LPS and S100β are ligands for RAGE (Yamamoto et al., 2011), and that RAGE is expressed on astrocytes & microglia as well as dopamine neurons, the astrocytic input towards initiating neurodegenerative signalling cascades in the guise of overt S100β production, both exclusively, and in tandem with microglia, is of pre-eminent relevance to our current investigation. A recent finding by (Gasparotto et al., 2018) has shown that RAGE cellular localisation and expression changes from endothelial to neuronal cells & is vastly upregulated in dopamine neurons of the SNpc in particular (RAGE + TH co-localisation) 15 days post LPS administration (5 mg/kg i.p.), findings which were accompanied by decreases in nigral TH protein expression and concomitant increases in Iba1, GFAP, IL-1β, TNF-α and phosphorylated ERK1/2 protein levels. Importantly, concurrent, targeted multi-modal inhibition of RAGE in the SN with FPS-ZM1 (40µg) attenuates intra-nigral 6-OHDA (10µg)-mediated increases in RAGE, NfκB p65 nuclear translocation, $Iba1^{+}$ & $GFAP^{+}$ microglial and

astrocyte activation respectively, reduces circulating serum & CSF TNF-α & IL-1β levels and ameliorates TH^+ nigral cell loss & affiliated striatal denervation, findings which were accompanied by attenuated 6-OHDA-induced motor deficits in locomotion and exploratory behaviour (Gasparotto et al., 2017). Thus, astrocytes may indeed be covert usurpers of the reigns of the midbrain inflammatory axis in response to LPS by virtue of copious S100β production and ensuing RAGE-mediated neurotoxicity of dopamine neurons. Taken together, increases in astrocyte-derived S100B could have major implications for the propagation of reactive gliosis in response to brain injury & throughout the course of neurodegenerative disease. These findings support our data demonstrating that LPS-induced elevations in nigral S100B expression at the peak of astrogliosis is associated with robust microglial activation and dopaminergic neuronal loss, whereas L-AAA-induced astroglial dysfunction within the nigra lead to a diminished gliosis and limited dopaminergic neurodegeneration in response to lesioning the SNpc with LPS. Thus, the astrocytic contribution towards an LPS-induced Parkinsonian phenotype in the current study may be attributed at least in part, to raised S100B protein levels within the substantia nigra, and indeed the partial neuroprotection observed in the LPS+L-AAA group may also be attributed to the inability of astrocytes to produce high levels of this protein.

An intricate study by (Mallajosyula et al., 2008) involving a genetically engineered adult mouse line in which MAO-B expression is increased specifically within astrocytes (mimicking the age-related increase in humans) has shown that elevations in murine astroglial MAO-B expression leads to the selective, progressive loss of dopaminergic neurons in the SNpc, local nigral microglial activation, increases in mitochondrial oxidative stress in dopaminergic neurons and decreased locomotor activity *in vivo*. The authors show that the elevations in astrocytic MAO-B expression was induced globally throughout the brain and yet lead to the preferential death of dopaminergic neurons within the substantia nigra, implying that dopamine itself may be involved in this process. Indeed, substrate oxidation by MAO-B within astrocytes leads to membrane-permeant H_2O_2 production, which can diffuse into nearby dopaminergic structures and oxidize dopamine to dopaminochrome (DACHR) which in turn, by interacting with mitochondrial complex I can increase superoxide production. Given that MAO-B is primarily expressed in astrocytes (Levitt et al., 1982);(Westlund et al., 1985) and that it's activity levels are doubled within the SN of Parkinson's disease patients and correlate with dopaminergic neuronal loss (Damier et al., 1996), midbrain astrocytic derived MAO-B catalysed reactive oxygen species production poses an encroaching threat to the dopaminergic system, particularly when dopamine neurons are inherently vulnerable to oxidative stress due to low antioxidant levels within the SN (Perry et al., 1982). Interestingly, data from the same study revealed that co-treatment with minocycline (a microglial activation inhibitor) attenuated the loss of dopamine neurons within the SNpc, indicating that microglial activation plays a role in the demise of dopaminergic neurons associated with astrocytic MAO-B elevations and ensuing Parkinson's pathology in mice (i.e. H_2O_2 generated by MAO-B oxidation can stimulate microglia as well, secondary microglial

activation is enhanced due to the death of DAergic neurons, which leads to copious ROS production and exacerbated damage to local dopaminergic neurons). These results, along with that of (Liddelow et al., 2017) are in tandem with our observations from the current study indicating that when the immune-competency of nigral astrocytes is compromised due to L-AAA-induced astrocytic dysfunction, LPS-activated microglia fail to induce the formation of neurotoxic astrocytes and thus, the effects of LPS on the nigrostriatal dopaminergic system are deputised exclusively to microglia alone, and by virtue of which, the impact of the lesion is suppressed.

In summary, L-AAA mediated acute astrocytic impairment diminished LPS-induced neurotoxicity of dopamine neurons in the SNpc, preserved striatal nerve terminal integrity and limited motor dysfunction, findings which were underpinned by attenuated gliosis and reductions in nigral S100β expression. LPS-activated nigral microglia may be incapable of inducing the formation of A1 neurotoxic astrocytes in the presence of L-AAA. Moreover, under the premise of an astrocytic deficit L-AAA may be inadvertently rendering nigral microglia less capable of responding to an inflammatory stimulus when the functional immune-competency of nigral astrocytes is compromised (see schematic, below, as a diagrammatic illustration of potential routes of L-AAA induced impediment of glial crosstalk at the interface of dopaminergic neurodegeneration).

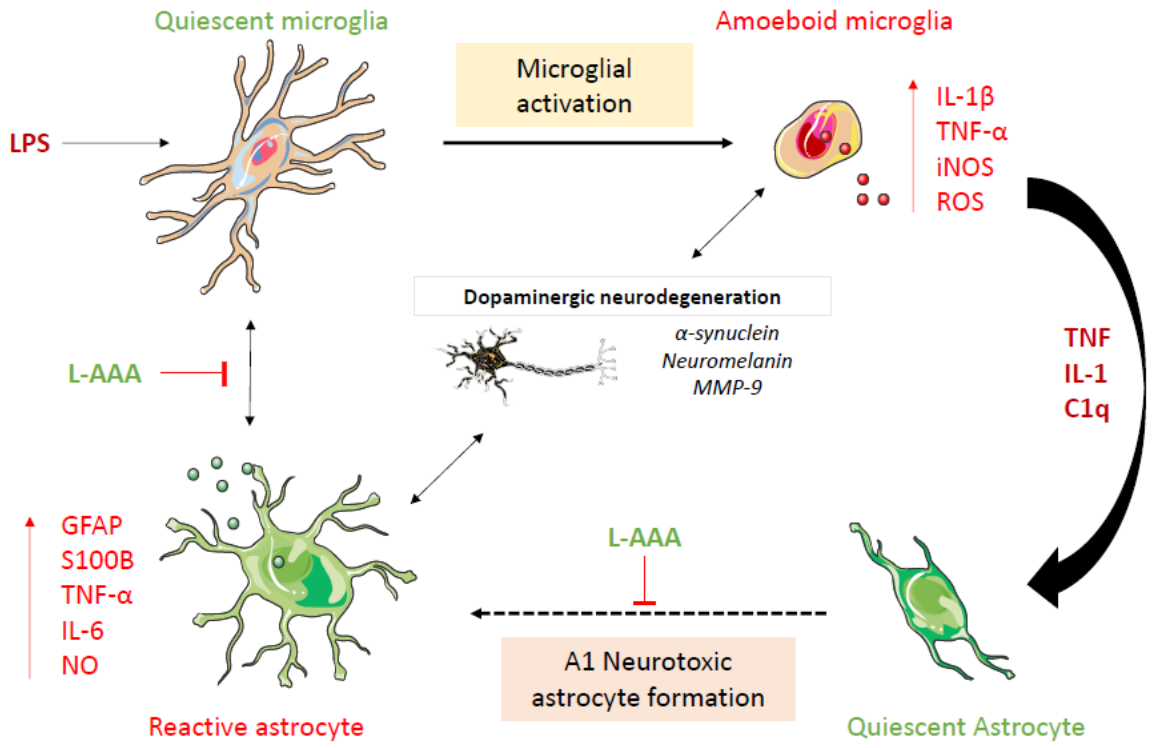


Figure 4.12 Glial crosstalk at the interface of dopaminergic neurodegeneration; routes of interference for L-AAA. LPS-activated microglia induce the formation of A1 neurotoxic astrocytes via the secretion of TNF, IL-1 & C1q. Reactive astrocytes promote the neurotoxicity of dopamine neurons via the overt production of soluble, transferrable factors (e.g. S100β, TNF-α, IL-6, and nitric oxide). This highlights a primary route of interference for L-AAA to exert neuroprotective effects in response to an immune stimulus. Reactive astrocytes may also actively sustain microglial activation within the SNpc (e.g. astrocyte derived S100β acts on microglial RAGE receptors to mediate apoptosis of dopamine neurons via downstream signalling through the RAGE/TNF-α pathway), a second route of interference for L-AAA to mitigate LPS-induced dopaminergic neuronal loss. Moreover, damaged/dying dopamine neurons release immunogenic factors (e.g. α-synuclein, neuromelanin, MMP-9) that can further precipitate reactive gliosis and ongoing dopaminergic neurotoxicity. Thus, a bidirectional glial crosstalk between nigral microglia & reactive astrocytes exists at the interface of dopaminergic neurodegeneration which may be a crucial cellular process underlying the chronic inflammatory component of Parkinson's disease progression.

Thus, taken together with our current data, a mechanistic interplay exists between reactive astrocytes and microglia at the interface of nigrostriatal neurodegeneration and motor dysfunction, which opens promising avenues for pharmacological intervention strategies aimed at modulating glial cell activation states to exert anti-inflammatory and potential neuroprotective effects to treat the human condition. Furthering the well documented implications of microglial activation in the pathogenesis of PD, here we have now demonstrated in our current study, that reactive astrocytes are indeed also, dark horses of

dopaminergic neurodegeneration in the LPS model of Parkinson's disease. Future studies should focus on identifying soluble mediators that astrocytes exclusively release under inflammatory conditions that damage local dopaminergic neurons directly and/or contribute to microglial activation and subsequent neuronal loss.

Chapter 5

Enhancing noradrenergic tone inhibits microglial activation and attenuates dopaminergic neurodegeneration in the intra-nigral LPS model of Parkinson's disease

5.1 Introduction

The locus coeruleus (LC), lying within the outermost layer of the pontine tegmentum, is the primary source hub of noradrenergic cell bodies in the CNS, their neuronal projections innervating multiple central brain regions, including the substantia nigra (SN) and the striatum (Gesi et al., 2000). At autopsy, neuronal cell bodies are reduced to a greater extent in the LC (63%) than in the substantia nigra of Parkinson's disease patients and in the nucleus basalis in Alzheimer's disease patients (Zarow et al., 2003b). Given that degeneration of the LC-noradrenergic system is concomitant with dopaminergic neurodegeneration in PD patients, it's evident that disturbances in CNS noradrenaline (NA) levels due to LC noradrenergic cell loss, as occurs in Parkinson's disease can exacerbate PD-related neuropathology (Mavridis et al., 1991), whilst pharmacological agents that increase extra-synaptic NA bioavailability may provide neuroprotection.

The loss of LC noradrenergic neurons is a pathological commonality in Alzheimer's and Parkinson's disease brains (German et al., 1992). Noradrenergic depletion elevates inflammatory mediator expression, inhibits recruitment of microglia to sites of Aβ plaque deposition and impairs microglial phagocytosis of Aβ, leading to elevated amyloid plaque burden (Heneka et al., 2010). The authors' further show that NA-stimulation of murine microglia suppresses Aβ-induced increases in cytokine & chemokine gene expression *in vitro* and that pharmacological treatment of NA-depleted APP-transgenic mice with the NA precursor L-threo-DOPS restores microglial clearance of Aβ *in vivo*. Treatment of 5 month old 5xFAD transgenic mice with L-threo-DOPS decreases plaque load in the cortex and hippocampus, increases neprilysin mRNA levels & improves spatial learning in the Morris water maze, findings which were associated with reduced astrocytic activation and increased cortical and hippocampal BDNF protein expression (Kalinin et al.). Taken together, pharmacological intervention strategies aimed at raising CNS noradrenaline may be beneficial in alleviating AD-related neuropathology and cognitive dysfunction.

Studies by (Li et al., 2018) in the PD field have shown that administration of DSP4 (50 mg/kg i.p.) induced a 34% loss of DβH-positive cells in the LC, leading to spatial learning and memory deficits, aggravates striatal dopamine depletion and potentiates hypokinesia in response to a subsequent MPTP regimen (25 mg/kg/day for 5 days). Moreover, studies by (Rommelfanger et al., 2007) have shown that lesioning the LC-noradrenergic system with DSP4, or dopamine β-hydroxylase knockout (*Dbh*$^{-/-}$) mice (which lack NA altogether) display greater motor abnormalities than MPTP-injected mice with 80% dopaminergic nerve terminal loss. Data from the same study showed that acute pharmacological restoration of central NA levels with L-threo-DOPS however, improved motor function in *Dbh-/-* mice. Moreover, MPTP-induced degeneration of the nigrostriatal dopaminergic system and ensuing DA loss is attenuated in noradrenaline transporter knockout (NAT $^{-/-}$) mice, a protective effect similarly conferred upon treatment with the specific NAT inhibitor nisoxetine (Rommelfanger et al., 2004). Thus, loss of LC-noradrenergic cell bodies and ensuing cortical NA deficiency may exacerbate PD-related neuropathology and motor dysfunction, whereas augmentation of central noradrenergic tone may have therapeutic value in Parkinson's disease.

Atomoxetine (marketed under brand name: Strattera by Eli Lilly and Company) is a selective noradrenaline reuptake inhibitor (NRI) approved by the US Food and Drug Administration for the treatment of attention deficit hyperactivity disorder (ADHD). Atomoxetine blocks the noradrenaline transporter (NAT) and inhibits the reuptake of noradrenaline, resulting in an increase in extracellular noradrenaline concentration which further promotes noradrenergic signalling. Idazoxan is a selective α_2-adrenoceptor antagonist that can potentiate the bio-availability of noradrenaline by blocking presynaptic α_2-adrenoceptors (which normally act as auto-receptors to regulate noradrenaline release), and thus potentiate noradrenergic tone. An *in vivo* microdialysis study on freely moving rats by (Swanson et al., 2006) demonstrated that atomoxetine (3 mg/kg; i.p.) in combination with idazoxan (1 mg/kg; i.p.) induced a 7-fold increase in cortical noradrenaline efflux, greater than either compound alone, indicating a synergistic effect in terms of enhancing central noradrenergic tone.

Systemic pre-treatment with the FDA-approved serotonin-noradrenaline reuptake inhibitor (SNRI) duloxetine (10 mg/kg i.p.) for 5 days enhances Levodopa-induced abnormal involuntary movements (AIM) in 6-OHDA lesioned hemi-Parkinsonian rats, indicating that the increased effect of levodopa could be partially attributed to blockade of the noradrenaline transporter (Nishijima et al., 2016). The authors proclaim from this study however that despite notable enhancements in locomotor function, duloxetine administration could increase the risk of worsening levodopa-induced dyskinesia (LID) in PD patients. An 8-week open-label clinical trial however, on 13 PD patients receiving anti-Parkinsonian medication with at least 3 daily doses of levodopa (300mg) demonstrated an improvement in Unified Parkinson's Disease Rating Scale (UPDRS) part III (motor signs of PD) scores in response to treatment with duloxetine (40 mg/day), indicating that adjunctive noradrenergic enhancement strengthens the effects of levodopa on motor function in PD

patients (Nishijima et al., 2017). Interestingly, treatment with the α_2-adrenoceptor antagonist idazoxan (10 mg/kg i.p.) prior to L-DOPA administration (12 mg/kg s.c.) exerts an anti-dyskinetic effect in 6-OHDA lesioned hemi-parkinsonian rats (Barnum et al., 2012), highlighting the noradrenergic system as a modulator of LID, and enhancing noradrenergic tone as a plausible adjunctive therapeutic option for advanced PD patients suffering from dyskinesia. Treatment with idazoxan (2 mg/kg i.v.) has also shown efficacy in alleviating motor abnormalities (rigidity & akinesia) in MPTP-lesioned (0.5 mg/kg i.v.) Parkinsonian monkeys (Bezard et al., 1999).

In a randomized double-blind placebo-controlled study, an acute single oral dosage of idazoxan (20mg) has previously been shown to exert an anti-dyskinetic effect in 18 PD patients suffering from L-DOPA induced dyskinesia (Rascol et al., 2001). In a randomized double-blind placebo-controlled study, 19 mild-moderate idiopathic PD patients undergoing fMRI during a stop-signal task receiving 40mg of the NRI atomoxetine orally as an adjunctive treatment to their usual dopaminergic therapy exhibited restorations in functional interactions between the pre-supplementary motor cortex and the inferior frontal gyrus (iFG), a connection which was found to be absent in PD patients receiving dopaminergic therapy only (Rae et al., 2016). The authors show that the functional improvements in response inhibition (indicated by a reduced stop-signal reaction time) in response to treatment with atomoxetine correlated strongly with the structural connectivity within the white matter underlying the iFG. These findings are important in supporting the case for noradrenergic enhancement as an adjunctive treatment for PD, as prior diffusion tensor imaging studies have demonstrated that the white matter tracts connecting the frontal cortex to the basal ganglia are abnormal in PD patients (Rae et al., 2012; Agosta et al., 2013), and that abnormal connectivity in the response inhibition network together with difficulties in stopping movement is a salient feature of Parkinson's disease.

Noradrenaline transporters (NAT) are scarce in the basal ganglia, yet dense in the frontal cortex (Amunts et al., 2010) so it is likely that atomoxetine may exert its beneficial effects at the level of the iFG, enhancing sensitivity to neuronal inputs from the pre-supplementary motor area, leading to reductions in stop-signal reaction times. Moreover, a task-free fMRI study on 33 idiopathic PD patients receiving the same dose of atomoxetine (40mg) has previously been shown to restore connectivity between the right iFG and dorsolateral prefrontal cortex (PFC), a finding which was proportional to improvements in executive function as indexed by verbal fluency (Borchert et al., 2016). Thus, irrespective of the currently unknown, yet possible direct therapeutic effects of enhancing noradrenergic tone on damage done to the nigrostriatal dopaminergic system & ensuing motor deficits in Parkinson's disease, adjunctive treatment with atomoxetine may indirectly improve motor function in PD patients by strengthening neural connections associated with the response inhibition network (namely the iFG, pre-SMA & subthalamic nuclei) and thus restore inhibitory control over motor activity, which may be particularly beneficial in combating impulsivity in PD patients. Furthermore, in a recent randomized clinical trial for atomoxetine

in 30 PD patients with mild cognitive impairment (PD-MCI), oral atomoxetine treatment (40mg daily weeks 1-2, then 80mg daily weeks 3-10) lead to subjective improvements in executive function (inattention & impulsivity) as measured by the Conners Adult Attention Deficit Hyperactivity Disorder Rating Scale (CAARS) in patients with PD-MCI (Hinson et al., 2017).

Given that executive dysfunction and impairments in attention induced by PD-MCI have a strong impact on balance and gait abnormalities in PD (Yogev-Seligmann et al., 2008), patients with PD-MCI have higher postural instability & gait disorder subscale scores than that of cognitively intact individuals with PD (Sollinger et al., 2010). This highlights that treatment with atomoxetine could be beneficial for PD patients who suffer from MCI, which in turn could have a knock-on therapeutic effect on gait and postural instability. Given that in an 8-week open-label dose trial demonstrating the beneficial effects of atomoxetine on executive dysfunction in PD patients has shown that chronic administration of atomoxetine is well tolerated (up to 100mg/day) underscores the suitability of atomoxetine in particular as a potential drug candidate to pharmacologically enhance CNS noradrenergic tone for therapeutic effects (Marsh et al., 2009).

Thus, the premise for our current investigation into the effects of atomoxetine alone and in combination with idazoxan on motor dysfunction, nigrostriatal neurodegeneration and dopamine loss are bolstered by an array of preclinical and clinical data demonstrating an intriguing prospect of enhancing noradrenergic tone as a possible adjunctive therapy for Parkinson's disease patients. Evidence in the literature into whether these potential drug candidates are effective (and their mechanism of action) in clinically relevant animal models of PD however, is relatively sparse to date. Here we will assess the therapeutic efficacy of noradrenergic enhancement using the noradrenaline reuptake inhibitor (NRI) atomoxetine and/or the α_2-adrenoceptor antagonist idazoxan in combating LPS-driven microglial activation, dopaminergic neurodegeneration, nigrostriatal dopamine loss and motor dysfunction.

5.2.1 Study aims and objectives

The aim of this study was to pharmacologically target the noradrenergic system for anti-inflammatory and potential neuroprotective effects in the intra-nigral LPS model of Parkinson's disease. Specifically, we aimed to enhance noradrenergic tone within the brain by administering the noradrenaline reuptake inhibitor (NRI) atomoxetine and/or the α_2-adrenoceptor antagonist idazoxan to combat LPS-induced microglial activation, dopaminergic neurodegeneration, nigro-striatal dopamine loss and associated motor deficits in the rat. Thus, the main objective of this study was to assess the therapeutic efficacy of treatment with atomoxetine alone or in combination with idazoxan on PD-related neuropathology and motor dysfunction.

5.2.2 Experimental design

Adult male Wistar rats (n=48) aged 7-8 weeks (220-250g) were obtained from the Comparative Medicines Unit (CMU, TCD) and were group housed in cages of 3 in climate controlled rooms set to 21°C on a 12:12 hour light-dark cycle. Animals were allowed food and water *ad libitum* and were habituated to the animal housing facilities for 1 week prior to commencing any experimental procedures. All behavioural testing was conducted between 09:00 and 18:00. The experimental protocols involved were in compliance with the European directive 2010/63/EU on the protection of animals used for scientific purposes. Rats were sorted into the following eight treatment groups: (1) Vehicle + saline, (2) Vehicle + atomoxetine, (3) Vehicle + idazoxan, (4) Vehicle + atomoxetine + idazoxan, (5) LPS + saline, (6) LPS + atomoxetine, (7) LPS + idazoxan, (8) LPS + atomoxetine + idazoxan, *n* = 6 per group. Baseline behavioural testing was conducted 5 days prior to stereotactic surgery. Rats received a unilateral intra-nigral injection of LPS (10µg/2µl) or vehicle (2µl 0.89% sterile saline). The coordinates for SNpc infusion were as follows: anteroposterior (AP) -5.3 mm, mediolateral (ML) ± 2.0 mm and dorsoventral (DV) -8.5 mm. Treatment with atomoxetine (3 mg/kg; i.p.), idazoxan (1 mg/kg; i.p.) or saline control commenced 4 hours post lesioning and continued twice daily (b.i.d) for 7 days (morning drug treatment between 9-10 am; evening drug treatment between 5-6 pm). Behavioural testing in the staircase, stepping and cylinder tests was conducted 7 and 14 days post-surgical procedures between the hours of 10-5pm. The following day, rats were either euthanized by transcardial perfusion fixation in preparation for immunohistochemical analysis of nigrostriatal integrity and glial cell activation, or decapitated and their brains free-hand dissected on dry ice in preparation for HPLC analysis of biogenic amine concentrations in the midbrain and striatum.

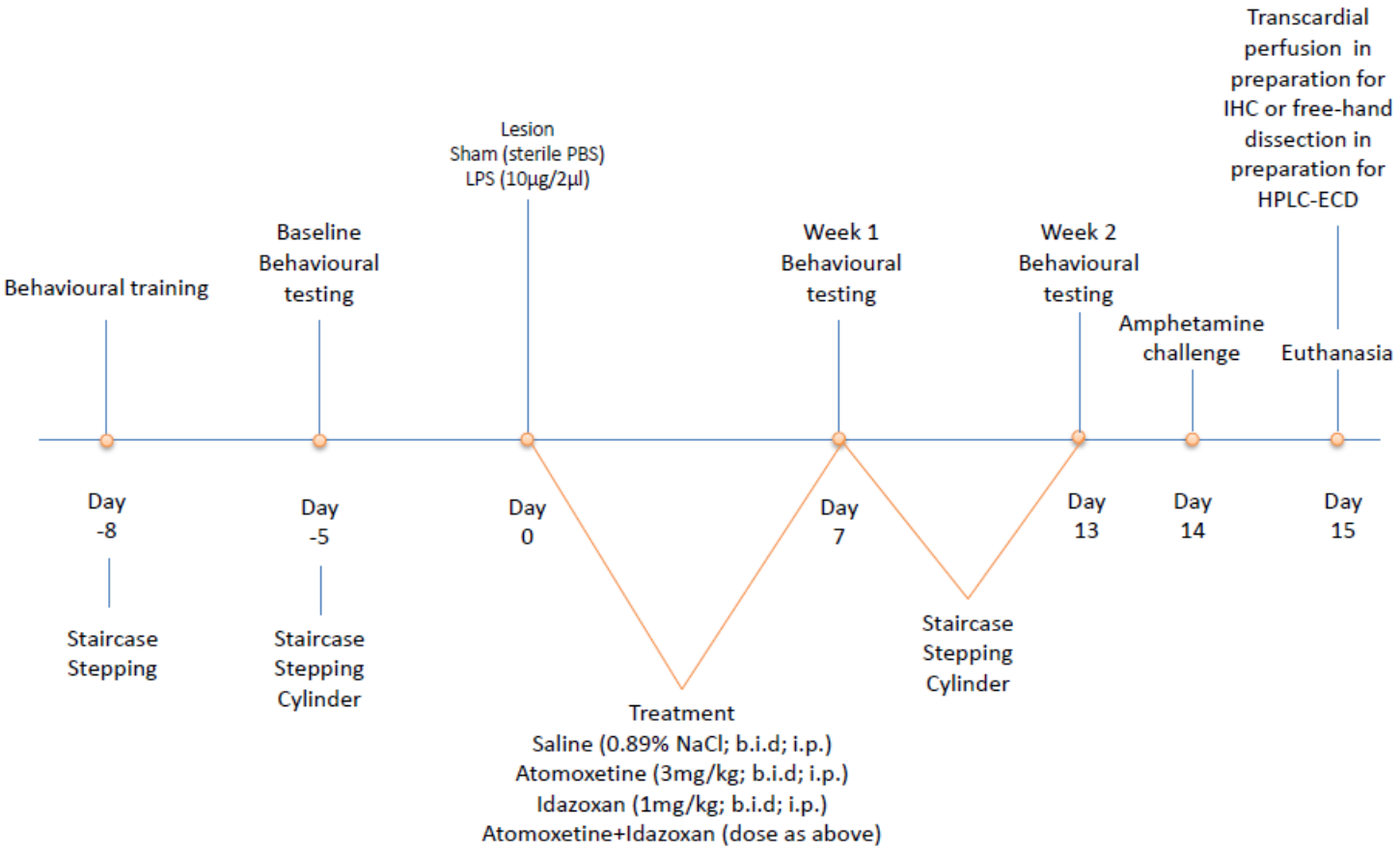


Figure 5.1 Experimental timeline of investigations.

5.3 Results

5.3.1 Treatment with atomoxetine alone or in combination with idazoxan does not attenuate LPS-induced deficits in skilled motor function in the staircase test.

The staircase test was used as a method to assess the impact of a unilateral intra-nigral LPS injection on skilled motor function. Intra-nigral LPS injection reduced the contralateral success rate at 7 ($P<0.05$) but not 13 days post lesioning relative to pre-lesion controls. Treatment with the NRI atomoxetine or the α_2-AR antagonist idazoxan had no effect on the contralateral success rate of vehicle or LPS lesioned animals.

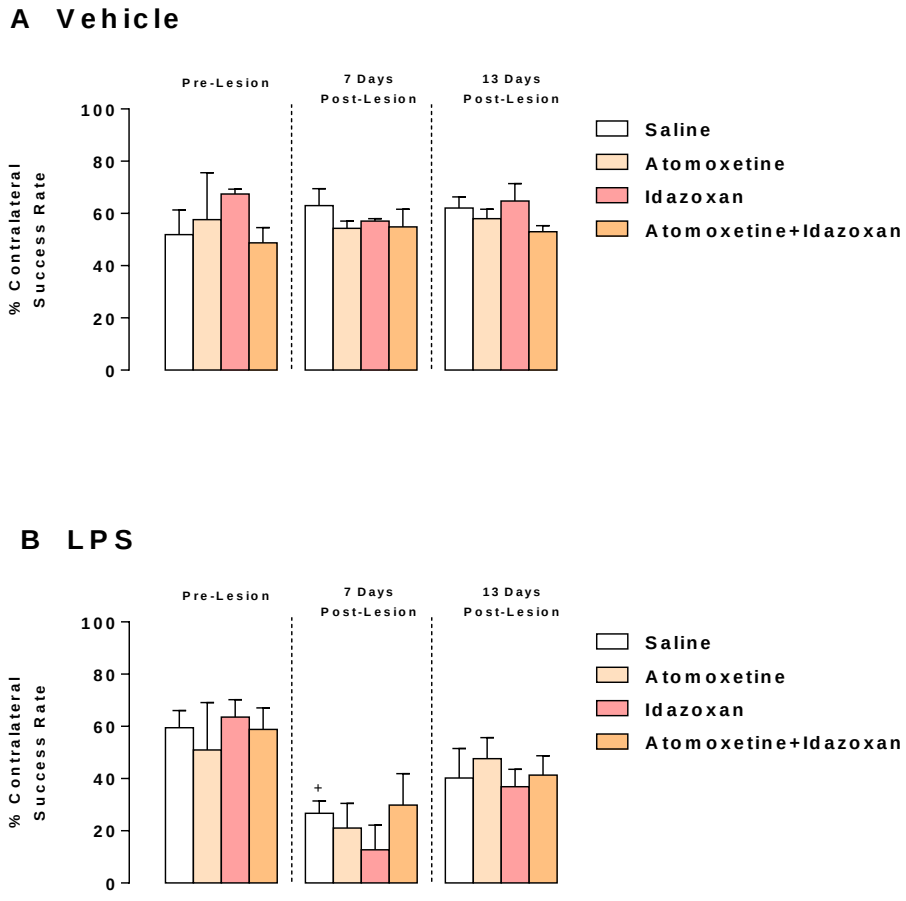


Figure 5.2 Treatment with atomoxetine alone or in combination with idazoxan fails to alleviate LPS-induced deficits in skilled motor function in the staircase test. The effect of a unilateral intra-nigral injection of LPS on skilled motor function was assessed using the staircase test. Treatment with the NRI atomoxetine (3mg/kg i.p.) and/or the α_2-AR antagonist idazoxan (1mg/kg i.p.) commenced 4 hours following surgery and continued once daily for 7 days post lesioning. Unilateral injection of LPS (10µg/2µl) into the substantia nigra reduced the contralateral success rate at 7, but not 13 days post lesioning. Treatment with atomoxetine alone or in combination with idazoxan failed to attenuate the LPS-induced deficits in skilled motor function. Data are expressed as mean ± S.E.M. (n=4-6) +$P<0.05$ vs. pre-lesion by 2-way repeated measures ANOVA with *post hoc* Newman-Keuls.

5.3.2 Treatment with atomoxetine alone and in combination with idazoxan ameliorates LPS-induced forelimb akinesia in the forehand direction.

The stepping test was used as an assessment of forelimb akinesia in response to lesioning with LPS. A two-way repeated measures ANOVA demonstrated an effect of time on the number of contralateral adjusting steps taken in response to lesioning with LPS ($F_{(2,8)}$ = 200.36, P<0.001). Newman-Keuls *post hoc* analysis revealed that LPS reduced the number of adjusting steps made using the contralateral limb in the forehand direction at 7 (P<0.01) and 13 (P<0.01) days post-surgical procedures relative to pre-lesion testing. A two-way repeated measures ANOVA demonstrated an effect of atomoxetine on the number of adjusting steps made using the contralateral limb in the forehand direction at 7 ($F_{(1,14)}$ = 18.20, P<0.001) and 13 ($F_{(1,14)}$ = 41.15, P<0.001) days post lesioning. Newman-Keuls *post hoc* analysis revealed an increase in the number of contralateral adjusting steps made in the forehand direction in response to treatment with atomoxetine alone (P<0.01) and in combination with idazoxan (P<0.01) at both 7 & 13 days post lesioning.

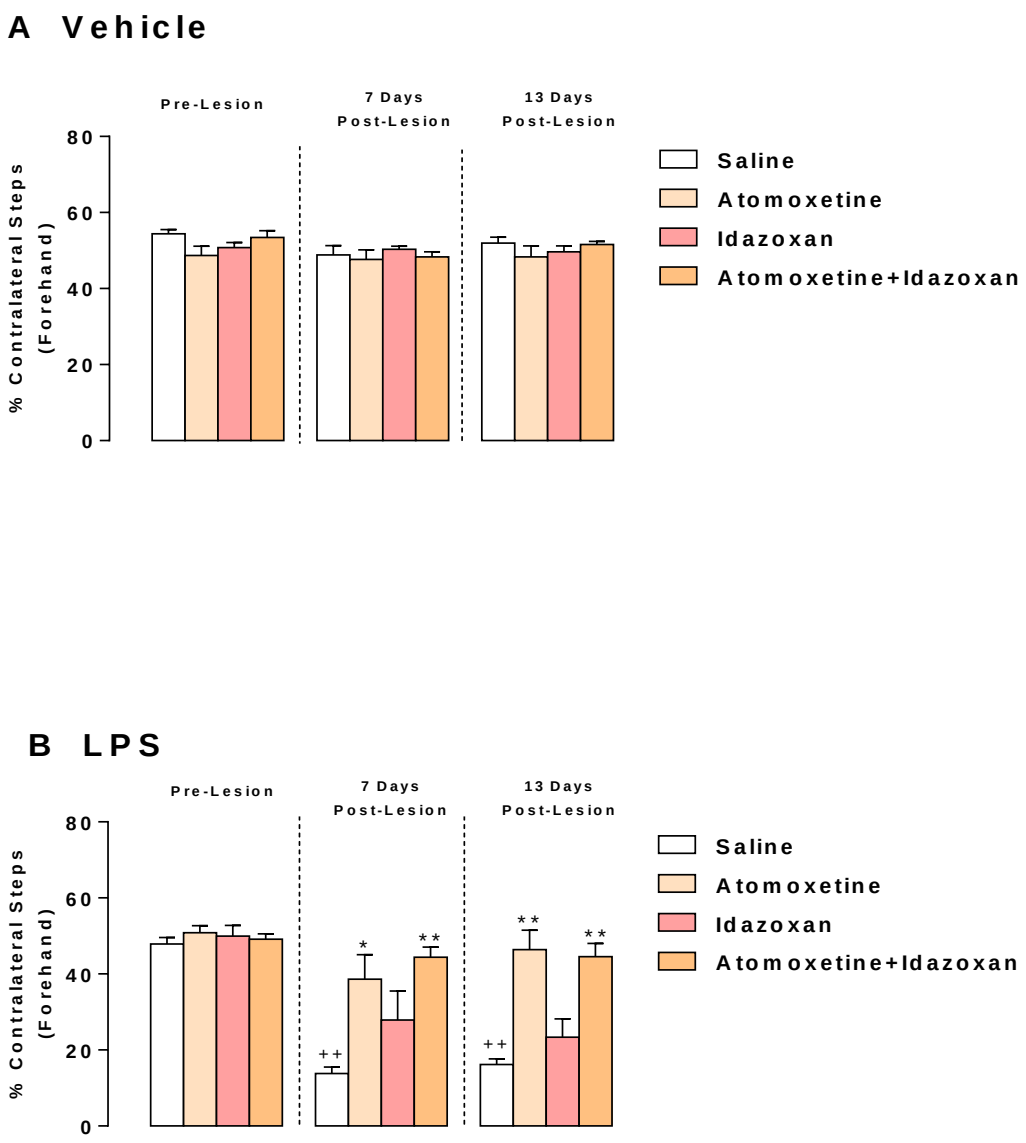


Figure 5.3 Treatment with atomoxetine alone or in combination with idazoxan attenuates LPS-driven forelimb akinesia in the forehand direction. The effect of a unilateral intra-nigral LPS injection on forelimb kinesis was assessed using the stepping test. Treatment with the NRI atomoxetine (3mg/kg i.p.) and/or the α_2-AR antagonist idazoxan (1mg/kg i.p.) commenced 4 hours following surgery and continued once daily for 7 days post lesioning. Unilateral injection of LPS (10µg/2µl) into the substantia nigra reduced the number of contralateral adjusting steps made in the forehand direction at 7 & 13 days post lesioning. Treatment with atomoxetine alone and in combination with idazoxan ameliorated LPS-induced akinetic behaviour in the forehand direction at 7 and 13 days post lesioning. Data are expressed as mean ± S.E.M. (n=4-6) ++P<0.01 vs. pre-lesion controls *P<0.05, **P<0.01 vs. pre-lesion controls by 2-way repeated measures ANOVA with *post hoc* Newman-Keuls.

5.3.3 Treatment with atomoxetine alone or in combination with idazoxan leads to an observed attenuation in forelimb akinesia in the backhand direction.

A two-way repeated measures ANOVA demonstrated an effect of time on the number of contralateral adjusting steps made in response to lesioning with LPS ($F_{(2,8)}$ = 84.54, P<0.001). Newman-Keuls *post hoc* analysis revealed an LPS-induced reduction in the number of adjusting steps made using the contralateral limb in the backhand direction at 7 (P<0.01) and 13 (P<0.01) days post lesioning with compared to pre-lesion controls. Treatment with atomoxetine and/or idazoxan had no significant effect on the number of contralateral steps taken in LPS-lesioned animals but a trend towards an attenuation of LPS-driven akinetic behaviour was observed.

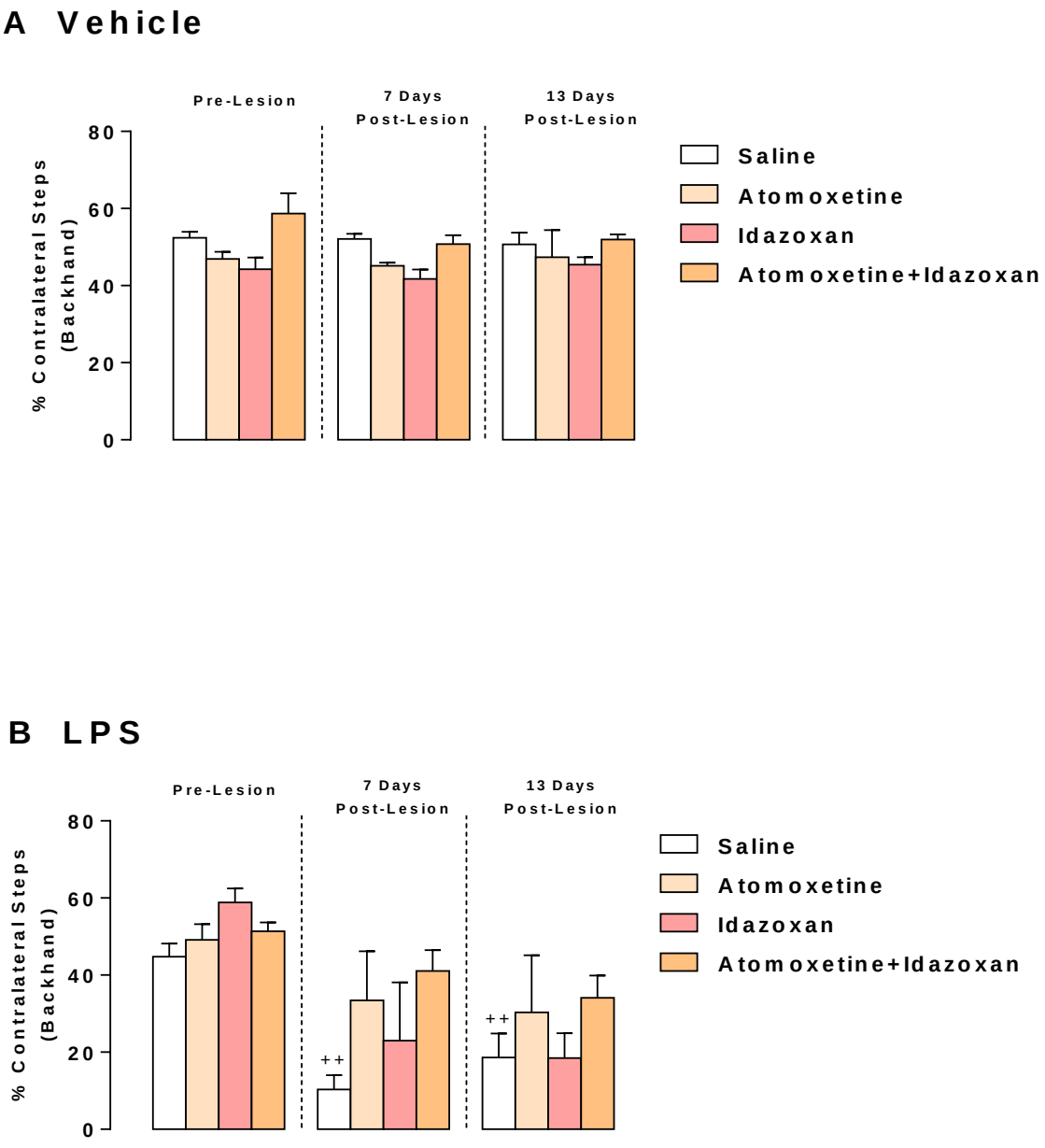


Figure 5.4 Treatment with atomoxetine alone or in combination with idazoxan fails to significantly restore forelimb kinesis in the backhand direction. The effect of a unilateral intra-nigral LPS injection on forelimb kinesis was assessed using the stepping test. Treatment with the NRI atomoxetine (3mg/kg i.p.) and/or the α_2-AR antagonist idazoxan (1mg/kg i.p.) commenced 4 hours following surgery and continued once daily for 7 days post lesioning. Unilateral injection of LPS (10µg/2µl) into the substantia nigra reduced the number of contralateral adjusting steps made in the backhand direction at 7 & 13 days post lesioning. Treatment with atomoxetine and/or idazoxan failed to significantly repress LPS-driven akinetic behaviour in the backhand direction. Data are expressed as mean ± S.E.M. (n=4-6) ++P<0.01, vs. pre-lesion controls by 2-way repeated measures ANOVA with *post hoc* Newman-Keuls.

5.3.4 Treatment with atomoxetine in combination with idazoxan ameliorates forelimb use asymmetry in the cylinder test

A two-way repeated measures ANOVA demonstrated an effect of time on the number of contralateral wall placements following an intra-nigral LPS injection in the cylinder test ($F_{(2,8)}$ = 47.74, P<0.001) Newman-Keuls post hoc analysis revealed a reduction in the number of wall placements made using the contralateral limb at 7 (P<0.01) and 13 (P<0.01) days post lesioning with LPS compared to pre-lesion testing. A two-way ANOVA demonstrated an effect of atomoxetine on the number of contralateral wall placements made of LPS-lesioned animals at 7 ($F_{(1,13)}$ = 10.32, P=0.007) and 13 ($F_{(1,14)}$ = 8.59, P=0.011) days post lesioning, and of idazoxan at 7 days post lesioning ($F_{(1,14)}$ = 8.07, P=0.014). Newman-Keuls *post hoc* analysis revealed an increase in the number of wall placements made using the contralateral limb of LPS-injected animals in response to treatment with atomoxetine in combination with idazoxan at 7 days (P<0.05) post lesioning relative to saline-treated controls.

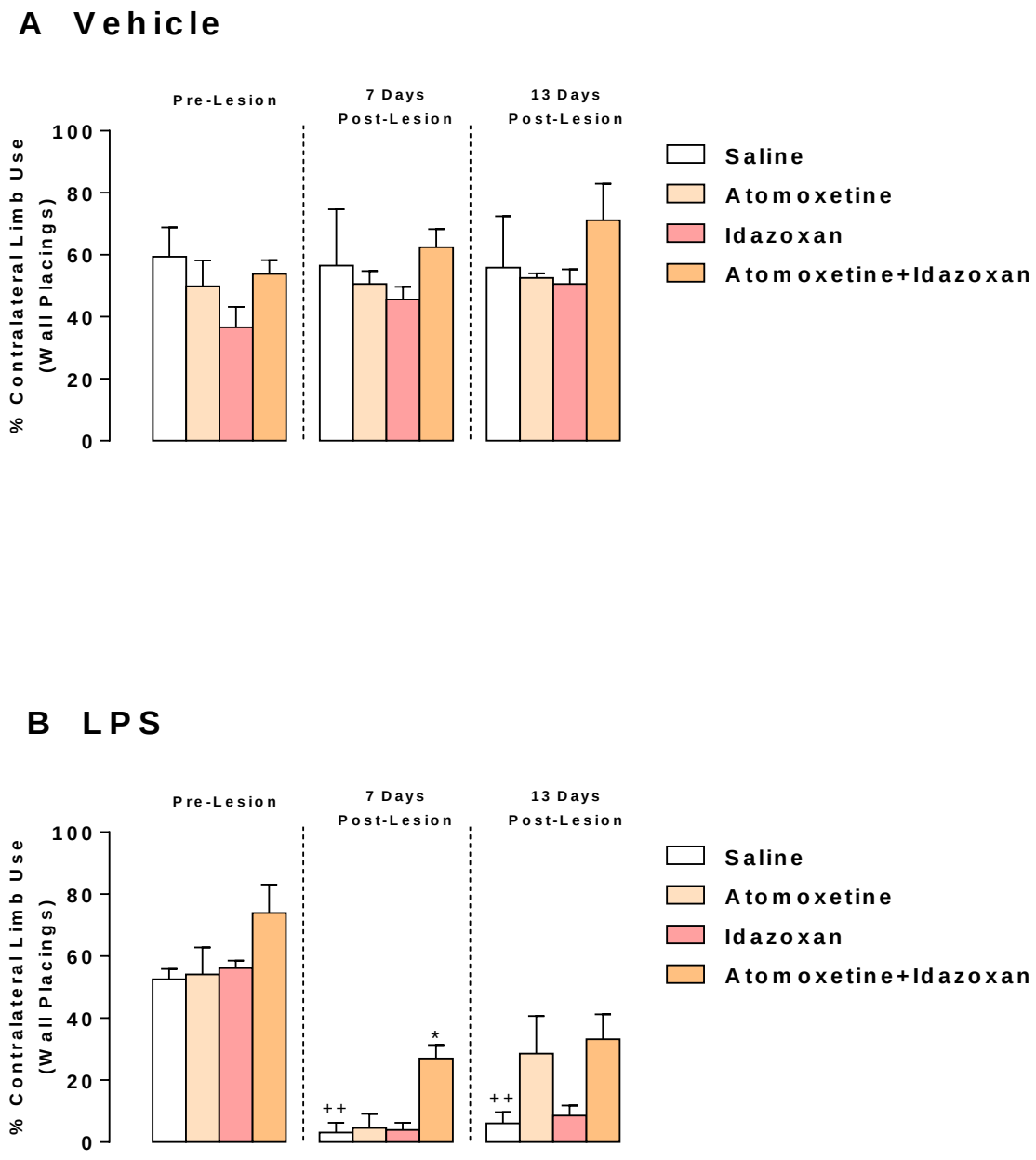


Figure 5.5 Treatment with atomoxetine in combination with idazoxan restores forelimb use symmetry in the cylinder test. The effect of a unilateral intra-nigral LPS injection on forelimb asymmetry was assessed using the cylinder test. Treatment with the NRI atomoxetine (3mg/kg i.p.) and/or the α_2-AR antagonist idazoxan (1mg/kg i.p.) commenced 4 hours following surgery and continued once daily for 7 days post lesioning. Unilateral injection of LPS (10µg/2µl) into the substantia nigra reduced the number of wall placements made using the contralateral limb at 7 & 13 days post lesioning. Treatment with atomoxetine in combination with idazoxan suppressed the LPS-driven forelimb use asymmetry at 7 days post lesioning. Data are expressed as mean ± S.E.M. (n=4-6) ++P<0.01 vs. pre-lesion controls, *P<0.05 vs. saline-treated controls by 2-way repeated measures ANOVA with *post hoc* Newman-Keuls.

5.3.5 Treatment with atomoxetine in combination with idazoxan reduces ipsiversive rotational behaviour in the amphetamine challenge.

A two-way ANOVA demonstrated an effect of atomoxetine on ipsilateral rotational asymmetry in response to lesioning with LPS ($F_{(1,16)}$ = 22.13, P<0.001). There was also an atomoxetine x idazoxan interaction effect ($F_{(1,16)}$ = 5.38, P=0.034). Newman-Keuls *post hoc* analysis revealed that atomoxetine in combination with idazoxan significantly reduced ipsiversive rotational behaviour of LPS-lesioned rats in response to amphetamine challenge (P<0.05).

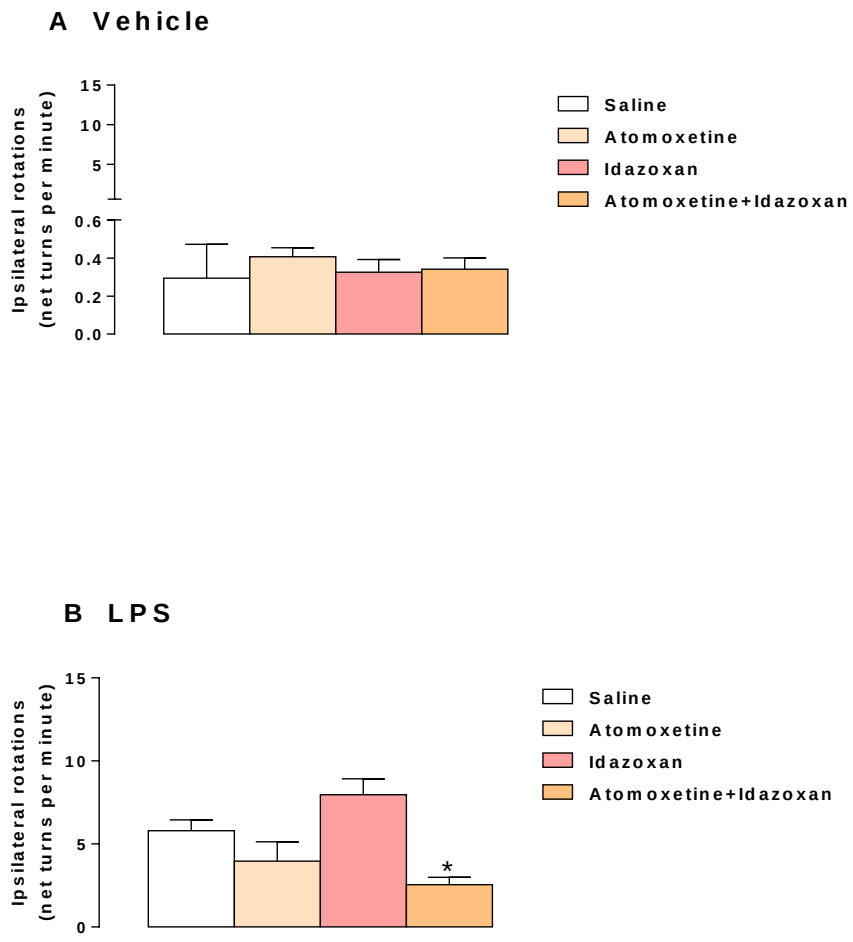


Figure 5.6 Treatment with atomoxetine in combination with idazoxan ameliorates amphetamine-induced rotational asymmetry in LPS-lesioned rats. The effect of a unilateral intra-nigral LPS injection on rotational asymmetry was assessed using the amphetamine challenge. Treatment with the NRI atomoxetine (3mg/kg i.p.) and/or the α_2-AR antagonist idazoxan (1mg/kg i.p.) commenced 4 hours following surgery and continued once daily for 7 days post lesioning. Rats were administered d-amphetamine (5mg/kg i.p.) and their home-cage activity was recorded for 40 minutes. The number of ipsilateral & contralateral rotations was scored and expressed as net rotations per minute. Data are expressed as mean ± S.E.M. (n=4-6) *P<0.05 vs. LPS-lesioned rats treated with saline control by 2-way ANOVA with *post hoc* Newman-Keuls.

5.3.6 Treatment with atomoxetine alone or in combination with idazoxan attenuates LPS-induced reductions in nigrostriatal dopamine content.

A Student's t-test demonstrated a decrease in midbrain and striatal dopamine concentrations in LPS-lesioned animals treated with saline relative to vehicle + vehicle treated controls ($P < 0.01$). A two-way ANOVA demonstrated an effect of atomoxetine on midbrain dopamine content in LPS-lesioned rats ($F_{(1,12)} = 17.04$, $P=0.001$). Newman-Keuls *post hoc* analysis revealed that treatment with atomoxetine ameliorates the LPS-induced loss of midbrain dopamine levels when administered alone ($P<0.05$) or in combination with idazoxan ($P<0.05$) relative to LPS-lesioned rats treated with saline. A two-way ANOVA demonstrated an effect of atomoxetine on striatal dopamine content in LPS-lesioned rats ($F_{(1,11)} = 16.57$, $P=0.002$). Newman-Keuls *post hoc* analysis revealed that treatment with atomoxetine increased striatal dopamine levels when administered alone ($P<0.05$) and in combination with idazoxan ($P<0.05$) when compared to LPS-lesioned rats treated with saline.

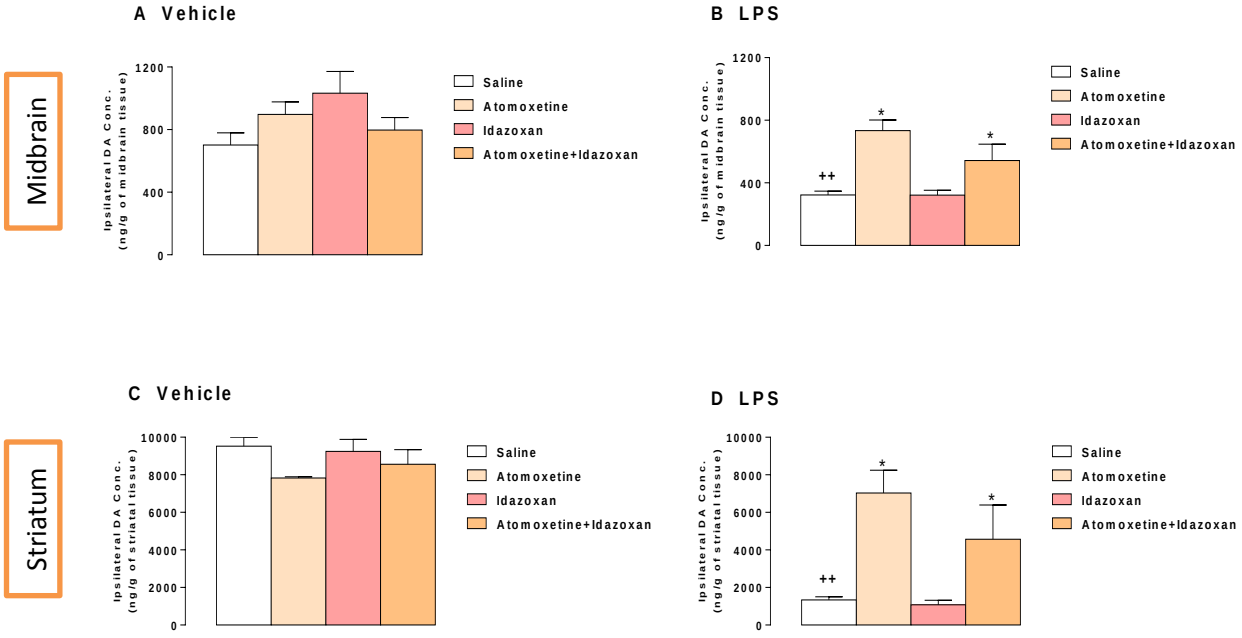
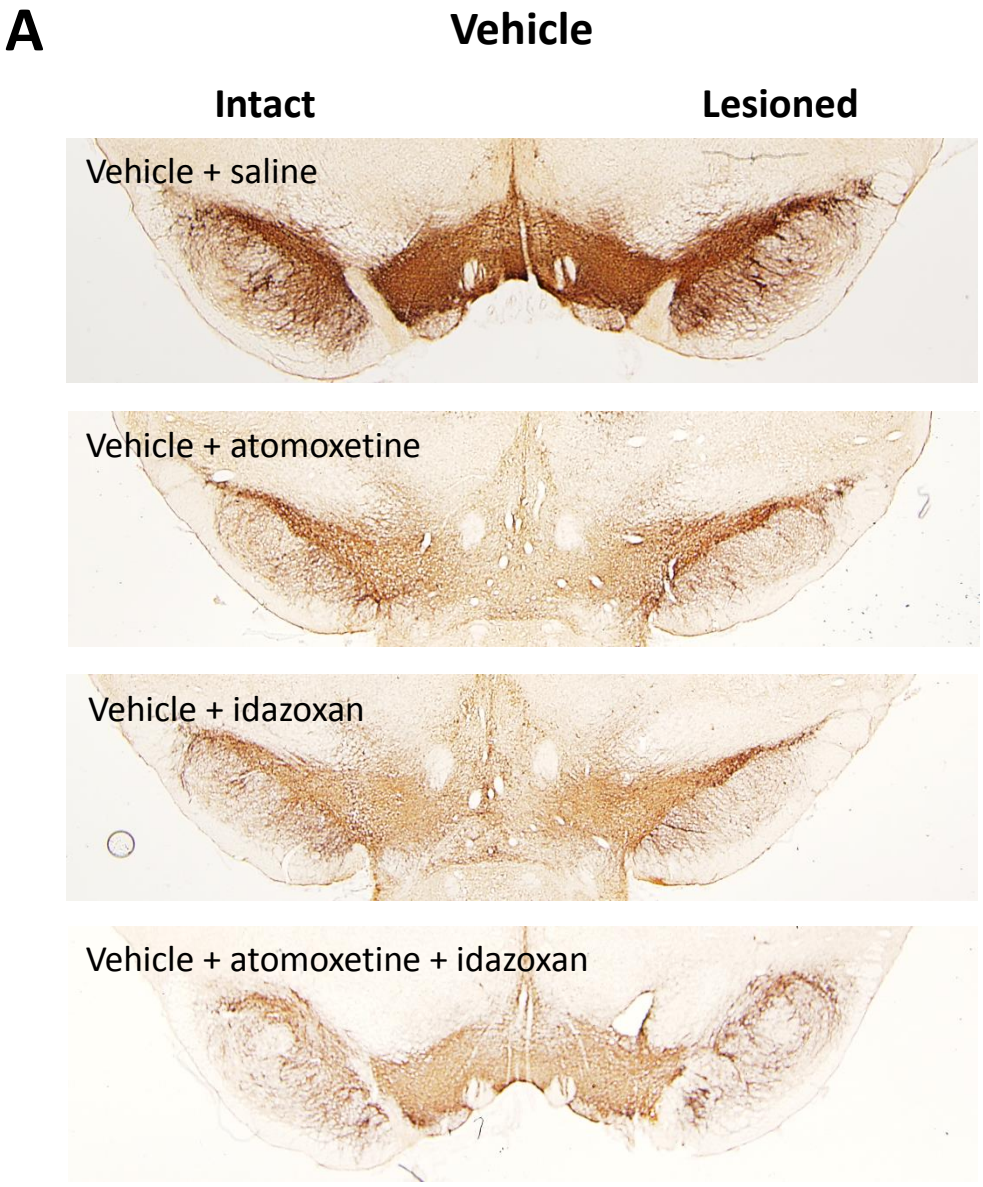


Figure 5.7 Treatment with atomoxetine alone and in combination with idazoxan protects against LPS-induced nigrostriatal dopamine loss. The effect of a unilateral intra-nigral LPS injection on nigrostriatal DA concentrations was assessed via HPLC-ECD. Treatment with the NRI atomoxetine (3mg/kg i.p.) and/or the α_2-AR antagonist idazoxan (1mg/kg i.p.) commenced 4 hours following surgery and continued once daily for 7 days post lesioning. Rats were euthanized 15 days post lesioning and midbrain & striatal tissue was harvested and prepared for HPLC analysis. Data are expressed as mean ± S.E.M. (n=3-6) ++P<0.01 vs. Vehicle-injected controls treated with saline, *P<0.05 vs. saline-treated controls by 2-way ANOVA with *post hoc* Newman-Keuls.

5.3.7 Treatment with atomoxetine alone and in combination with idazoxan attenuates LPS-induced dopamine neuron loss in the substantia nigra.

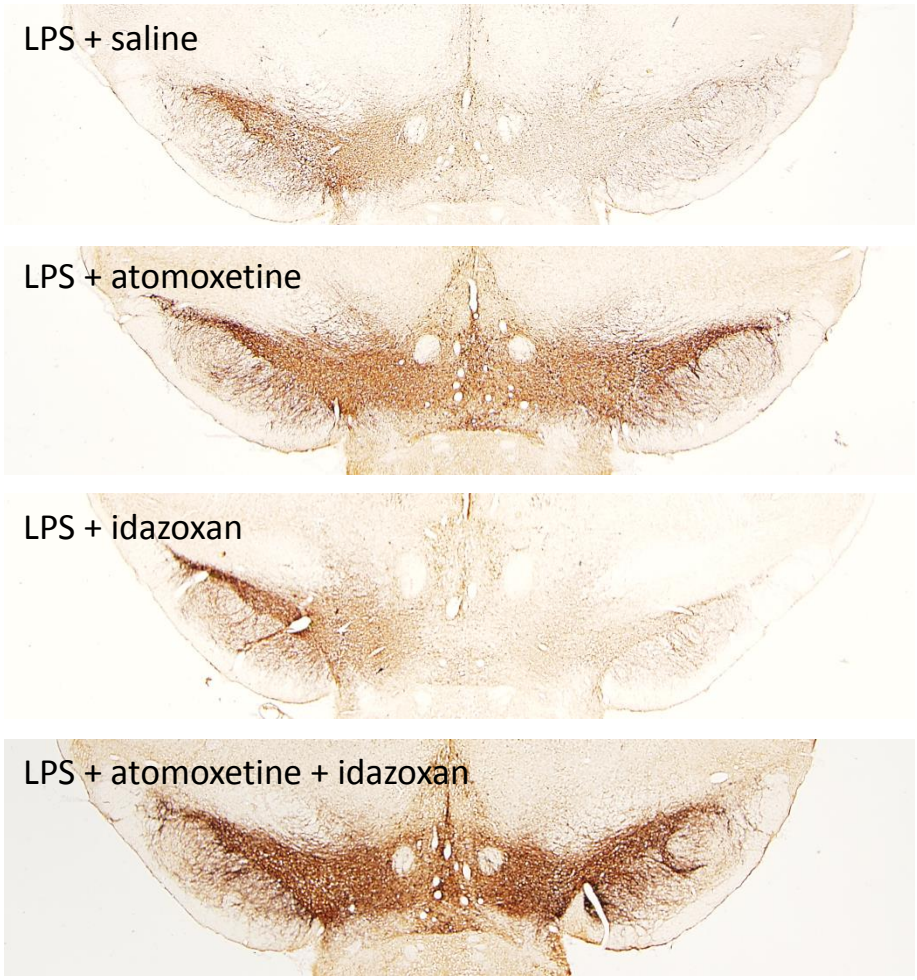
A three-way ANOVA demonstrated an effect of LPS ($F_{(1, 24)}$ = 115.3, P<0.0001), an effect of atomoxetine ($F_{(1, 24)}$ = 81.8, P<0.0001) and an interaction between LPS x atomoxetine ($F_{(1, 24)}$ = 92.75, P<0.0001) on nigral TH^+ cell counts in the SNpc. Newman-Keuls *post hoc* analysis revealed that intra-nigral LPS injection induced a severe loss of dopamine neurons in the SNpc relative to vehicle-injected controls (P<0.001). Treatment with idazoxan alone did not attenuate the LPS-induced loss of dopamine neurons in the SNpc. Treatment with atomoxetine alone or in combination with idazoxan largely attenuated the loss of TH^+ dopamine neurons in the SNpc of LPS-lesioned rats (P<0.001).



B

LPS

Intact **Lesioned**



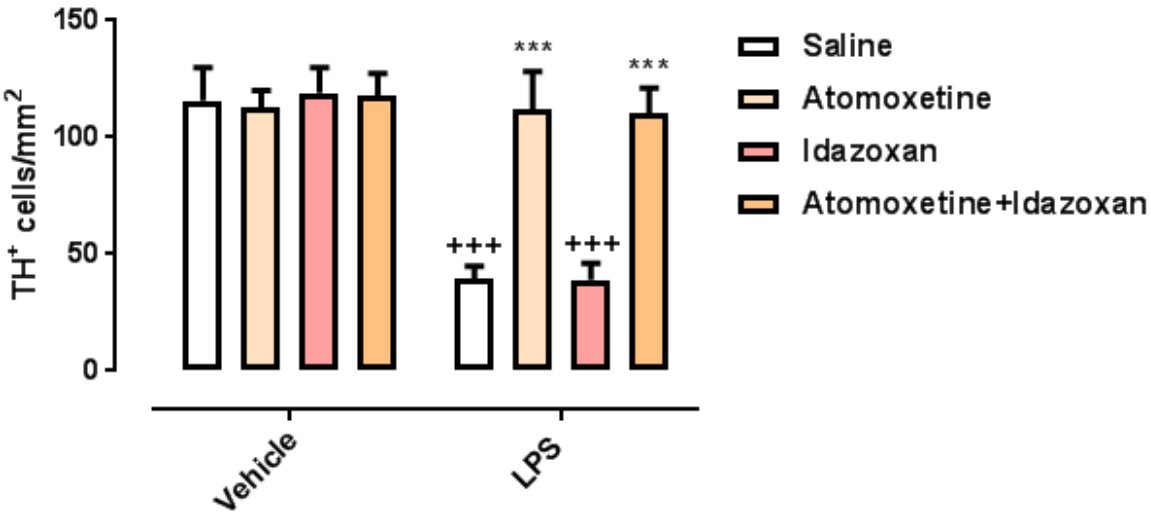
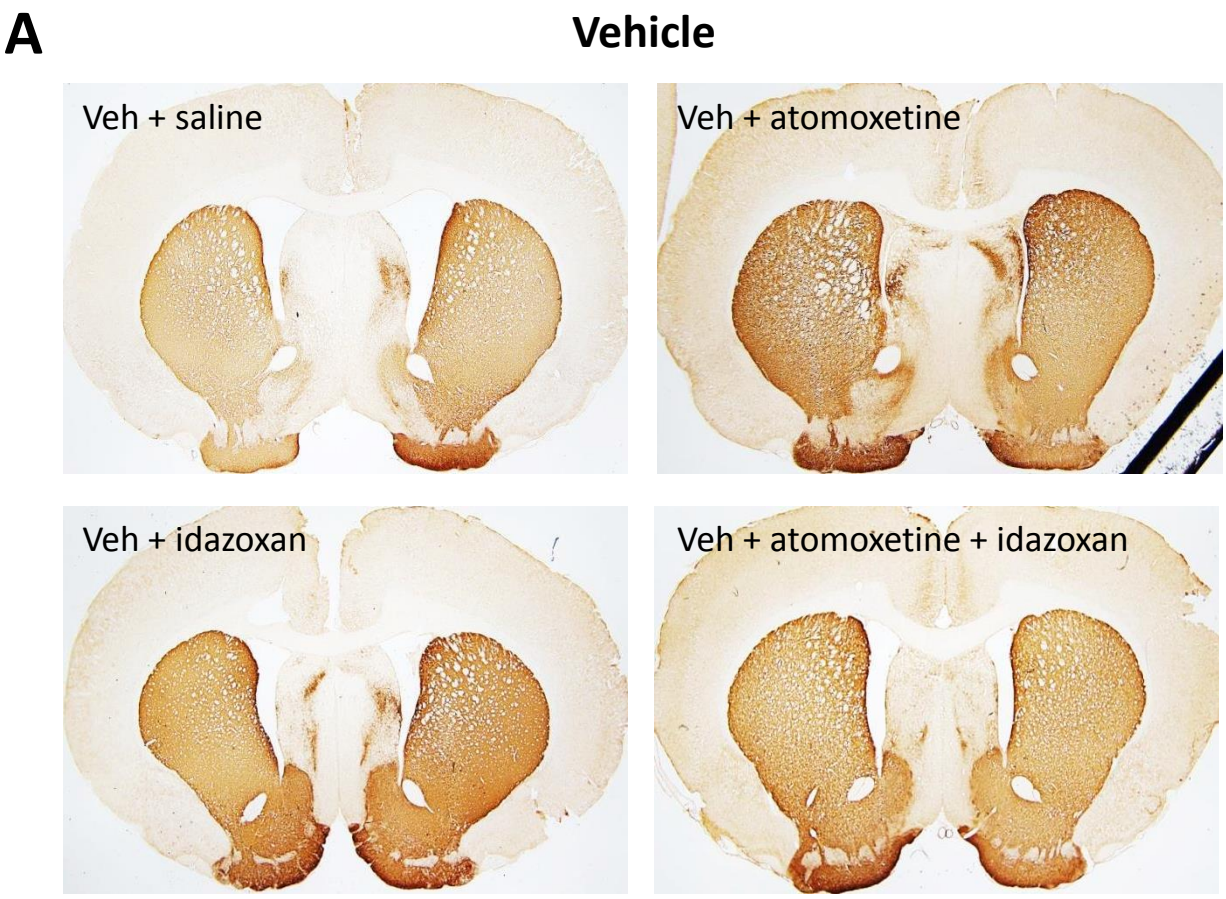


Figure 5.8 Treatment with atomoxetine attenuates dopaminergic neuronal loss within the SNpc. The effect of a unilateral intra-nigral LPS injection on dopaminergic neurons within the substantia nigra was assessed via anti-tyrosine hydroxylase immunohistochemistry. Treatment with the NRI atomoxetine (3mg/kg i.p.) and/or the α_2-AR antagonist idazoxan (1mg/kg i.p.) commenced 4 hours following surgery and continued once daily for 7 days post lesioning. Treatment with atomoxetine alone or in combination with idazoxan ameliorated LPS-induced loss of dopamine neurons within the SNpc. Data are expressed as mean ± S.E.M. (n=4) +++P<0.001 vs. Vehicle-injected controls, ***P<0.001 vs. LPS-lesioned rats treated with saline control by 3-way ANOVA with *post hoc* Newman-Keuls.

5.3.8 Treatment with atomoxetine protects against LPS-induced dopaminergic nerve terminal loss in the ipsilateral striatum.

A three-way ANOVA demonstrated an effect of LPS ($F_{(1, 24)}$ = 432.2, P<0.0001), an effect of atomoxetine ($F_{(1, 24)}$ = 58.69, P<0.0001) and an interaction effect between LPS x atomoxetine ($F_{(1, 24)}$ = 36.72, P<0.0001) on striatal TH-immunoreactivity. Newman-Keuls post hoc analysis revealed that intra-nigral LPS injection reduced TH^+ nerve fibers in the ipsilateral striatum relative to vehicle-injected controls (P<0.001). Treatment with idazoxan failed to attenuate the LPS-mediated striatal denervation, these rats also displayed robust deficits in TH-immunoreactivity relative to vehicle-injected controls (P<0.001). Treatment with atomoxetine alone or in combination with idazoxan attenuated the loss of TH^+ striatal dopaminergic nerve fibers in LPS-lesioned animals (P<0.001).



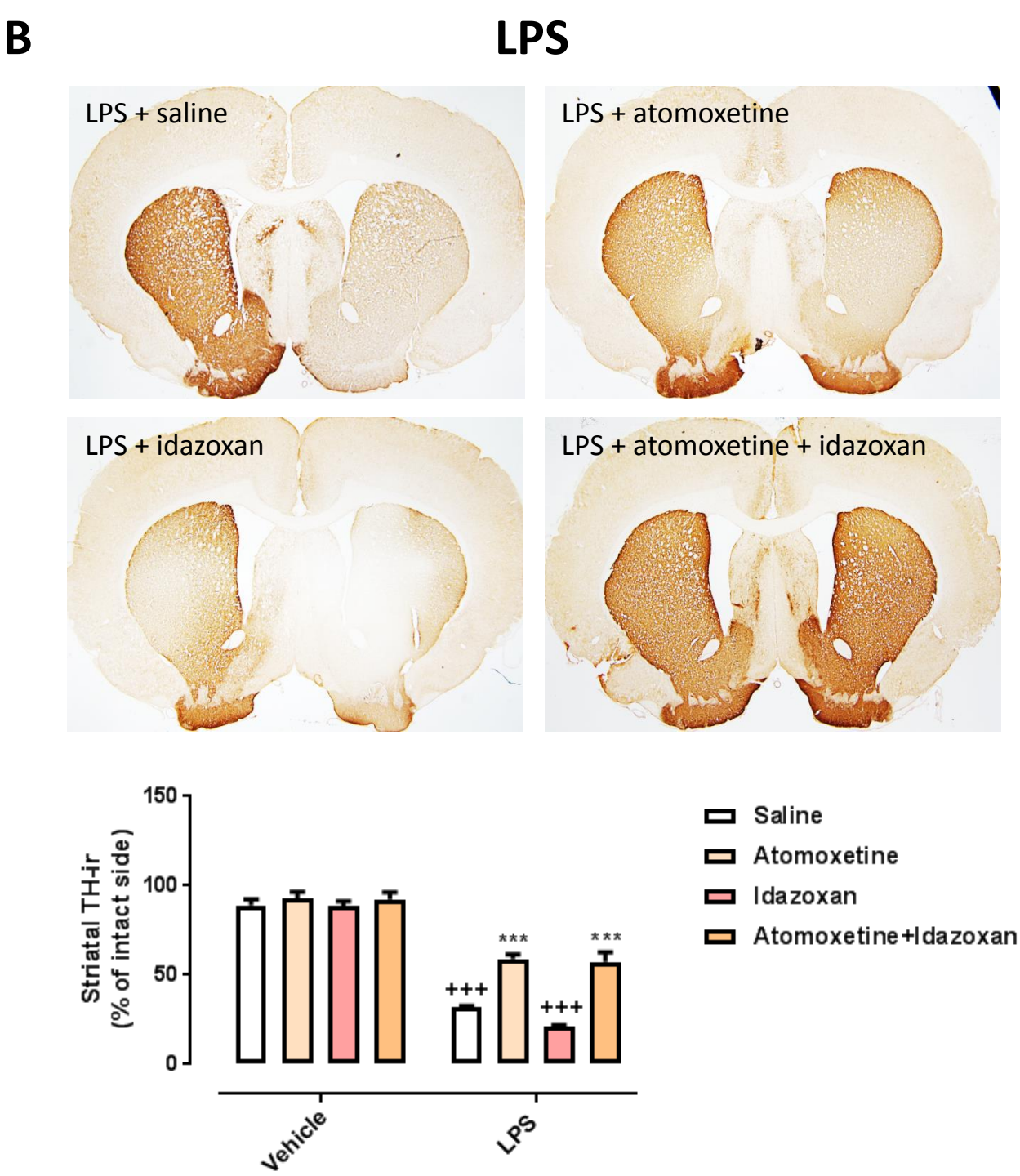
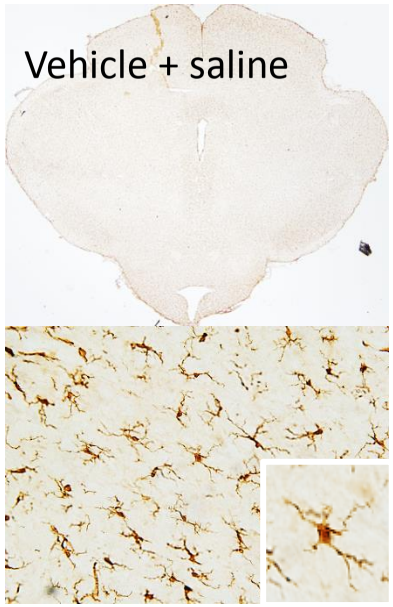
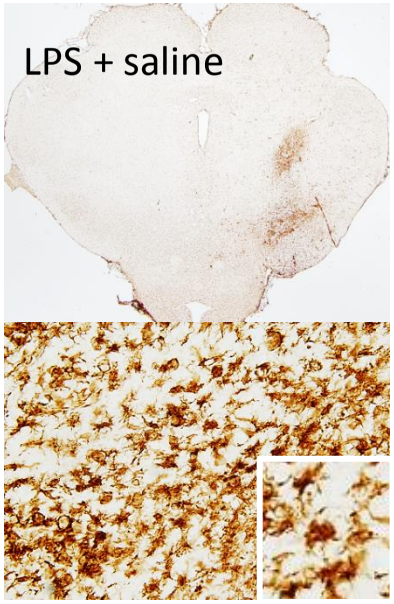


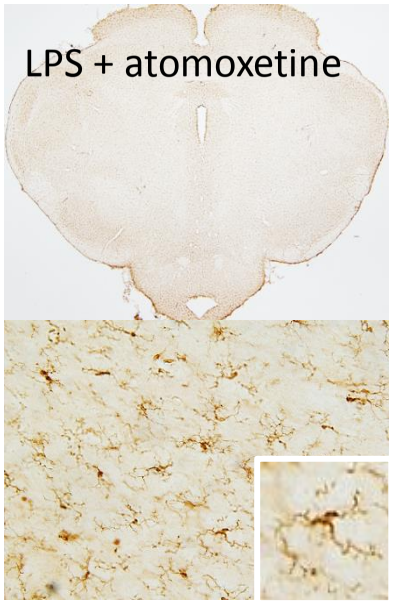
Figure 5.9 Treatment with atomoxetine alone or in combination with idazoxan protects dopaminergic nerve terminals from LPS-induced denervation in the ipsilateral striatum. The effect of a unilateral intra-nigral LPS injection on dopaminergic nerve terminals within the striatum was assessed via anti-tyrosine hydroxylase immunohistochemistry. Treatment with the NRI atomoxetine (3mg/kg i.p.) and/or the α_2-AR antagonist idazoxan (1mg/kg i.p.) commenced 4 hours following surgery and continued once daily for 7 days post lesioning. Treatment with atomoxetine alone or in combination with idazoxan ameliorated LPS-induced loss of dopaminergic nerve terminals within the ipsilateral striatum. Data are expressed as mean ± S.E.M. (n=4) +++P<0.001 vs. Vehicle-injected controls, ***P<0.001 vs. LPS-lesioned rats treated with saline control by 3-way ANOVA with *post hoc* Newman-Keuls.

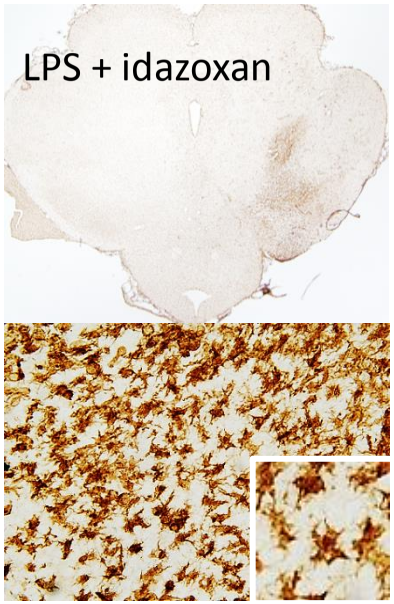
5.3.9 Treatment with atomoxetine suppresses LPS-induced Iba1^{+} microgliosis within the substantia nigra.

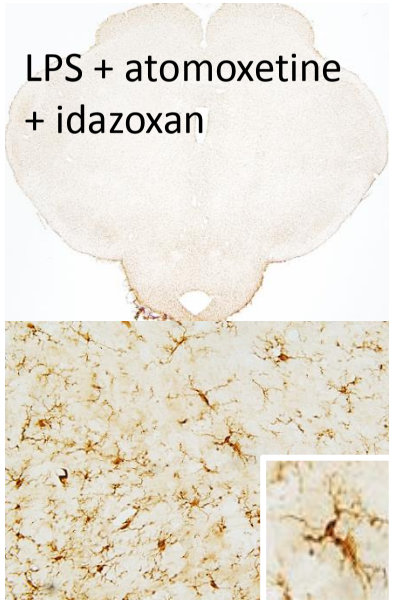
A three-way ANOVA demonstrated an effect of LPS ($F_{(1, 24)}$ = 43.45, P<0.0001), an effect of atomoxetine ($F_{(1, 24)}$ = 68.43, P<0.0001) and an interaction effect between LPS x atomoxetine ($F_{(1, 24)}$ = 80.39, P<0.0001) on nigral Iba1-immunoreactivity. Newman-Keuls post hoc analysis revealed a dramatic increase in the number of Iba1^{+} cells / nigral field in response to intra-nigral lesioning with bacterial LPS relative to vehicle-injected controls (P<0.001). Treatment with idazoxan failed to attenuate LPS-mediated increases in Iba1^{+} microglial cell counts, these animals displayed evidence of robust microgliosis relative to vehicle-injected controls (P<0.001). Treatment with atomoxetine alone or in combination with idazoxan ameliorated the LPS-induced microgliosis within the substantia nigra (P<0.001).


Vehicle + saline


LPS + saline


LPS + atomoxetine


LPS + idazoxan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+ idazoxan

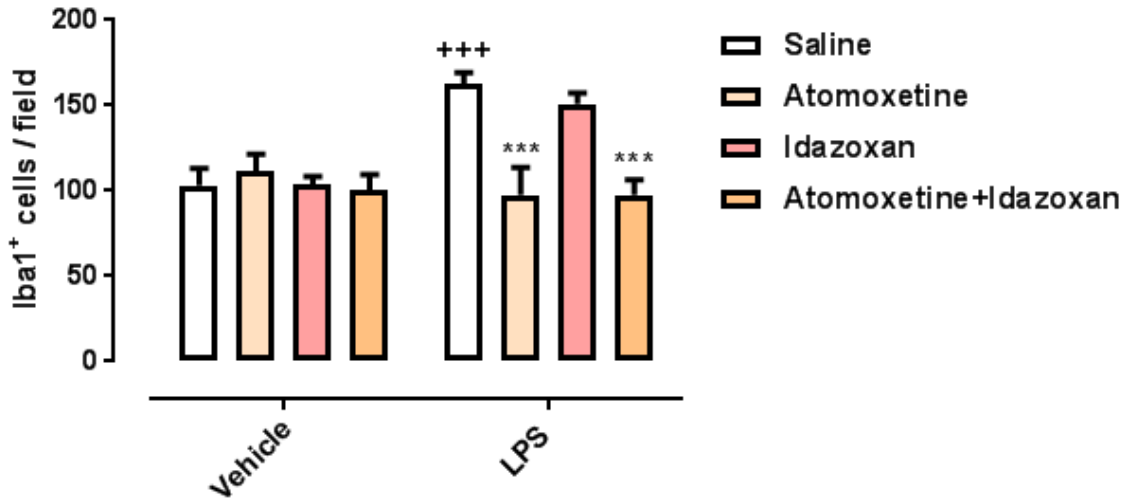
200
150
100
50
0
Iba1+ cells / field
+++
***
***
Vehicle
LPS
Saline
Atomoxetine
Idazoxan
Atomoxetine+Idazoxan

Figure 5.10 Treatment with atomoxetine inhibits microglial activation within the SNpc. The effect of a unilateral intra-nigral LPS injection on microglial reactivity within the substantia nigra was assessed via anti-Iba1 immunohistochemistry. Treatment with the NRI atomoxetine (3mg/kg i.p.) and/or the α_2-AR antagonist idazoxan (1mg/kg i.p.) commenced 4 hours following surgery and continued once daily for 7 days post lesioning. Treatment with atomoxetine alone or in combination with idazoxan suppressed LPS-induced nigral microgliosis. Data are expressed as mean ± S.E.M. (n=4) +++P<0.001 vs. Vehicle-injected controls, ***P<0.001 vs. LPS-lesioned rats treated with saline control by 3-way ANOVA with *post hoc* Newman-Keuls.

5.4 Discussion

Here we show that treatment with the noradrenaline reuptake inhibitor atomoxetine alone and in combination with the α_2-adrenoceptor antagonist idazoxan attenuates LPS-induced nigral microgliosis and dopaminergic neurodegeneration, suppresses nigrostriatal dopamine loss and provides partial protection from associated motor deficits. Enhancing noradrenergic tone inhibited LPS-induced microglial activation within the substantia nigra and protected dopaminergic neurons within the SNpc against LPS-mediated neurotoxicity. Treatment with atomoxetine but not idazoxan, also prevented ensuing dopaminergic nerve terminal degeneration in the ipsilateral striatum and associated nigrostriatal dopamine loss, findings which underpinned partial improvements in behavioural tests of motor function.

Motor deficits are a salient feature of Parkinson's disease and become apparent in patients with ongoing dopaminergic neurodegeneration after an approximate 80% loss of striatal dopamine content. Thus, we decided to use a battery of motor function tests to assess the impact of pharmacologically enhancing noradrenergic tone on different aspects of PD-related motor dysfunction relative to the human condition, namely forelimb akinesia (stepping test), asymmetric limb use (cylinder test & D-amphetamine challenge) and skilled motor function (staircase test). Accurate detection of the various motor deficits depended on the behavioural test employed. Behavioural deficits relate to damage of the nigro-striatal tract and begin to manifest when the severity of the lesion reaches a critical threshold i.e. extent of damage to nigral cell bodies and their axon extensions and terminals. Automatic vertical exploration in the cylinder test is largely controlled at the level of the spinal cord and brain stem (Metz et al., 1998; Muir and Whishaw, 1999; Piecharka et al., 2005) whereas skilled reaching requires integration of segmental and supra-spinal descending pathways with propriospinal neurons further co-ordinating postural adjustments to successfully execute directed forepaw retrieval (Weidner et al., 2001). With respect to the differences in sensitivity across tests, it is likely that motor deficits are dependent on the extent of damage imposed in the neural circuitry involved in either automatic limb movements (stepping, cylinder) and/or skilled forelimb executions (staircase) consistent with the extent of dopamine loss observed in both the midbrain and striatum following LPS administration. It is also evident that there are differences across the testing paradigms in relation to the

efficacy of treatment with atomoxetine and idazoxan alone or in combination. The fact that idazoxan exerted a therapeutic effect on motor function only when atomoxetine was co-administered as well highlights that the protective effects of enhancing noradrenergic tone against LPS-driven motor deficits are more attributable to inhibition of NAT due to treatment with atomoxetine, rather than blockade of the α_2-AR downstream of idazoxan administration. This observation is bolstered by our HPLC analysis of nigrostriatal DA content demonstrating an increase in dopamine concentrations within the midbrain & striatum of LPS-lesioned rats in response to treatment with atomoxetine alone or in combination with idazoxan, but not when idazoxan was administered alone.

Idazoxan and the α_2-AR antagonist fipramezole have consistently demonstrated reductions in abnormal involuntary movements in rodent models (Lundblad et al., 2002), primate models (Grondin et al., 2000a; Grondin et al., 2000b; Fox et al., 2001) and human PD patients (Rascol et al., 2001; LeWitt et al., 2012). Additionally, idazoxan administration delayed the onset of dyskinesia in MPTP-induced Parkinsonism and reduced levodopa-induced dyskinesia severity (Grondin et al., 2000b). Therefore, idazoxan may have a dual effect; firstly, it may facilitate NA release by binding to pre-synaptic α_{2a}- inhibitory autoreceptors resulting in the regulation of DAergic firing and second; it may bind to post-synaptic α_{2c}-ARs to influence DAergic signalling (Buck et al., 2010). Indeed this may be the mechanism underlying partial improvements in forelimb asymmetry observed in LPS lesioned rats. Here, treatment with idazoxan, in combination with atomoxetine lead to substantial improvements in impaired limb use in the cylinder test and attenuated the increase in ipsiversive rotations in response to d-amphetamine challenge in LPS-lesioned rats. Idazoxan has a similar affinity for α_{2a}, $_{2b}$ and $_{2c}$ subtypes (Ki 10nM, 37nM and 20nM respectively), however, α_{2c}-ARs are highly expressed in the striatum and this subtype most likely accounts for idazoxan's effects (Zhang et al., 1999). LPS-lesioned rats treated with idazoxan alone however, failed to display increases in midbrain and striatal DA concentrations signifying a lack of protection to the nigro-striatal dopaminergic tract and potentially accounting for the absence of improvements in motoric behaviour.

The rescue in motor function following treatment with atomoxetine/idazoxan in each behavioural paradigm (bar the staircase test) as well as an increase in nigral and striatal DA concentration following intra-nigral LPS injection prompted further investigation to investigate if TH and IBA-1 immunoreactivity would be complimentary to the behavioural and neurochemical results generated. Atomoxetine treatment alone and in combination with idazoxan attenuated the LPS-driven deficits in TH^+ nigral cell counts and striatal TH^+ immunoreactivity, and reduced nigral microgliosis. This is in accordance with the literature demonstrating the ability of NA to suppress microglial activation and subsequent inflammatory mediator production (Feinstein et al., 2002b; Heneka et al., 2002b; Heneka et al., 2002a; Marien et al., 2004). The atomoxetine-induced improvements in motor deficits, DA neuronal loss and neuroinflammation are consistent with the outcomes of reported *in vitro* studies; NA suppresses microglial activation and inflammatory gene expression in the

brain resulting in reduction of the synthesis and release of multiple cytokines, chemokines and other inflammatory agents, particularly following a challenge with an inflammatory stimulus (Feinstein et al., 2002a; Heneka et al., 2002a; Marien et al., 2004). Given that the midbrain contains the highest density of microglial cells in the entire rodent brain (Kim et al., 2000), the root, and indeed progression of this inflammatory-derived neuropathology and ensuing motor dysfunction is likely to be congruent on the activation state of nigral microglia. In essence, nigral microgliosis & subsequent pro-inflammatory mediator production is a cellular prerequisite for LPS-mediated neurotoxicity of dopamine neurons. Treatment with atomoxetine alone or in combination with idazoxan however, inhibited nigral microglial activation in response to lesioning with LPS. Intra-nigral LPS injection lead to a robust increase in the number of $Iba1^{+}$ microglial cells within the substantia nigra with a morphologically distinct phenotype (retracted processes & enlarged cell soma) from quiescent microglia (extended processes & smaller cell soma). Treatment with atomoxetine alone or in combination with idazoxan inhibited the LPS-induced $Iba1^{+}$ nigral microgliosis and rendered the nigral microglia morphologically indicative of a quiescent phenotype. In support of the well documented anti-inflammatory effect of noradrenaline on microglial activation and pro-inflammatory cytokine secretion (Dello Russo et al., 2004b; McNamee et al., 2010a), in the present study, treatment with atomoxetine also attenuated the LPS-induced increases in TNF-α and IL-1β pro-inflammatory gene expression within the midbrain, a finding which is likely to have underpinned the attenuated loss of nigrostriatal dopamine neurons and DA concentrations (see Yssel et al., 2018 for full mRNA data).

NA may also act extra-synaptically on neurons themselves to mediate neuroprotective effects. Co-incubation with NA partially reduced Aβ-induced damage through increased glutathione (GSH) and PPARγ production (Madrigal et al., 2007). Treating mesencephalic neuronal cultures (which die spontaneously and progressively as the cultures mature) with low levels of NA (0.3-10 μM) promotes long-term survival and functionality while caspase inhibition mimics the effects of NA, independent of the GSH antioxidant system (Troadec et al., 2001). Furthermore, direct application of NA to murine primary astrocytes and neurons increased neuronal longevity through activation of the anti-inflammatory group of nuclear hormones, PPARγ (Klotz et al., 2003), which is also under the control of cAMP. NA may also confer neuroprotection through the production of neurotrophic factors. Of particular importance is GDNF whose neuroprotective potential was first documented in 1993 where it promoted the survival of midbrain DAergic neurons, which displayed increased cell body size and neurite length, and increased DA uptake (Lin et al., 1993a). Furthermore, GDNF has conferred neuroprotection in various animal models of PD, encouraging neuronal outgrowth and improving motor dysfunction when administered directly into the striatum, nigra or ventricular system (Björklund et al., 2000; Siegel and Chauhan, 2000; Peterson and Nutt, 2008; Sullivan and Toulouse, 2011). Quite remarkably, GDNF delivery into the rat nigra (once a day for 30 days) following striatal 6-OHDA administration, completely prevented nigral neuronal death while a single injection 7 days post-lesion had a substantial

regenerative effect (Sauer et al., 1995). Furthermore, *in vivo* gene therapy expressing GDNF has demonstrated efficacy in PD animal models. For example, injection of adenoviral GDNF vector increased nigral cell survival following striatal 6-OHDA in rats and mice (Bilang-Bleuel et al., 1997; Choi-Lundberg, 1997; Choi-Lundberg et al., 1997; Connor et al., 1999). Moreover, direct intra-putamenal infusion of GDNF in 5 Parkinson's disease patients increases dopamine storage in the putamen, improves UPDRS motor scores and reduces medication-induced dyskinesias (Gill et al., 2003). In the present study, enhancing noradrenergic bioavailability also induced a marked increase in growth factor production within the midbrain. Treatment with atomoxetine significantly increased GDNF and BDNF mRNA expression within the midbrain of LPS-lesioned rats. CDNF mRNA expression was also observably increased in these animals, albeit to a lesser extent to that of GDNF & BDNF (see (Yssel et al., 2018) for full details). Here, we propose that treatment with atomoxetine is exerting a glial-derived neurotrophic response to raised extra-synaptic noradrenergic tone on foot of blockade of the NAT. As reported above, the increases in GDNF are particularly promising from a neuro-protective perspective, as delayed intra-striatal delivery of this growth factor has previously been shown to prevent dopaminergic neurodegeneration within the SNpc, increases striatal dopamine concentration and promotes functional recovery in 6-OHDA-lesioned rats (Wang et al., 2002). Thus, in conjunction with the anti-inflammatory effect of atomoxetine treatment on activated microglia, we now show that atomoxetine also induces a spurt in growth factor expression within the midbrain, most likely from astrocytes in an adrenergic receptor-dependent manner. Thus, atomoxetine has a dual effect on midbrain glial cells, inducing a tonic inhibition on microglial activation & pro-inflammatory gene expression, whilst concurrently promoting the synthesis of neurotrophic factors from neighbouring astrocytes, with both aspects likely to be major contributors to neuroprotection against LPS-mediated neurotoxicity.

In summary, treatment with the NRI atomoxetine, with and without idazoxan, conferred neuroprotection in the LPS model of PD. The data gathered herein promotes the use of atomoxetine in particular as a potential treatment in PD. Rats treated with atomoxetine exhibited a suppression in nigral microgliosis following LPS administration, an amelioration of LPS-driven degeneration of the nigrostriatal dopaminergic system, higher dopamine levels and partial improvements in motor function. Treatment with idazoxan alone failed to exert the same therapeutic effects however, albeit when administered in combination with atomoxetine contributed towards protection against LPS-induced motoric asymmetry as evident in the cylinder test and amphetamine challenge. Considering PD, and many other neurodegenerative diseases incorporate a chronic inflammatory component that is thought to account for the progressive nature of the disease (Bartels and Leenders, 2007), the ability of atomoxetine to suppress microglial activation and regulate the inflammatory response in a clinically relevant animal model of PD, promotes its use as a prospective treatment to prevent further degeneration and sheds insight into the endogenous immunomodulatory potential of NA in regulating CNS inflammation. Significant added value is to be gained by

either pre-treating with NA agents in advance of the lesion (with time allowed for drug clearance prior to lesion) and by post-treating with NA treatments following a delayed period of dopamine cell degeneration to better represent the clinical presentation. Moreover, the augmentation of central NAergic tone through blockade of the NAT represents a clinically feasible neuroprotective strategy in PD as these agents are currently used in the treatment of depression and ADHD, and clinical data demonstrates that these agents are safe when taken for prolonged periods (Zhou, 2004).

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Chapter 6

Targeting β_2-adrenoceptors as a neuroprotective strategy in the treatment of Parkinson's disease.

6.1 Introduction

Higher levels of activated microglia are evident in the post mortem substantia nigra of intra-nigral LPS (Hoban et al., 2013), intra-striatal 6-OHDA (Marinova-Mutafchieva et al., 2009) and MPTP (Barcia et al., 2004) experimental models of PD, and in human PD brains (Imamura et al., 2003). Expression levels of the inflammatory cytokines IL-1β, TNF-α, IL-2, IL-6, IL-8 & IFN-γ are elevated in the brain, CSF and blood of PD patients (Lee et al., 2017). Moreover, infiltration of peripheral $CD4^+$ & $CD8^+$ T-cells into the midbrain of MPTP-intoxicated mice and in PD patients due to abnormal BBB permeability contributes to dopaminergic neurotoxicity, thus implicating the adaptive arm of the immune system in PD pathogenesis (Brochard et al., 2009). Also, a possible role for humoral immunity in genetic & idiopathic PD cases was elucidated in studies by (Orr et al., 2005) demonstrating an increase in IgG-immunolabelled pigmented dopamine neurons and an increase in activated microglia expressing the IgG receptor FcγRI within the substantia nigra, findings which were associated with numerous FcγRI microglia within the SN containing pigment granules, thus indicative of a targeted phagocytic attack of FcγRI-positive microglia on IgG-immunopositive dopamine neurons. Thus, interplay between peripheral & CNS immune signalling in response to an immune stressor (as instigated by bacterial / viral infection of peripheral origin or the release of immunogenic factors from damaged / dying dopaminergic neurons of central origin) may play a vital role in the process of Inflammation-mediated neurodegeneration of the nigrostriatal dopaminergic system and the pathophysiology of Parkinson's disease.

Studies by (Qin et al., 2007b) have demonstrated that systemic LPS treatment induces chronic brain inflammation and the progressive loss of dopamine neurons in the SNpc. The authors show that peripheral immune challenge with bacterial LPS (5 mg/kg .i.p) activates microglia and elevates brain TNF-α, IL-1β, MCP-1 & NF-κB p65 (subunit involved in Nf-κB heterodimer formation) expression in wildtype, but not in TNF $R1/R2^{-/-}$ mice leading to a 23% loss of TH-immunopositive neurons in the SNpc at 7 months post-treatment, progressing to a 47% loss after 10 months, findings which were accompanied by a high density of $F4/80^+$ (cell surface glycoprotein on macropahges) nigral microglia. The authors propose that systemic LPS induces the hepatic synthesis of TNF- α which increases serum TNF-α that subsequently crosses the BBB and activates microglia, promoting sustained pro-

inflammatory mediator release and neuroinflammation, leading to progressive dopaminergic neurodegeneration in a TNF-α dependent manner. Further studies by this group have shown that NADPH oxidase (NOX) drives age-related persistent increases in microglial activation, nigral ROS production and ensuing dopaminergic neurodegeneration (when assessed at 12 & 22 months) in response to systemic LPS (5 mg/kg i.p.) administration (Qin et al., 2013). Data from the same study shows that systemic LPS failed to induce a significant loss of TH-ir neurons within the SNpc of NOX-2 deficient (NOX-$2^{-/-}$) mice and that treatment with the NOX inhibitor diphenyliodonium (DPI) blocks LPS-induced microglial activation, ROS production and pro-inflammatory mediator expression (decreases in TNF-α, IL-1β, MCP-1), thus indicating a driving role for LPS-induced increases in NADPH oxidase expression in neuroimmune activation and ensuing dopaminergic neurotoxicity during aging. Taken together, a single occurrence of sepsis can lead to the delayed and progressive loss of dopamine neurons within the SNpc, highlighting a possible link between patients surviving septic shock and the incidence of PD.

Interestingly, a non-toxic dose of intra-nigral LPS (0.09 μg) increases susceptibility of dopaminergic neurons to intra-striatal 6-OHDA-induced degeneration (Koprich et al., 2008). The authors demonstrate that intra-nigral LPS induced microglial activation and prominent increases in IL-1β production which rendered nigral dopamine neurons more vulnerable to a subsequent low dose of 6-OHDA. Continuous infusion of an interleukin-1 receptor antagonist (3.64 mg/kg/hr IL-1Ra, s.c.) however, suppressed TNF-α and IFN-γ levels and abrogated the augmented dopaminergic neuronal loss within the substantia nigra mediated by LPS-sensitisation to dopaminergic neurodegeneration when assessed 21 days post intra-nigral 6-OHDA injection. Similar studies by (Deleidi et al., 2010) have revealed that a prior intra-nigral injection of the TLR3 agonist poly(I:C) induced a long-term neuroinflammatory reaction within the SN and dorsolateral striatum which predisposed rats to exacerbations of midbrain dopaminergic neuronal loss and striatal nerve terminal degeneration in response to subsequent exposure to a low dose of intra-striatal 6-OHDA (5μg). As before, systemic administration of IL-1ra neutralised exacerbations in nigral dopamine cell loss and striatal nerve terminal degeneration in response to combined intra-nigral poly(I:C)-induced neuroinflammation & intra-striatal 6-OHDA-mediated oxidative stress. This ''multiple hit'' hypothesis of sporadic PD may be explained at least partially by a phenomenon known as microglial priming, whereby activated microglia (by virtue of accumulating misfolded proteins during ongoing neurodegenerative disease or changes in their cellular microenvironment for example) are susceptible to a secondary inflammatory stimulus and thereby trigger an exaggerated inflammatory response, with potent neurotoxic consequences (Perry and Holmes, 2014). In the elderly population, the secondary inflammatory stimulus driving an exaggerated inflammatory response from primed microglia may also be of central origin, albeit is more common to arise in these individuals due to a systemic infection harbouring an inflammatory component (Norden et al., 2015). Hence, pharmacologically targeting systemic disease directly, or disrupting signalling

pathways that instigate the CNS response to systemic infection may be a vital therapeutic window to slow/halt disease progression in instances where peripheral and/or CNS inflammation are known to contribute to neuropathology.

The β_2-adrenoceptor (β_2-AR) is a G protein-coupled receptor GPCR involved in a variety of roles including the regulation of airway and vasculature smooth muscle activity, and is expressed on immune cells such as macrophages, T-cells, B-cells and more highly on the cell surface of brain-resident microglia (Tanaka et al., 2002). Downstream signalling through β_2-AR activation can influence the inflammatory response & immune function of these cells (van der Poll et al., 1994; Farmer and Pugin, 2000b; Kin and Sanders, 2006) and thus, pharmacologically targeting this receptor for immunomodulation may be of therapeutic value in conditions where inflammation contributes to neuropathology. Clenbuterol is a brain penetrant β_2-adrenoceptor agonist used in the treatment of respiratory disorders including asthma and chronic obstructive pulmonary disease (Baronti et al., 1980; Papiris et al., 1986; Boner et al., 1988) and has been shown to have neuroprotective properties both *in vitro* and *in vivo* by reducing apoptosis induced by the excitotoxin kainic acid (KA) (Semkova et al., 1996a; Gleeson et al., 2010), in rodent models of cerebral ischaemia (Semkova et al., 1996b; Zhu et al., 1998; Culmsee et al., 1999b; Junker et al., 2002; Culmsee et al., 2004), and in a murine model of motor neuron disease (Teng et al.). Administration of clenbuterol (0.5 mg/kg i.p.) to rats suppresses NfκB activity and ameliorates expression of the NfκB-inducible genes TNF-α and ICAM-1 in response to bacterial LPS (1 μg/5μl; icv), whilst concurrently elevating the temporal expression of the NfκB-inhibitory protein IκBα (Ryan et al., 2013). Moreover, clenbuterol (0.5 mg/kg; i.p.) has been reported to induce expression of the negative regulators IL-1ra and IL-1RII *in vivo*, and also increases central expression of the broad spectrum anti-inflammatory cytokine IL-10 and its downstream signalling molecule suppressor of cytokine signalling-3 (SOCS-3) thereby contributing to its anti-inflammatory effects in the brain (Ryan et al., 2011). Studies by (Farmer and Pugin, 2000a) have demonstrated that β_2-adrenoceptor stimulation curtails LPS-induced TNF-α and IL-8 production from human pro-monocytic THP-1 cells, and that the β_2-adrenoceptor agonist terbutaline suppresses LPS-driven p38MAPK activation and subsequent TNF-α production in renal mesangial cells (Nakamura et al., 2003). Moreover, the long acting β_2-agonists salmeterol and clenbuterol attenuate IL-1β-induced ICAM-1 expression in human airway smooth muscle cells (Kaur et al., 2008). Thus, β_2-adrenoceptor stimulation drives an anti-inflammatory phenotype in the CNS that may be of therapeutic benefit in conditions where central and/or peripheral inflammation contributes to neuropathology.

In the field of Alzheimer's disease research, evidence indicates that prior DSP4-induced (2 x 50 μg/kg i.p. set 1 week apart) locus coeruleus (LC) degeneration and ensuing noradrenaline depletion exacerbates intra-cortical $A\beta_{1\text{-}42}$-dependent induction of IL-1β, IL-6 and iNOS expression in mice, findings which were attenuated by co-injection with noradrenaline or the β-AR agonist isoproterenol (Heneka et al., 2002b). This indicates that degeneration of the LC-noradrenergic system, as occurs in Alzheimer's disease (Grudzien et al., 2007), is

permissive for enhanced neuroinflammation and AD-related neuropathology, whereas enhancing central NAergic bioavailability or mimicking its endogenous activity with a β-agonist may curtail pro-inflammatory events & slow disease progression. Furthermore, chronic treatment with the selective β_1-AR partial agonist xamoterol (ADRB1) attenuates neuroinflammation in the 5XFAD transgenic mouse model of Alzheimer's disease (diminished regional AD-related gliosis & reductions in Iba1, CD74, CD14 and TGF-β expression), findings which were accompanied by ameliorated amyloid & tau pathology (Ardestani et al., 2017). Similarly, in the Parkinson's disease field, treatment with DSP4 (50 mg/kg i.p injection every 2 weeks) commencing 6 months post systemic LPS challenge (5 mg/kg i.p.) exacerbates the loss of dopaminergic neurons in the rodent SNpc (Jiang et al., 2015). Interestingly, the same authors demonstrate that pre-treatment of LPS-challenged mesencephalic neuron/glia cultures derived from β_2-AR-deficient mice with NA does not totally abolish the neuroprotection observed, indicating that at a submicromolar level (10- to 100-fold lower than its K_i value of binding affinity to adrenergic receptors) the neuroprotection afforded by NA occurs at least partially, in a β_2-AR-independent fashion. Indeed, data derived from the same studies show that NA, at submicromolar concentrations, exerts an anti-inflammatory effect on activated microglia by attenuating LPS-induced pro-inflammatory mediator production (such as TNF-α & Nitric oxide) and inhibiting microglial NADPH oxidase-mediated superoxide production. Hence, degeneration of LC noradrenergic neurons (up to 80%), as occurs in Parkinson's disease even before the onset of dopaminergic neuronal loss (Zarow et al., 2003a; Baloyannis et al., 2006) is likely to enhance neuroinflammation & PD-related neuropathology whereas noradrenergic augmentation strategies could curtail these inflammatory processes & slow/halt disease progression.

Moreover, studies by (Qian et al., 2011) has shown that continuous infusion of the β_2-AR agonist salmeterol (1 or 10 μg/kg/day) for 2 weeks commencing 2 days prior to lesioning, prevents dopaminergic neurotoxicity when assessed 3 weeks later in response to a 6-day consecutive lesioning regimen with MPTP (15 mg/kg s.c.). Similarly, data from the same study has shown that treatment with salmeterol (10 μg/kg/day) for 2 weeks, commencing 3 months after a single systemic injection of LPS (5 mg/kg i.p.) protected against motor deficits in the rotarod test when assessed at 8 & 10 months post lesioning, and prevented the loss of dopaminergic cells in the SNpc. The authors demonstrate that the neuroprotection afforded by glial β_2-AR activation occurred through the inhibition of microglial activation and ensuing pro-inflammatory mediator production (including TNF-α, superoxide & NO) in conjunction with the inhibition of TAK1-mediated phosphorylation of MAPK and p65 NfκB in a β_2-AR/β-arrestin2-dependent signalling pathway. It is of considerable interest to note however, that despite the well documented anti-inflammatory effect of glial β_2-AR stimulation on activated microglia and ensuing pro-inflammatory mediator production, the endogenous ligand for the β_2-AR receptor, noradrenaline, may also exert a neuroprotective role in the brain via inducting increased trophic factor synthesis

from astrocytes. Studies by (Junker et al., 2002) have demonstrated that clenbuterol activates astrocytes & protects cultured hippocampal cells against glutamate toxicity, but not in the presence of the β-blocker propranolol or the specific β_2-AR antagonist ICI 118551 (10 μM), findings which were replicated with the specific β_2-AR agonist salmeterol (0.001-1 μM). The specific β_1-AR antagonist metoprolol (10-100 μM) however, failed to alter the neuroprotective effect of clenbuterol, indicating that neuronal protection against glutamate induced excitotoxicity was attributed to glial β_2-AR stimulation. Moreover, the same authors show that pre-treatment with clenbuterol (0.3 mg/kg i.p.) 5 hours prior to middle cerebral artery occlusion decreased the infarct area in a murine model of focal cerebral ischaemia, a finding which was blocked by co-administration of the selective β_2-AR antagonist butoxamine (5 mg/kg), but not the selective β_1-AR antagonist metoprolol (0.5-5 mg/kg), thus further indicating that neuroprotection afforded by clenbuterol *in vivo* occurs exclusively via the activation of β_2-AR's. The authors propose that increases in the synthesis of growth factors in activated astrocytes may underlie the neuroprotection observed downstream of glial β_2-AR stimulation.

Indeed, studies on the comparative effects of CDNF & GDNF in a nonhuman primate model of Parkinson's disease by (Garea-Rodríguez et al., 2016) have demonstrated using ^{123}I-FP-CIT (DaTSCAN) SPECT that intra-cerebral delivery of CDNF (20 μg) increases dopamine transporter binding activity in 6-OHDA-lesioned marmoset monkeys and that intra-cerebral delivery of GDNF (20 μg) in particular, induced an increase in TH-positive neurons, structures & varicosities in the 6-OHDA-lesioned caudate nucleus, indicating the strong neuro-restorative potential of GDNF *in vivo*. Moreover, prior chronic running exercise for 4 weeks attenuates the LPS (1 mg/kg i.p.)-induced loss of dopaminergic neurons in the SNpc, striatal DA loss and motor impairment on the rotarod test in a manner that was dependent on the BDNF signalling pathway, as blockade of the BDNF signalling pathway with the TrkB antagonist K252a prevented the exercise-induced protection against LPS-mediated neurotoxicity (Wu et al., 2011). Thus, noradrenaline may exert a bimodal neuroprotective role in the brain by (a) inhibiting microglial activation, anchoring NfκB activity in place and suppressing pro-inflammatory mediator production, and (b) providing trophic support via inducing the synthesis of growth factors from astrocytes, with both mechanisms likely to be dependent on glial β_2-AR activation in order to protect/restore dopaminergic structures from neuronal insult.

A recent breakthrough discovery by (Mittal et al., 2017) has highlighted that the β_2-adrenoceptor is a regulator of the α-synuclein gene (*SNCA*). The authors studied the pharmaceutical history of 4 million Norwegians who were taking one of the β-AR agonists for other medical problems over an 11 year period and found that usage of the β_2-AR agonist salbutamol (a brain-penetrant asthma medication) was associated with a decreased risk of developing Parkinson's disease, and conversely, that blockade of the β_2-AR with the antagonist propranolol was associated with an increased risk of developing PD. Interestingly, out of 1126 FDA-approved drugs & compounds screened, 4 significantly

reduced the *SNCA* mRNA & α-Syn protein levels in SK-N-MC cells; three of them being the selective β_2-AR agonists metaproterenol, clenbuterol & salbutamol. Furthermore, treatment with clenbuterol (20μM) reduced *SNCA* mRNA expression and α-Syn protein levels in *SNCA*-triplication patient iPSC-derived neuronal precursor cells and attenuated the loss of TH^+ dopaminergic neurons in the SNpc of MPTP-lesioned mice *in vivo*. Moreover, the authors demonstrate that the β_2-AR regulates the transcription of the human α-synuclein gene *SNCA* through H3K27 acetylation (H3K27ac) of promoters & enhancers in the human *SNCA* locus and that treatment with clenbuterol is correlated with a decrease in H3K27ac levels and relative expression of *SNCA* mRNA levels. Data derived from the same study also shows that knockout of the β_2-AR gene (*Adrb2*) in murine primary neurons, RNA interference-induced silencing of the β_2-AR in human SK-N-MC cells or chemical antagonism of the β_2-AR with the β-blocker propranolol in SK-N-MC cells consistently increases *SNCA* mRNA expression and α-Syn protein abundance. Conversely, transfection of SK-N-MC cells with *ADRB2* constructs reduces α-Syn *SNCA* mRNA levels and genetic silencing of the β_2-AR with siRNA's or its blockade with propranolol abrogates the SNCA expression-lowering effects induced by treatment with the β_2-AR agonist clenbuterol. Taken together, these results are strong evidence that the β_2-AR is linked to transcription of α-synuclein and risk of developing Parkinson's disease, and that pharmacological stimulation of glial β_2-AR's may provide neuroprotection.

Recent studies from our laboratory involving the LPS rat model of Parkinson's disease have demonstrated that treatment with the noradrenaline reuptake inhibitor (NRI) atomoxetine (3 mg/kg i.p.) alone or in combination with the α_2-adrenoceptor antagonist idazoxan (1 mg/kg i.p.) inhibits LPS-induced microglial activation within the substantia nigra, protects against dopaminergic neurodegeneration, attenuates nigrostriatal dopamine loss and confers partial protection against the associated motor deficits (Yssel et al., 2018). These findings implore future studies investigating which adrenergic receptor(s) can be targeted for activation in order to elicit the anti-inflammatory & neuroprotective effects afforded by pharmacologically enhancing central noradrenergic tone. Currently, we have gathered strong preliminary data to suggest that glial β_2-adrenoceptor stimulation (commencing 4 hours post intra-nigral LPS lesioning) with the brain penetrant, highly selective β_2-AR agonist clenbuterol or formoterol exerts potent anti-inflammatory & neuroprotective effects in the intra-nigral LPS model of PD (unpublished results). Given that PD-related neuropathology has progressed to an advanced stage prior to clinical diagnosis of Parkinson's disease in patients however, the therapeutic efficacy of targeting glial β_2-AR's in a more clinically relevant scenario to the human condition (i.e. allowing PD-related neuropathology and motor dysfunction to become apparent prior to commencing a treatment regimen) remains to be established.

6.2.1 Study aims & objectives

The aim of this study was to assess the therapeutic efficacy of glial β_2-adrenoceptor stimulation in the intra-nigral LPS model of Parkinson's disease. We aimed to mimic the effect of the endogenous neurotransmitter noradrenaline on glial β_2-AR's using the brain penetrant and selective β_2-adrenoceptor agonist formoterol for anti-inflammatory and neuroprotective effects. Previously we have shown that enhancing noradrenergic tone with the NRI atomoxetine protects against neuroinflammation, nigrostriatal neurodegeneration & motor dysfunction in the intra-nigral LPS model of PD (Yssel et al., 2018). Recently, preliminary data from our laboratory has demonstrated that pharmacological blockade of β_2-AR's with the β_2-AR antagonist ICI 118,551 (5 mg/kg; b.i.d; i.p.) abolishes the anti-inflammatory & neuroprotective effects afforded by treatment with atomoxetine. Moreover, treatment with either of the highly selective, lipophillic β_2-AR agonists clenbuterol or formoterol inhibits LPS-induced microglial activation within the SN, suppresses nigrostriatal neurodegeneration whilst attenuating striatal dopamine loss and improving motor function in rats (unpublished results). This treatment regimen commenced 4 hours post intra-nigral injection of LPS however, and is arguably suppressing an LPS effect that hasn't been given sufficient time to induce nigrostriatal neurodegeneration to levels comparable to that of the human condition, and thus, the clinical relevance of these findings in terms of promoting the use of a β_2-AR agonist as a potential PD pharmacotherapy remains to be established.

Here, we aim to address this issue by commencing formoterol treatment 4 weeks post the initial intra-nigral LPS lesion to allow PD-related neuropathology & motor dysfunction to progress to a more clinically relevant setting, where nigral microglial activation, dopaminergic neurodegeneration, nigrostriatal DA loss & associated motor deficits are already apparent. Moreover, we aimed to establish if systemic inflammatory events as occurs in PD patients (via contracting a bacterial infection for example) can augment microglial activation & exacerbate ongoing dopaminergic neurodegeneration along the nigrostriatal tract and the associated motor deficits. Therefore, the experimental aims of the current study are two-fold: (1) Assess whether or not prior LPS-mediated nigrostriatal dopaminergic neuropathology can predispose to a heightened degree of inflammation-induced Parkinsonian neuropathology & motor deficits when rodents are subsequently exposed to bacterial LPS of systemic origin, and (b) evaluate the anti-inflammatory & neuroprotective efficacy of glial β_2-AR stimulation on halting potential exacerbations of PD-related neuropathology & motor dysfunction in response to peripheral immune challenge. Thus, the main objective of this study was to assess the neuroprotective potential of glial β_2-AR stimulation post-onset of an LPS-induced Parkinsonian state in the rat (see schematic of experimental aims & objectives, below).

The long-acting, lipophilic, and highly selective β_2-AR agonist formoterol was selected for use in this study instead of clenbuterol as we have previously shown that it is more effective

at inhibiting microglial activation and protecting against striatal dopamine loss in response to intra-nigral LPS lesioning (unpublished results) and it is also currently a vital partition of an FDA-approved metered dose inhaler marketed under brand name Symbicort® for the treatment of asthma and COPD in humans so it is safe to use for prolonged periods of time in humans.

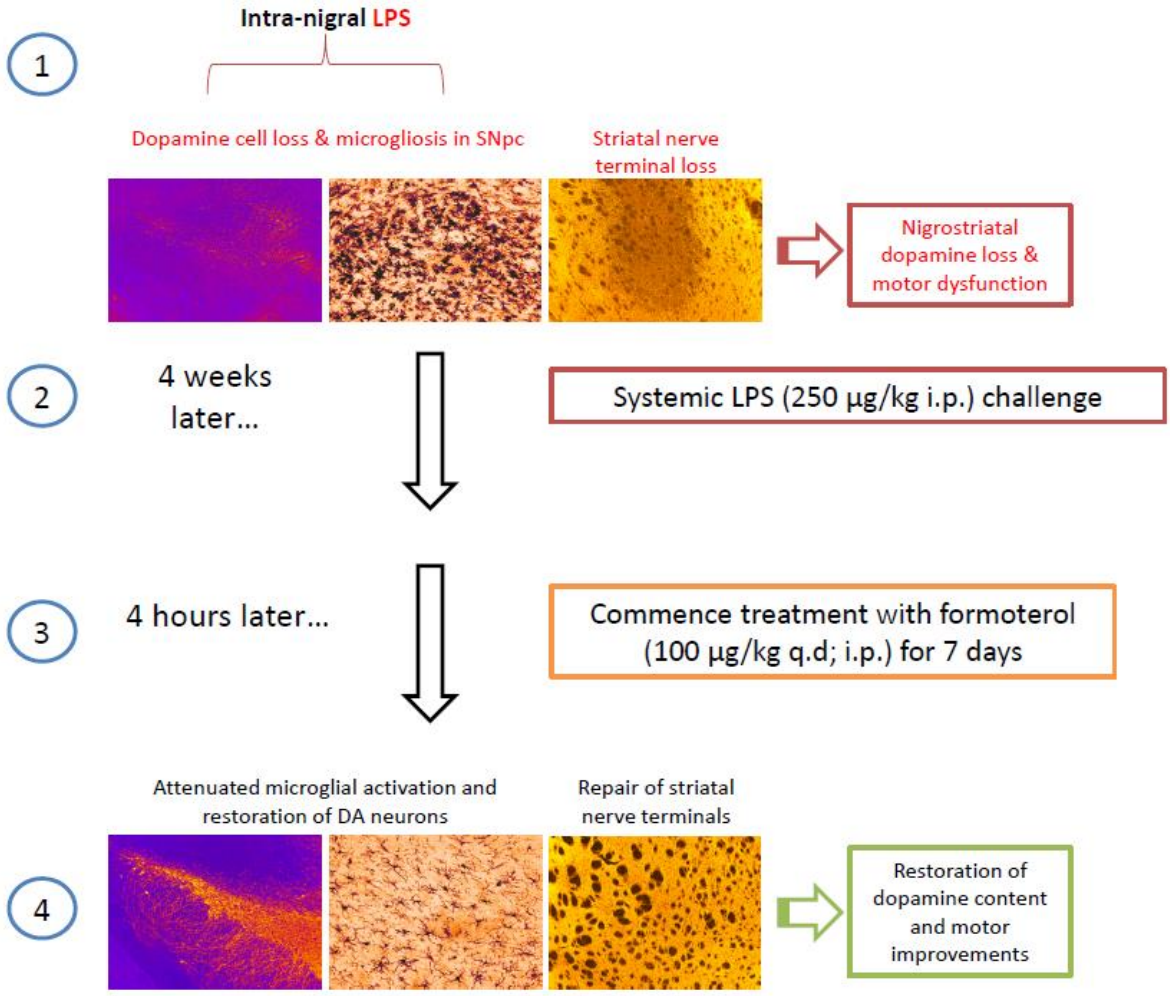


Figure 6.1 Diagrammatic summary of experimental aims & objectives. Unilateral intra-nigral injection of LPS to induce neuro-inflammation, nigrostriatal neurodegeneration, dopamine loss and associated motor deficits (1). Systemic immune challenge with the bacterial endotoxin LPS (250 µg/kg i.p.) 4 weeks later to assess the impact of peripheral immune challenge on PD-related neuropathology & motor dysfunction (2). Treatment commencement 4 hours later with the β_2-AR agonist formoterol once daily for 7 days (3). Assessing the therapeutic efficacy of glial β_2-AR stimulation in combating microglial activation, dopaminergic neurodegeneration and motor deficits in the LPS model of Parkinson's disease (4).

6.2.2 Experimental design

Adult male Wistar rats (n=64) aged 6-8 weeks (220-250g) were obtained from the comparative medicines unit (CMU, TCD) and were group housed in cages of 2 in climate controlled rooms set to 21°C on a 12:12 hour light-dark cycle. Animals were allowed food and water *ad libitum* and were habituated to the animal housing facilities for 1 week prior to commencing any experimental procedures. All behavioural testing was conducted between 09:00 and 18:00. The experimental protocols involved were in compliance with the European directive 2010/63/EU on the protection of animals used for scientific purposes. Rats were sorted into the following eight treatment groups: (1) Vehicle + saline + saline, (2) Vehicle + LPS + saline, (3) LPS + saline + saline, (4) LPS + LPS + saline, (5) Vehicle + saline + formoterol, (6) Vehicle + LPS + formoterol, (7) LPS + saline + formoterol, (8) LPS + LPS + formoterol n = 8 per group as listed in the following table.

Lesion	Systemic challenge	Treatment
Vehicle	saline	saline
Vehicle	LPS	saline
LPS	saline	saline
LPS	LPS	saline
Vehicle	saline	formoterol
Vehicle	LPS	formoterol
LPS	saline	formoterol
LPS	LPS	formoterol

Table 6.2 Experimental treatment groups

Behavioural training in the staircase test was conducted 5 days prior to stereotactic surgery. Rats received a unilateral intra-nigral injection of LPS (10µg/2µl) or vehicle (2µl 0.89% sterile saline). The coordinates for SNpc infusion were as follows: anteroposterior (AP) -5.3 mm, mediolateral (ML) ± 2.0 mm and dorsoventral (DV) -8.5 mm. Behavioural testing in the staircase, stepping and cylinder tests was conducted at 35 (week 5) and 42 (week 6) days post-surgical procedures between the hours of 10-5pm. Rats were systemically challenged with the bacterial endotoxin LPS (250 µg/kg i.p.) or sterile saline solution 4 weeks post intra-nigral LPS injection (day 28). Treatment with formoterol (100 µg/kg i.p.) or saline control commenced 4 hours post systemic challenge with LPS or sterile saline and continued once daily (i.p.) for 7 days. After conducting week 6 post-lesioning behavioural testing (day 42), rats were euthanized by transcardial perfusion fixation in preparation for immunohistochemical analysis of nigrostriatal integrity and glial cell activation.

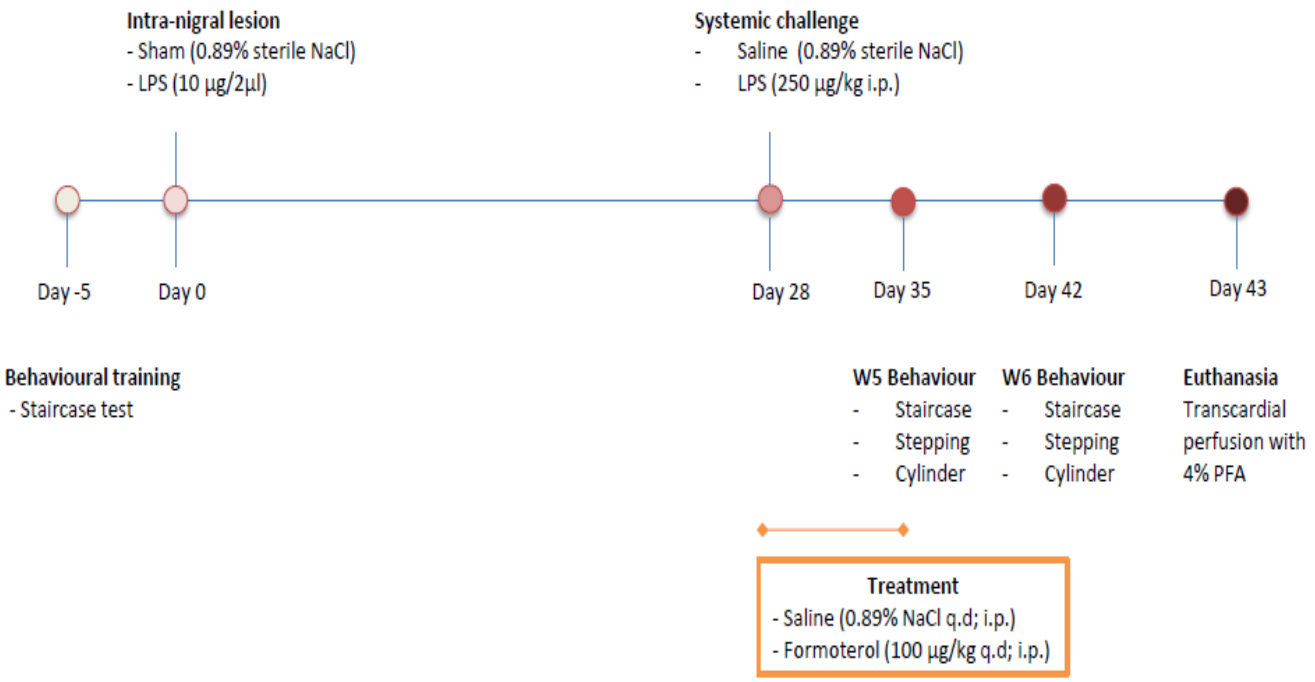


Figure 6.2 Experimental timeline of investigations.

6.3 Results

6.3.1 Treatment with formoterol protects against advancements in skilled motor dysfunction in the staircase test.

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 56)}$ = 74.42, P<0.0001) and an interaction effect between formoterol x systemic LPS ($F_{(1, 56)}$ = 6.678, P=0.0124) on the forelimb contralateral success rate in the staircase test of skilled motor function at week 5 post lesioning. Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 56)}$ = 288.8, P<0.0001), systemic LPS ($F_{(1, 56)}$ = 7.232, P=0.0094) and an interaction effect of intra-nigral LPS x systemic LPS ($F_{(1, 56)}$ = 5.6, P=0.0214) on contralateral success rate at week 6 post lesioning. Bonferroni *post hoc* analysis revealed that intra-nigral LPS injection reduced the contralateral success rate when assessed at both 5 weeks (P<0.05) and 6 weeks (P<0.001) post-lesioning relative to sham-lesioned controls. Systemic LPS challenge alone had no effect on contralateral success rate at either time-point tested. Systemic LPS challenge lead to an observed exacerbation of skilled motor impairments in animals previously lesioned with intra-nigral LPS at week 5 post lesioning, and further augmented skilled motor deficits at 6 weeks post lesioning relative to LPS-lesioned rats challenged systemically with vehicle control (P<0.01). Treatment with formoterol did not improve skilled motor function in animals lesioned centrally with intra-nigral LPS at either time-point tested, these animals also displayed clear motor deficits in the staircase test at both 5 and 6 weeks post-lesioning relative to Sham-lesioned animals (P<0.001). Skilled motor deficits were also apparent in intra-nigral LPS-lesioned animals subsequently challenged with systemic LPS and treated with formoterol at both week 5 (P<0.05) and week 6 (P<0.001) post-lesioning relative to Sham-lesioned controls. The contralateral success rate however, was observably higher in formoterol treated animals previously lesioned with intra-nigral LPS and subsequently challenged with LPS systemically relative to the LPS+LPS treatment group treated with saline control at both week 5 & week 6 post-lesioning, thus indicating a partial protective effect of formoterol treatment against exacerbations in skilled motor deficits in response to a peripheral immune stressor, albeit these results were not deemed statistically significant.

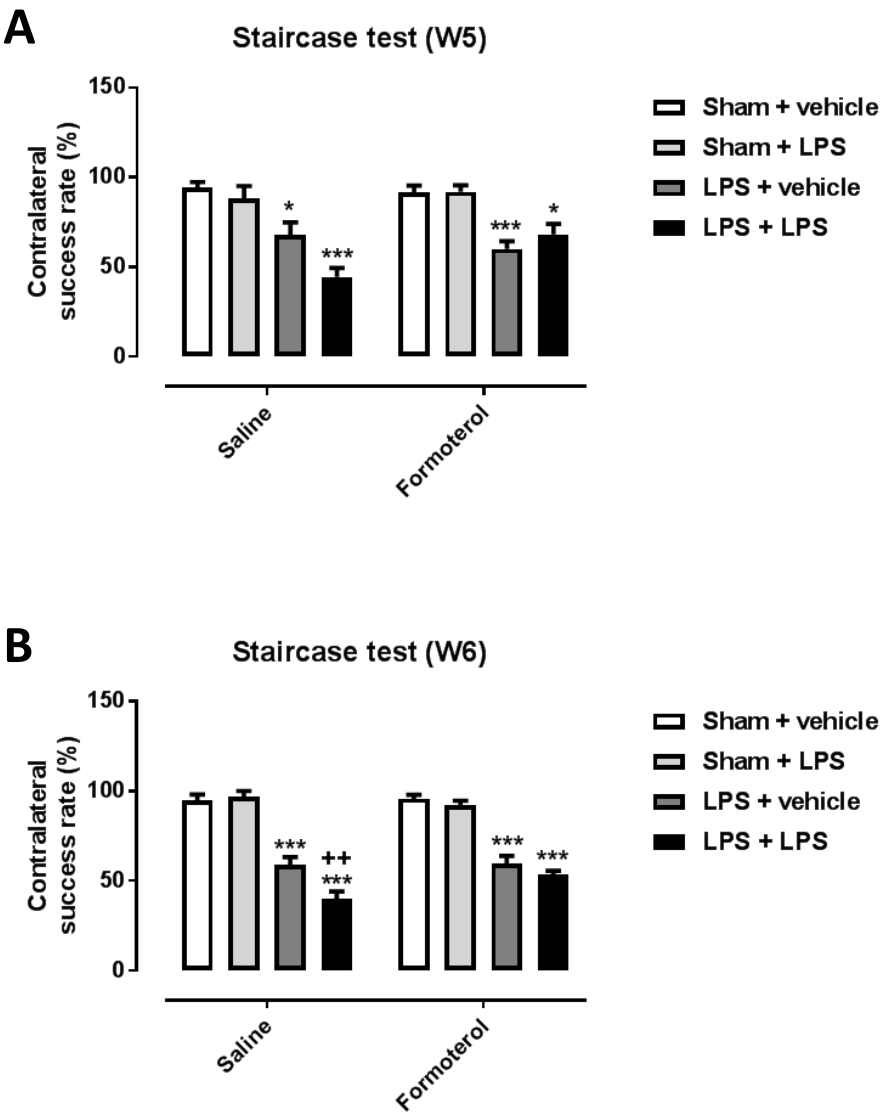


Figure 6.3 Formoterol provides partial protection against advancements in skilled motor deficits. Intra-nigral LPS reduced the contralateral success rate in the staircase test of skilled motor function. Systemic LPS alone had no effect on the contralateral success rate but exacerbated skilled motor deficits particularly at 6 weeks post-lesioning in animals previously lesioned with intra-nigral LPS. Treatment with formoterol failed to restore skilled motor function in LPS-lesioned rats but partially inhibited advancements in skilled motor deficits in response to a subsequent peripheral immune challenge with bacterial LPS (A+B). Data expressed as mean ± S.E.M. (n=8) * ($P<0.05$), *** ($P<0.001$) vs. Sham-lesioned animals with systemic vehicle challenge treated with saline, ++ ($P<0.01$) vs. LPS-lesioned rats with systemic vehicle challenge treated with saline by Three-way ANOVA with *post hoc* Bonferroni.

6.3.2 Treatment with formoterol protects against advancements in forelimb akinesia in the forehand direction in the stepping test.

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 56)}$ = 297.9, P<0.0001), an interaction effect of formoterol x systemic LPS ($F_{(1, 56)}$ = 10.74, P=0.0018) and an interaction effect of formoterol x intra-nigral LPS x systemic LPS ($F_{(1, 56)}$ = 7.562, P=0.0080) on forelimb kinesis in the forehand direction at week 5 post-lesioning. Bonferroni *post hoc* analysis revealed that intra-nigral LPS reduced the number of contralateral adjusting steps made in the forehand direction when assessed 5 weeks post-lesioning (P<0.001). Systemic LPS challenge alone had no effect on forelimb kinesis, but the no. of adjusting steps made with the contralateral limb was lower in response to peripheral LPS injection in animals previously lesioned with intra-nigral LPS relative to intra-nigral LPS-lesioned rats challenged with vehicle control, albeit this result was not statistically significant. Treatment with formoterol did not restore forelimb kinesis in the forehand direction at W5 post-lesioning, the deficits in the number of contralateral adjusting steps made in the FH direction were equally apparent relative to Sham-lesioned controls (P<0.001). Treatment with formoterol however completely prevented systemic LPS-mediated exacerbations in forelimb akinesia in animals previously lesioned with intra-nigral LPS at week 5 post-lesioning (P<0.01).

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 56)}$ = 163.8, P<0.0001), an interaction effect between formoterol x systemic LPS ($F_{(1, 56)}$ = 4.95, P=0.0301) and an interaction effect between formoterol x intra-nigral LPS x systemic LPS ($F_{(1, 56)}$ = 9.018, P=0.0040) on forelimb kinesis in the forehand direction at week 6 post-lesioning. Bonferroni *post hoc* analysis revealed that intra-nigral LPS reduced the number of contralateral adjusting steps made in the forehand direction when assessed 6 weeks post-lesioning relative to sham-lesioned controls (P<0.01). Systemic LPS challenge alone had no effect on forelimb kinesis. Systemic LPS challenge exacerbated forelimb akinesia in animals previously lesioned with intra-nigral LPS relative to LPS-lesioned animals challenged systemically with vehicle control (P<0.05). Treatment with formoterol did not restore forelimb kinesis in animals lesioned with intra-nigral LPS, these rats displayed clear deficits in adjusting steps relative to sham-lesioned controls (P<0.001). Treatment with formoterol attenuated systemic LPS-induced exacerbations in forelimb akinesia in the forehand direction by approximately 22% at week 6 post-lesioning relative to intra-nigral LPS lesioned rats that were subsequently exposed to peripheral immune challenge with bacterial LPS (P<0.05).

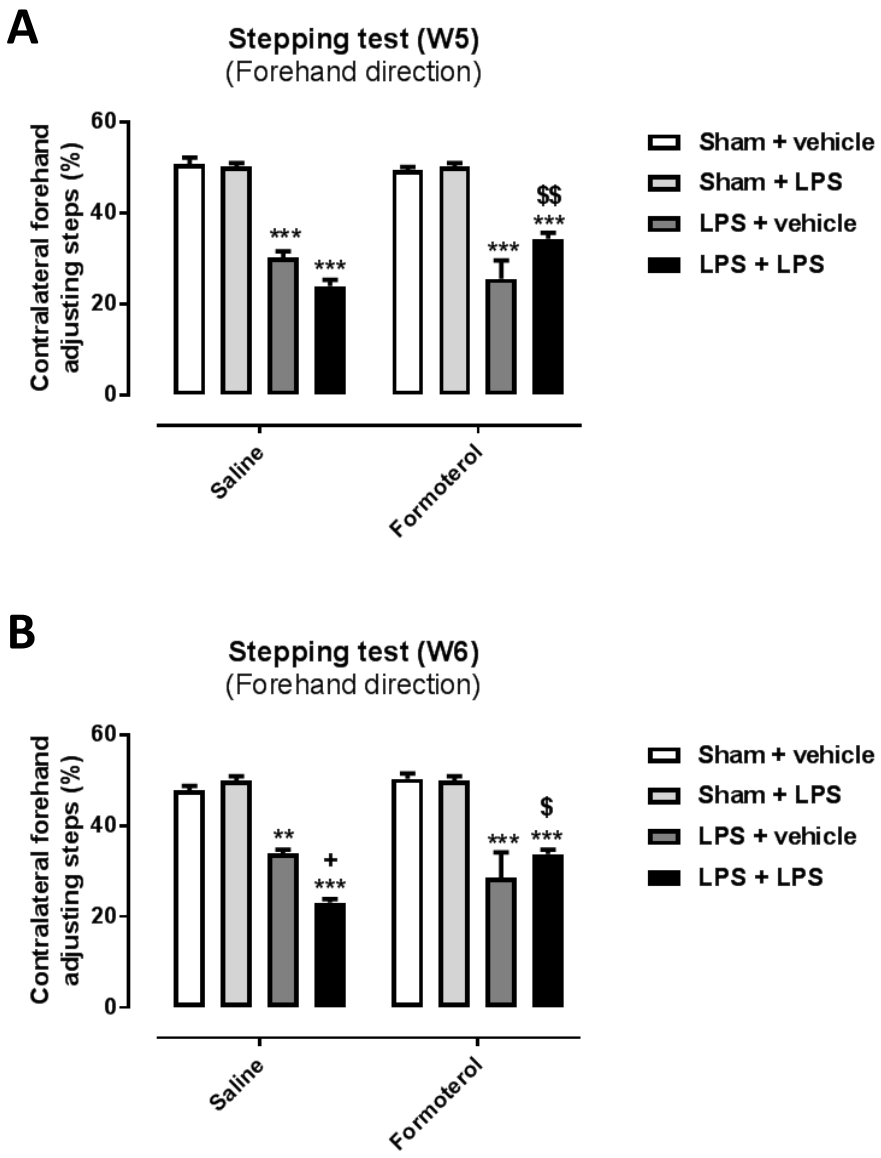


Figure 6.4 Formoterol curtails the development of forelimb akinesia in the forehand direction of the stepping test. Intra-nigral LPS injection reduced the number of adjusting steps made in the forehand direction at both W5 & W6 post lesioning. Systemic LPS alone had no effect on forelimb kinesis at either time-point tested. Systemic LPS exacerbated forelimb akinesia at W6 post-lesioning. Treatment with formoterol did not restore intra-nigral LPS-induced forelimb kinesis in the FH direction of the stepping test. Formoterol treatment mitigated exacerbations in forelimb akinesia in the forehand direction in intra-nigral LPS lesioned rats subsequently exposed to systemic LPS challenge (A+B). Data expressed as mean ± S.E.M. (n=8) ** (P<0.01), *** (P<0.001) vs. Sham-lesioned animals with systemic vehicle challenge treated with saline, + (P<0.05) vs. LPS-lesioned rats with systemic vehicle challenge treated with saline, $ (P<0.05), $$ (P<0.01) vs. LPS-lesioned rats with systemic LPS challenge treated with saline control by Three-way ANOVA with *post hoc* Bonferroni.

6.3.3 Treatment with formoterol protects against advancements in forelimb akinesia in the backhand direction in the stepping test.

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 56)}$ = 213.7, P<0.0001) on forelimb kinesis in the backhand direction at week 5 post-lesioning. Bonferroni *post hoc* analysis revealed that intra-nigral LPS alone and in combination with systemic LPS reduced the number of contralateral backhand adjusting steps made at W5 post-lesioning (P<0.001). Systemic LPS challenge alone had no effect on forelimb kinesis, albeit the number of contralateral adjusting steps made in the backhand direction was observably lower in systemic LPS challenged animals that were previously lesioned centrally with intra-nigral LPS. Treatment with formoterol did not restore forelimb kinesis in intra-nigral LPS-lesioned rats, these animals displayed clear deficits in the number of contralateral adjusting steps made in the backhand direction at week 5 post-lesioning relative to sham-lesioned rats (P<0.001).

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 56)}$ = 243.7, P<0.0001), an effect of formoterol ($F_{(1, 56)}$ = 11.28, P=0.0014) an interaction effect between formoterol x intra-nigral LPS ($F_{(1, 56)}$ = 5.185, P=0.0266), between formoterol x systemic LPS ($F_{(1, 56)}$ = 8.514, P=0.0051) and between formoterol x intra-nigral LPS x systemic LPS ($F_{(1, 56)}$ = 9.44, P=0.0033) on forelimb kinesis in the backhand direction at week 6 post-lesioning. Bonferroni *post hoc* analysis revealed that intra-nigral LPS reduced the number of contralateral adjusting steps made in the backhand direction relative to sham-lesioned controls (P<0.001). Systemic LPS alone had no effect on forelimb kinesis. Systemic LPS exacerbated forelimb akinesia in the backhand direction in animals previously lesioned with intra-nigral LPS (P<0.05). Treatment with formoterol did not restore forelimb kinesia in intra-nigral LPS-lesioned rats, these animals were clearly akinetic relative to sham-lesioned controls (P<0.001). Treatment with formoterol however ameliorated systemic LPS-mediated advancements in forelimb akinesia in the backhand direction by approximately 31% at week 6 post-lesioning relative to saline treated controls (P<0.001).

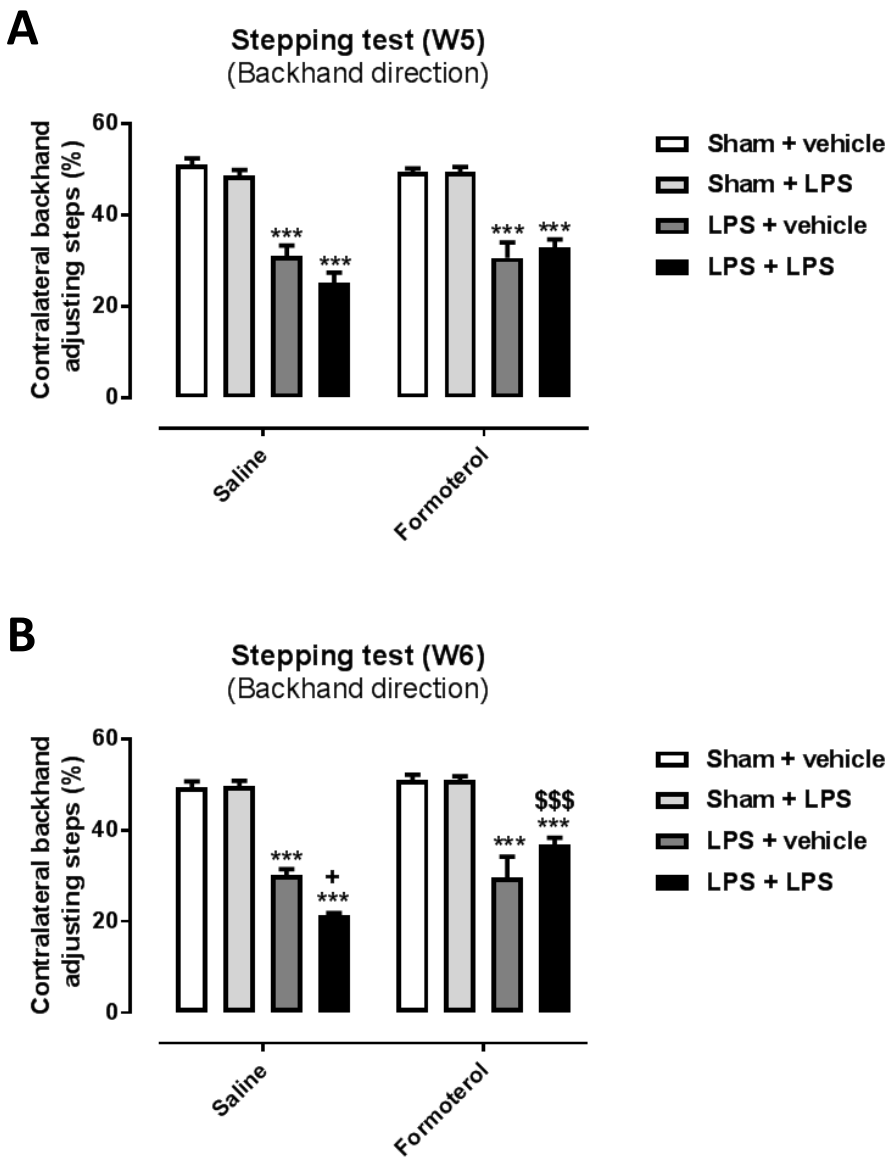


Figure 6.5 Formoterol curtails the development of forelimb akinesia in the backhand direction of the stepping test. Intra-nigral LPS injection reduced the number of adjusting steps made in the backhand direction at both W5 & W6 post lesioning. Systemic LPS alone had no effect on forelimb kinesis at either time-point tested. Systemic LPS exacerbated forelimb akinesia in the backhand direction at W6 post-lesioning. Treatment with formoterol did not restore intra-nigral LPS-induced forelimb kinesis in the BH direction of the stepping test. Formoterol treatment mitigated exacerbations in forelimb akinesia in the backhand direction in intra-nigral LPS-lesioned rats subsequently exposed to systemic LPS challenge (A+B). Data expressed as mean ± S.E.M. (n=8) *** ($P<0.001$) vs. Sham-lesioned animals with systemic vehicle challenge treated with saline, + ($P<0.05$) vs. LPS-lesioned rats with systemic vehicle challenge treated with saline, $$$ ($P<0.001$) vs. LPS-lesioned rats with systemic LPS challenge treated with saline control by Three-way ANOVA with *post hoc* Bonferroni.

6.3.4 Treatment with formoterol protects against advancements in asymmetric limb use in the cylinder test.

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 56)}$ = 130.8, P<0.0001) and systemic LPS ($F_{(1, 56)}$ = 8.213, P=0.0058) and an interaction effect of intra-nigral LPS x systemic LPS ($F_{(1, 56)}$ = 6.38, P=0.0144) on forelimb use asymmetry at week 5 post-lesioning in the cylinder test. Bonferroni *post hoc* analysis revealed that intra-nigral LPS reduced the number of contralateral wall placements made at W5 post-lesioning relative to sham-lesioned controls (P<0.001). Systemic LPS injection alone had no effect on forelimb use asymmetry. Systemic LPS challenge exacerbated deficits in contralateral wall placements made in animals that were previously lesioned with intra-nigral LPS (P<0.05). Treatment with formoterol did not restore intra-nigral LPS-induced forelimb use asymmetry in the cylinder test, these animals exhibited deficits in the number of wall placements made with the contralateral forelimb at W5 post-lesioning relative to sham-lesioned controls (P<0.01). No exacerbations in intra-nigral LPS-mediated forelimb use asymmetry in response to a subsequent systemic LPS challenge were evident in rats that received treatment with formoterol at week 5 post-lesioning.

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 56)}$ = 114.4, P<0.0001) and an interaction effect of formoterol x intra-nigral LPS ($F_{(1, 56)}$ = 4.549, P=0.0373) and between formoterol x systemic LPS ($F_{(1, 56)}$ = 8.833, P=0.0044) on the number of contralateral wall placements made at week 6 post-lesioning in the cylinder test. Bonferroni *post hoc* analysis revealed that intra-nigral LPS alone and in combination with systemic LPS reduced the number of contralateral wall placements at W6 post-lesioning relative to sham-lesioned animals in the cylinder test (P<0.001). Systemic LPS challenge exacerbated forelimb use asymmetry in animals previously lesioned with intra-nigral LPS at W6 in the cylinder test (P<0.05). Treatment with formoterol did not restore symmetrical forelimb use in intra-nigral LPS lesioned rats, these animals displayed clear deficits in the number of contralateral wall placements made at W6 post-lesioning relative to sham-lesioned controls (P<0.001). Treatment with formoterol attenuated systemic LPS-induced overt exacerbations in asymmetric limb use in rats that were previously lesioned with intra-nigral LPS at W6 post-lesioning (P<0.05).

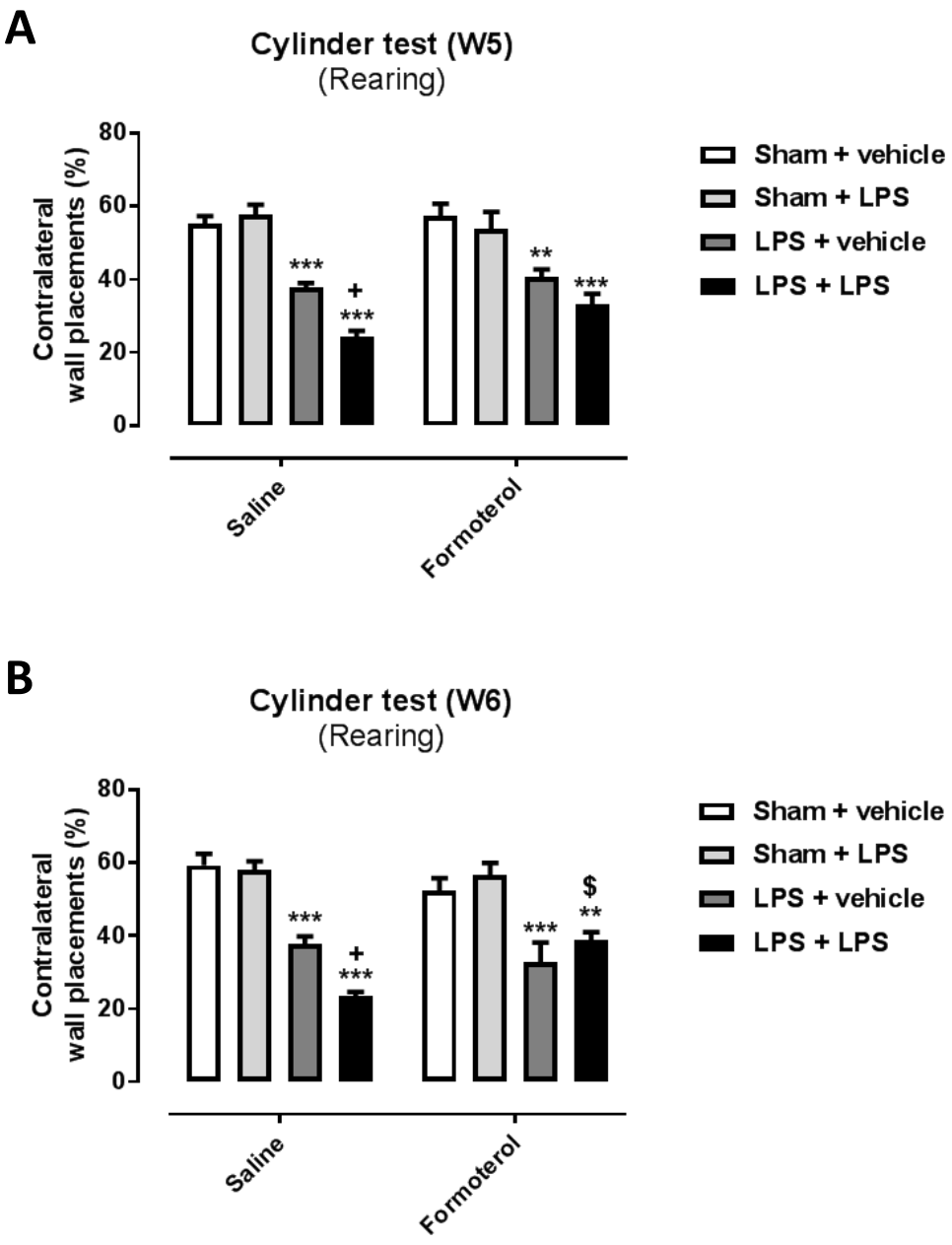
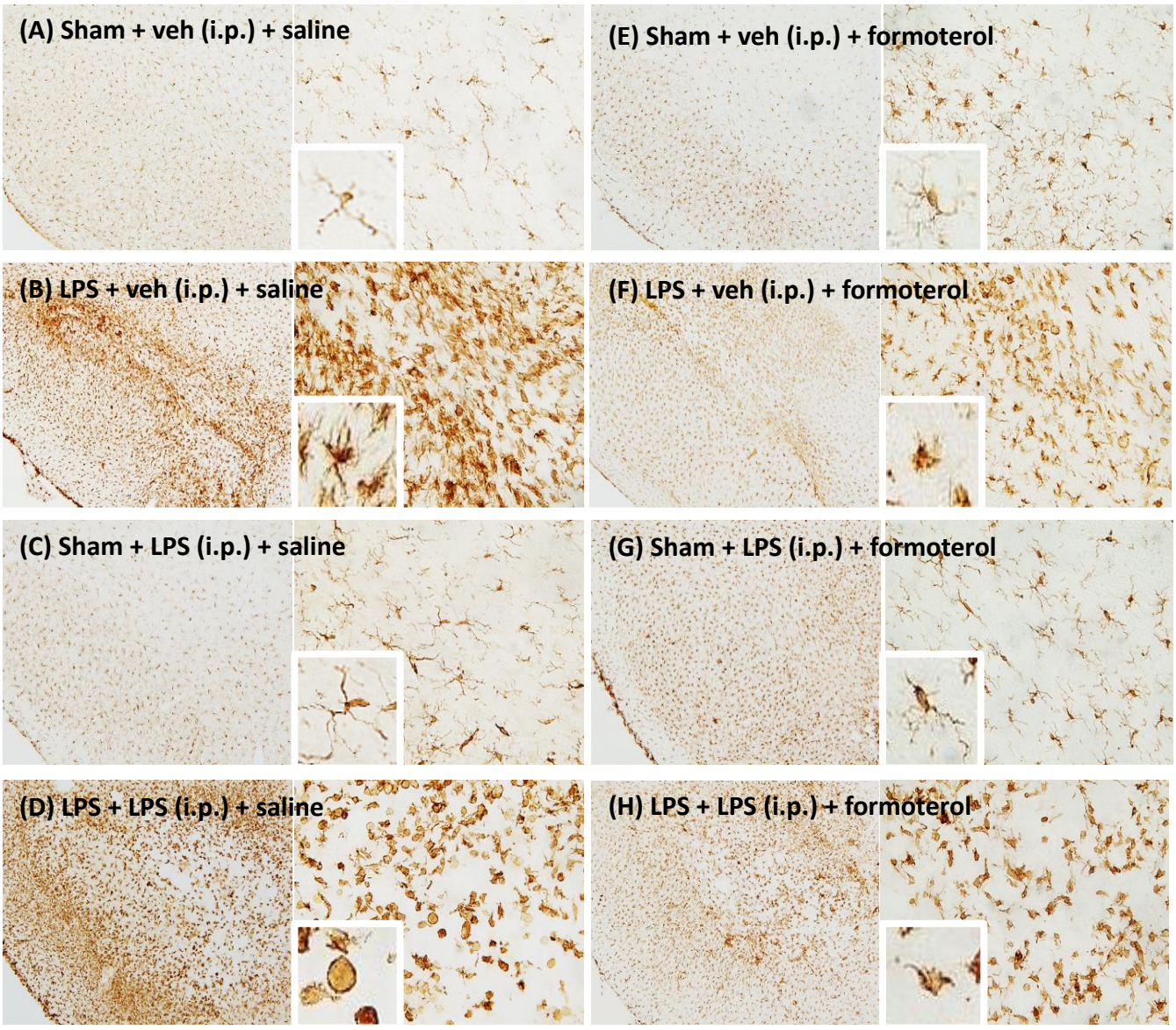


Figure 6.6 Formoterol attenuates advancements in forelimb use asymmetry in the cylinder test. Intra-nigral LPS reduced the number of contralateral wall placements made upon rearing at both W5 & W6 post-lesioning in the cylinder test. Systemic LPS challenge had no effect on forelimb preference. Systemic LPS exacerbated forelimb use asymmetry in LPS-lesioned rats. Formoterol treatment did not restore symmetrical forelimb use in LPS-lesioned animals in the cylinder test. Formoterol treatment ameliorated advancements in forelimb use asymmetry in response to peripheral immune challenge in animals that were previously lesioned with intra-nigral LPS (A+B). Data expressed as mean ± S.E.M. (n=8) ** (P<0.01), *** (P<0.001) vs. Sham-lesioned animals with systemic vehicle challenge treated with saline, + (P<0.05) vs. LPS-lesioned rats with systemic vehicle challenge treated with saline, $ (P<0.05) vs. LPS-lesioned rats with systemic LPS challenge treated with saline control by Three-way ANOVA with *post hoc* Bonferroni.

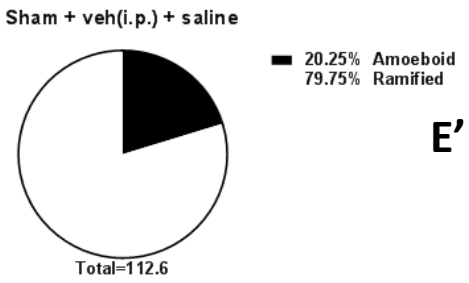
6.3.5 Treatment with formoterol curtails LPS-induced microglial activation within the SNpc and attenuates exacerbations in nigral microgliosis in response to peripheral immune challenge.

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 32)}$ = 98.47, P<0.0001) an effect of systemic LPS ($F_{(1, 32)}$ = 5.318, P=0.0277) and an effect of formoterol ($F_{(1, 32)}$ = 19.55, P=0.0001) on nigral Iba1-immunoreactivity. There was also an interaction effect between intra-nigral LPS x systemic LPS ($F_{(1, 32)}$ = 8.334, P=0.0069) and formoterol x intra-nigral LPS ($F_{(1, 32)}$ = 32.13, P<0.0001). Bonferroni *post hoc* analysis revealed that intra-nigral LPS injection alone and in combination with systemic LPS challenge induced a robust $Iba1^+$ microgliosis in the lesioned SN relative to Sham-lesioned animals challenged with vehicle control (P<0.001). Systemic LPS challenge alone had no effect on nigral Iba1-immunoreactivity. Systemic LPS challenge exacerbated the $Iba1^+$ microgliosis in rats previously lesioned intra-nigrally with LPS relative to LPS-lesioned rats challenged subsequently with vehicle control (P<0.05). Treatment with formoterol curtailed Iba1-immunoreactivity in the LPS-lesioned SN relative to LPS-lesioned rats treated with saline control (P<0.01). Treatment with formoterol prevented systemic LPS-mediated increases in nigral Iba1-immunoreactivity in animals previously lesioned intra-nigrally with LPS relative to LPS-lesioned animals challenged with systemic LPS and treated with saline control (P<0.001).

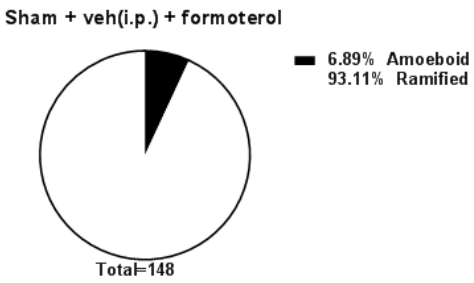
Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 32)}$ = 1331, P<0.0001) an effect of systemic LPS ($F_{(1, 32)}$ = 58.72 P<0.0001) and an effect of formoterol ($F_{(1, 32)}$ = 1333, P<0.0001) on the number of $Iba1^+$ amoeboid cells within the SN (see pie charts, below, for proportional differences). There was an interaction between intra-nigral LPS x systemic LPS ($F_{(1, 32)}$ = 44.74, P<0.0001), an interaction between formoterol x intra-nigral LPS ($F_{(1, 32)}$ = 618.3, P<0.0001) an interaction between formoterol x systemic LPS ($F_{(1, 32)}$ = 7.176, P=0.0116) and an interaction between formoterol x intra-nigral LPS x systemic LPS ($F_{(1, 32)}$ = 12.75, P=0.0011). Bonferroni *post hoc* analysis revealed that intra-nigral LPS injection alone or in combination with systemic LPS challenge increased the number of $Iba1^+$ amoeboid cells in the SN relative to sham-lesioned animals challenged with saline control (P<0.001). Systemic LPS alone had no effect on the number of $Iba1^+$ amoeboid cells in the SN. Superimposing a sub-toxic, low dose of bacterial LPS (250 µg/kg i.p.) 4 weeks post intra-nigral lesioning with LPS however, increased the proportion of $Iba1^+$ amoeboid cells in the substantia nigra by 29% when assessed at 6 weeks post-lesioning relative to LPS-lesioned rats challenged with saline control (P<0.001). Treatment with formoterol reduced the number of $Iba1^+$ amoeboid cells in the SN of LPS-lesioned rats (P<0.001) and attenuated the exacerbated increases in $Iba1^+$ amoeboid cell counts within the nigra of LPS-lesioned rats challenged with bacterial LPS (P<0.001).


(A) Sham + veh (i.p.) + saline
(E) Sham + veh (i.p.) + formoterol
(B) LPS + veh (i.p.) + saline
(F) LPS + veh (i.p.) + formoterol
(C) Sham + LPS (i.p.) + saline
(G) Sham + LPS (i.p.) + formoterol
(D) LPS + LPS (i.p.) + saline
(H) LPS + LPS (i.p.) + formoterol

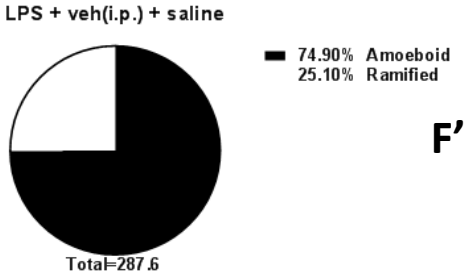
A’


Sham + veh(i.p.) + saline
20.25% Amoeboid
79.75% Ramified
Total=112.6

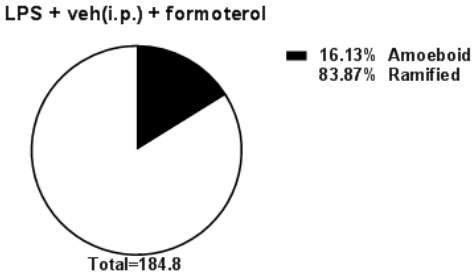
E’


Sham + veh(i.p.) + formoterol
6.89% Amoeboid
93.11% Ramified
Total=148

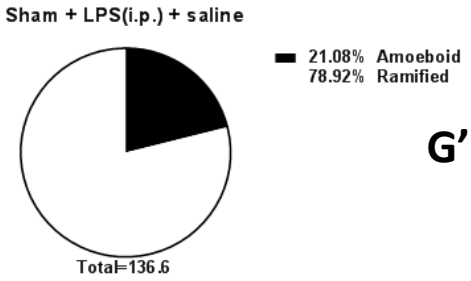
B’


LPS + veh(i.p.) + saline
74.90% Amoeboid
25.10% Ramified
Total=287.6

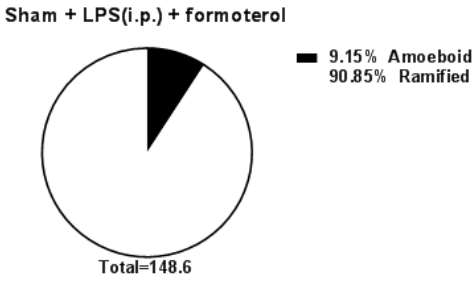
F’


LPS + veh(i.p.) + formoterol
16.13% Amoeboid
83.87% Ramified
Total=184.8

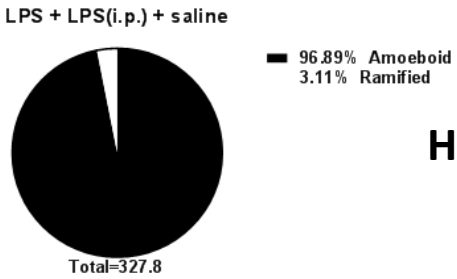
C’


Sham + LPS(i.p.) + saline
21.08% Amoeboid
78.92% Ramified
Total=136.6

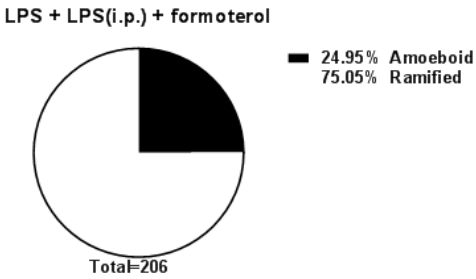
G’


Sham + LPS(i.p.) + formoterol
9.15% Amoeboid
90.85% Ramified
Total=148.6

D’


LPS + LPS(i.p.) + saline
96.89% Amoeboid
3.11% Ramified
Total=327.8

H’


LPS + LPS(i.p.) + formoterol
24.95% Amoeboid
75.05% Ramified
Total=206

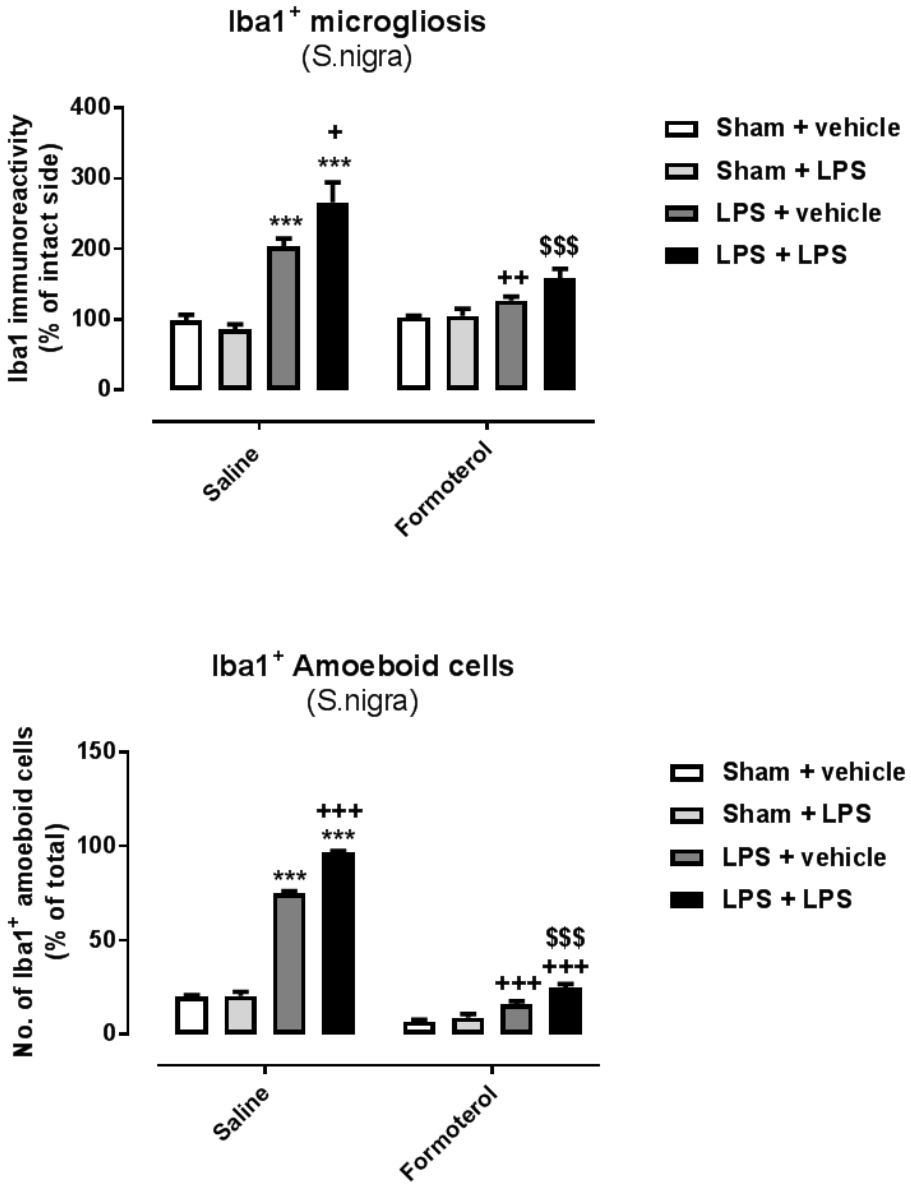
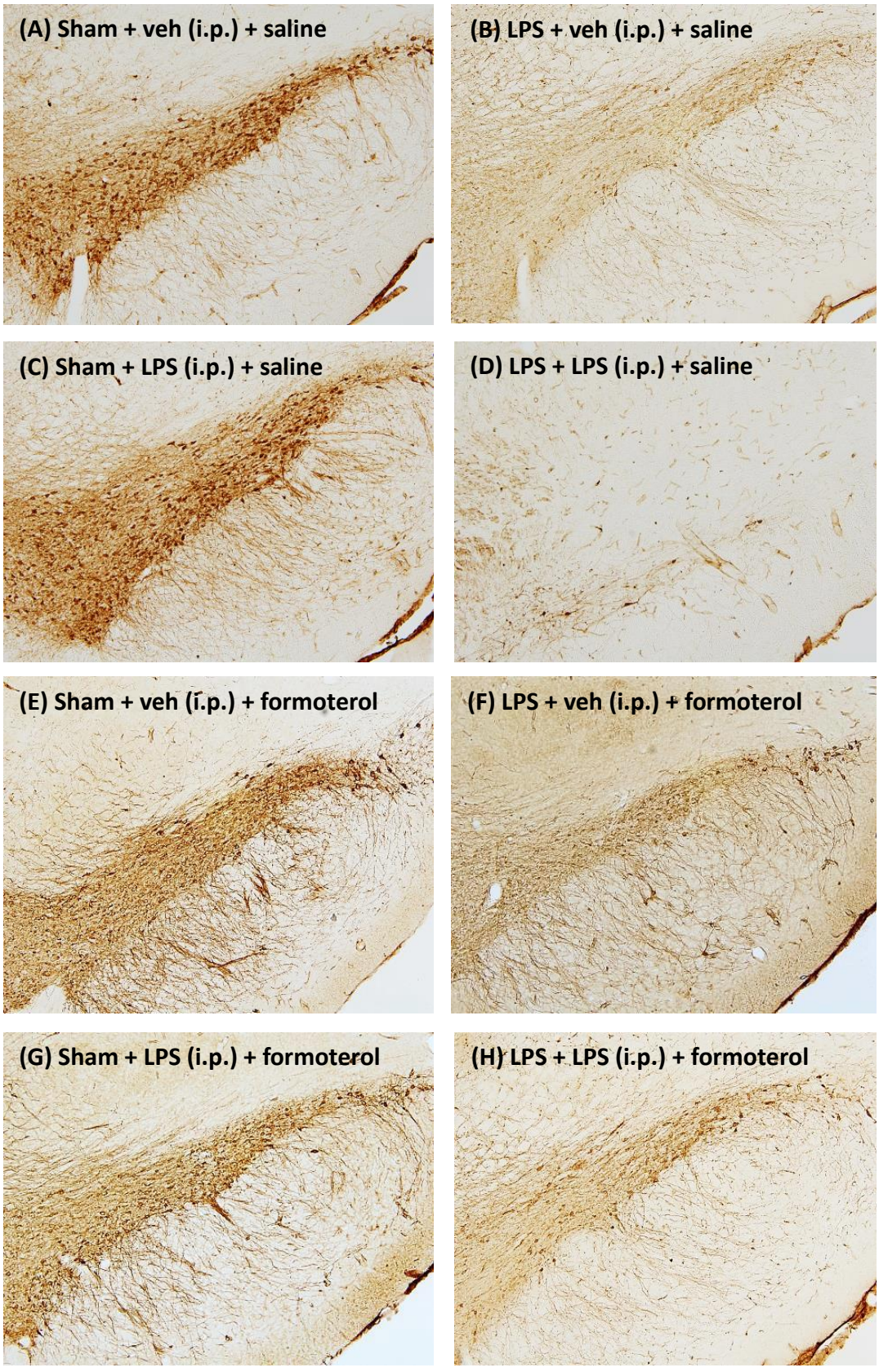


Figure 6.7 Formoterol attenuates microglial activation within the substantia nigra. Intra-nigral LPS injection induced robust microglial activation when assessed at 6 weeks post-lesioning. Systemic LPS administration alone had no effect on nigral Iba1-immunoreactivity or the number of Iba1$^+$ amoeboid cells within the SN but exacerbated nigral microgliosis and the number of Iba1$^+$ amoeboid cell counts in animals previously lesioned with LPS centrally. Treatment with formoterol restrained the intra-nigral LPS-induced increases in Iba1-immunoreactivity and suppressed exacerbations in nigral microgliosis (A-H) & the increases in the number of Iba1$^+$ amoeboid cells in response to peripheral immune challenge with bacterial LPS (A-H'). Data expressed as mean ± S.E.M. (n=5) *** (P<0.001) vs. Sham lesioned animals with systemic vehicle challenge treated with saline, + (P<0.05), ++ (P<0.01), +++ (P<0.001) vs. LPS-lesioned rats with systemic vehicle challenge treated with saline, $$$ (P<0.001) vs. LPS-lesioned rats with systemic LPS challenge treated with saline by Three-way ANOVA with *post hoc* Bonferroni.

6.3.6 Treatment with formoterol inhibits exacerbations in intra-nigral LPS-induced dopamine cell loss in the SNpc in response to peripheral immune challenge.

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 32)}$ = 770.1, P<0.0001) an effect of systemic LPS ($F_{(1, 32)}$ = 9.936, P=0.0035) and an effect of formoterol ($F_{(1, 32)}$ = 7.823, P=0.0087) on TH^+ cell counts within the SNpc. There was also an interaction effect between formoterol x intra-nigral LPS ($F_{(1, 32)}$ = 26.62, P<0.0001) and an interaction effect between formoterol x intra-nigral LPS x systemic LPS ($F_{(1, 32)}$ = 12.73, P=0.0012). Bonferroni *post hoc* analysis revealed that intra-nigral injection of LPS alone and in combination with systemic LPS challenge reduced the number of TH^+ dopamine cells in the SNpc by 61% & 83% respectively relative to sham-lesioned controls (P<0.001). Systemic administration of LPS alone had no effect on nigral TH^+ cell counts. Systemic administration of LPS exacerbated the TH^+ dopamine cell loss within the SNpc by 22% in animals previously lesioned with LPS centrally (P<0.001). Treatment with formoterol did not significantly restore the loss of dopamine neurons within the SNpc in response to intra-nigral lesioning with LPS. Treatment with formoterol prevented exacerbations in dopaminergic neuronal loss within the SNpc in LPS-lesioned rats subsequently exposed to systemic LPS relative to LPS+LPS lesioned rats treated with saline control (P<0.001).


(A) Sham + veh (i.p.) + saline
(B) LPS + veh (i.p.) + saline
(C) Sham + LPS (i.p.) + saline
(D) LPS + LPS (i.p.) + saline
(E) Sham + veh (i.p.) + formoterol
(F) LPS + veh (i.p.) + formoterol
(G) Sham + LPS (i.p.) + formoterol
(H) LPS + LPS (i.p.) + formoterol

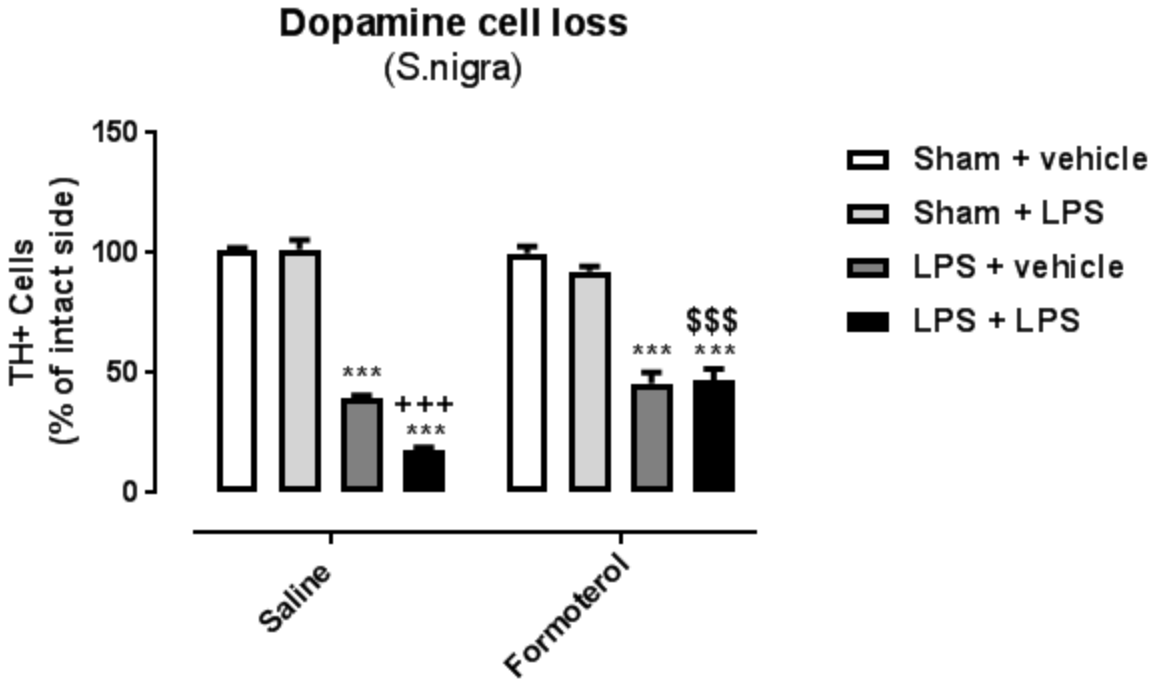
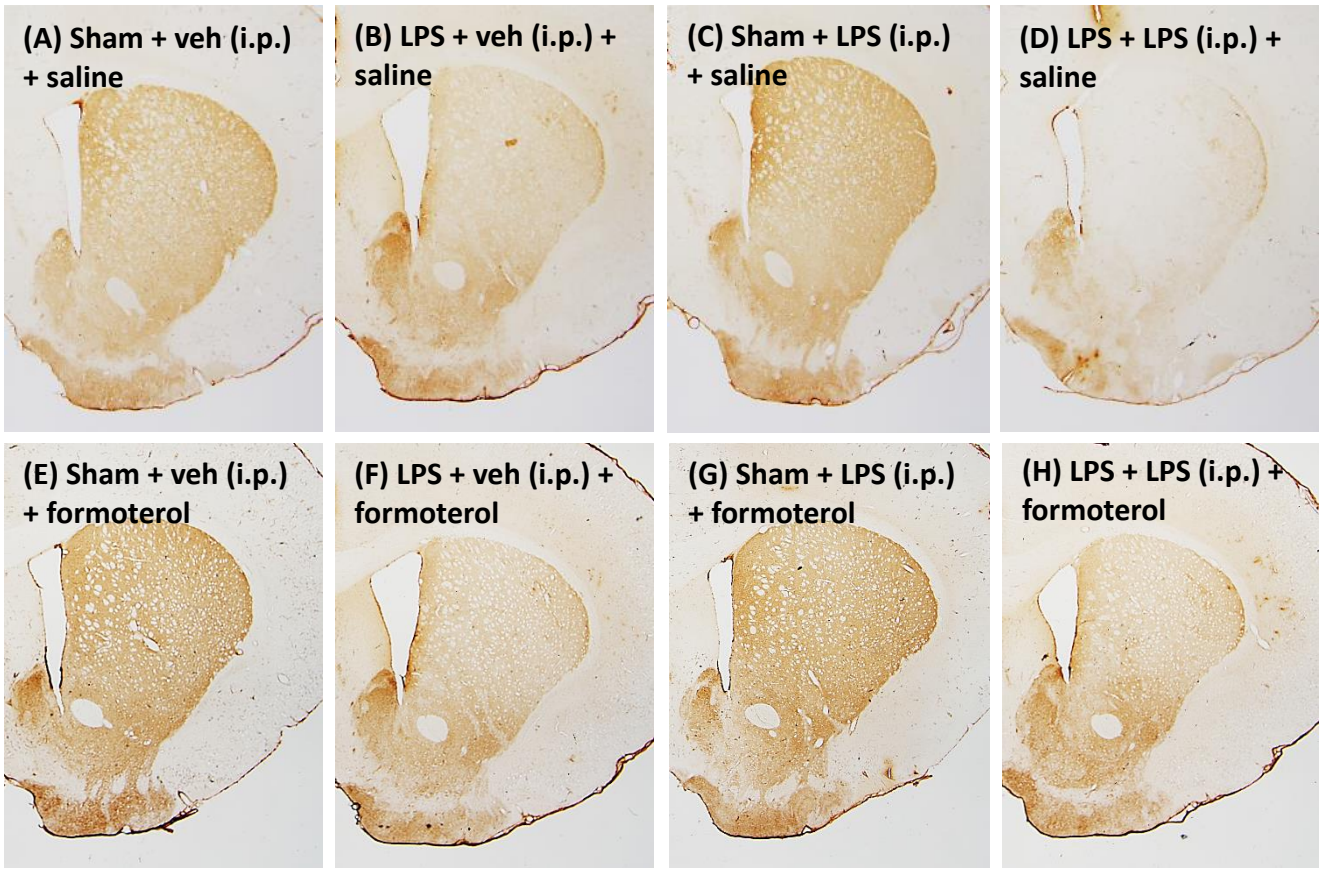


Figure 6.8 Formoterol halts ongoing intra-nigral LPS-induced dopaminergic neurodegeneration within the SNpc in response to peripheral immune challenge. Intra-nigral injection of LPS induced marked reductions in TH^+ dopamine cell counts at 6 weeks post lesioning. Systemic LPS administration exacerbated dopaminergic neurodegeneration within the SNpc in animals previously lesioned with LPS centrally. Treatment with formoterol failed to restore the loss of dopamine neurons within the SNpc, but completely attenuated the systemic LPS-induced exacerbations in the loss of dopamine cell bodies within the SNpc in animals previously injected with intra-nigral LPS. Data expressed as mean ± S.E.M. (n=5) *** ($P<0.001$) vs. Sham lesioned animals with systemic vehicle challenge treated with saline, +++ ($P<0.001$) vs. LPS-lesioned rats with systemic vehicle challenge treated with saline, $$$ ($P<0.001$) vs. LPS-lesioned rats with systemic LPS challenge treated with saline by Three-way ANOVA with *post hoc* Bonferroni.

6.3.7 Treatment with formoterol prevents exacerbations in intra-nigral LPS-induced nerve terminal degeneration in the striatum in response to peripheral immune challenge.

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 32)}$ = 354, P<0.0001) systemic LPS ($F_{(1, 32)}$ = 4.633, P=0.0390) and formoterol ($F_{(1, 32)}$ = 4.869, P=0.0346) on striatal TH-immunoreactivity. There was an interaction effect between intra-nigral LPS x systemic LPS ($F_{(1, 32)}$ = 6.694, P=0.0144) and between formoterol x intra-nigral LPS x systemic LPS ($F_{(1, 32)}$ = 4.91, P=0.0339). Bonferroni *post hoc* analysis revealed that intra-nigral LPS injection alone, or in combination with a subsequent peripheral immune challenge with bacterial LPS reduced dopaminergic nerve terminals in the striatum by 58% & 88% respectively relative to sham-lesioned animals challenged with saline control (P<0.001). Systemic LPS challenge alone had no effect on striatal nerve terminal integrity. Systemic LPS challenge exacerbated the loss of TH^+ dopaminergic nerve terminals by approximately 30% on average, in the striatum of rats previously exposed 6 weeks earlier to an intra-nigral LPS injection (P<0.01). Treatment with formoterol did not restore the intra-nigral LPS-induced loss of striatal nerve terminals relative to LPS-lesioned rats treated with saline control. Treatment with formoterol prevented exacerbations in intra-nigral LPS-induced striatal denervation in response to peripheral immune challenge with bacterial LPS (p<0.05).



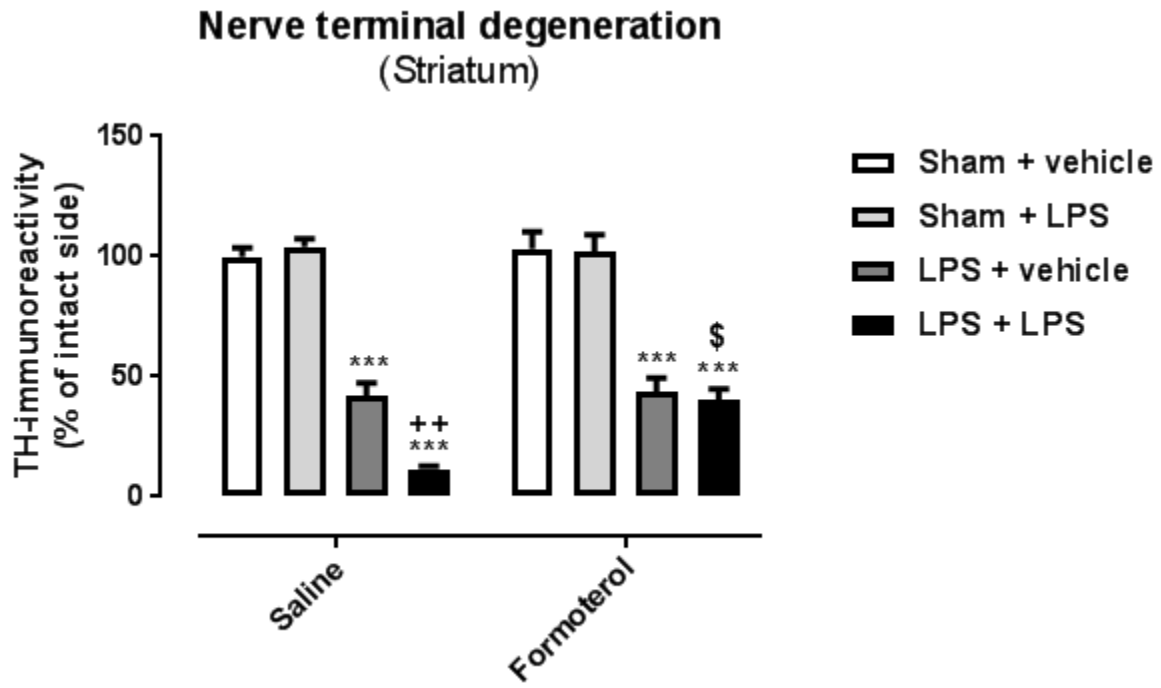
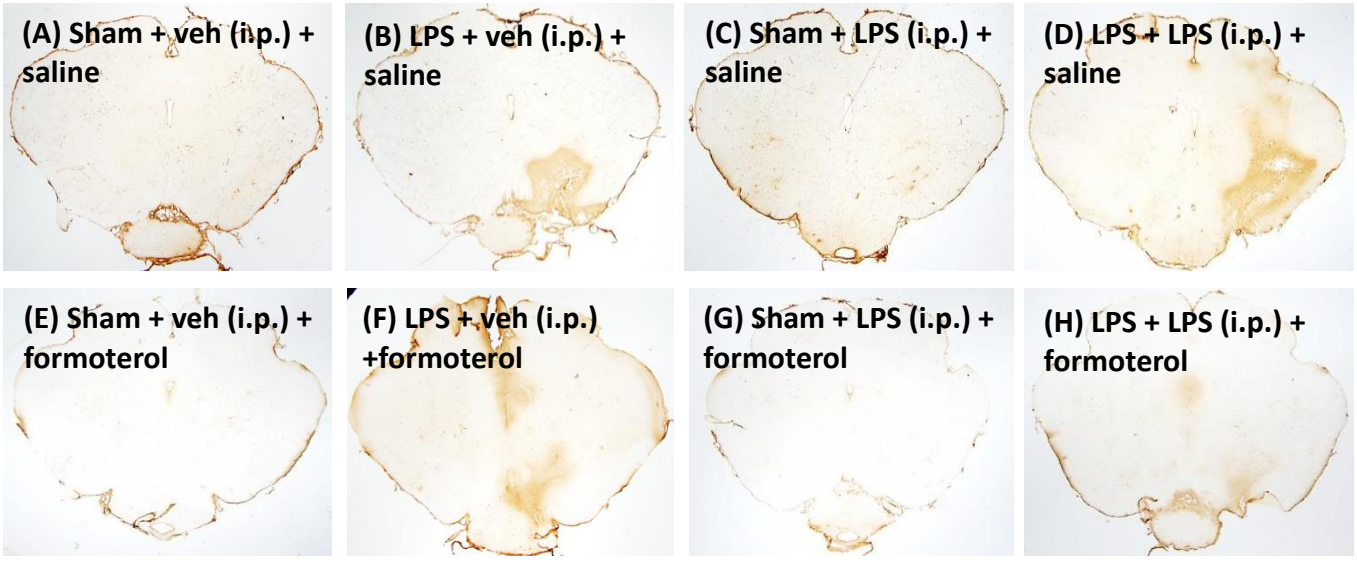


Figure 6.9 Formoterol halts progression in intra-nigral LPS-induced dopaminergic nerve terminal degeneration within the striatum in response to peripheral immune challenge. Intra-nigral injection of LPS induced marked reductions in TH+ dopaminergic nerve terminals at 6 weeks post lesioning. Systemic LPS administration exacerbated nerve terminal degeneration within the striatum in animals previously lesioned with LPS centrally. Treatment with formoterol failed to restore the loss of nerve terminals within the striatum, but completely attenuated the systemic LPS-induced exacerbations in the loss of dopaminergic nerve endings in animals previously injected with intra-nigral LPS 6 weeks earlier. Data expressed as mean ± S.E.M. (n=5) *** ($P<0.001$) vs. Sham-lesioned animals with systemic vehicle challenge treated with saline, ++ ($P<0.01$) vs. LPS-lesioned rats with systemic vehicle challenge treated with saline, $ ($P<0.05$) vs. LPS-lesioned rats with systemic LPS challenge treated with saline by Three-way ANOVA with *post hoc* Bonferroni.

6.3.8 Treatment with formoterol curtails exacerbations in intra-nigral LPS-induced increases in CD68 expression in response to peripheral immune challenge.

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 32)}$ = 67.8, P<0.0001) systemic LPS ($F_{(1, 32)}$ = 7.828, P=0.0086) and formoterol ($F_{(1, 32)}$ = 20.69, P<0.0001) on nigral CD68 expression. There was an interaction between formoterol x intra-nigral LPS ($F_{(1, 32)}$ = 14.94, P=0.0005) and formoterol x systemic LPS ($F_{(1, 32)}$ = 8.177, P=0.0074). Bonferroni *post hoc* analysis revealed that intra-nigral LPS injection alone (P<0.01), or in combination with a subsequent LPS challenge, increased CD68 expression within the SNpc relative to sham-lesioned controls (P<0.001). Systemic administration of LPS alone had no significant effect on nigral CD68 expression. Systemic LPS challenge exacerbated nigral CD68 expression in animals previously lesioned with an intra-nigral injection of bacterial LPS 6 weeks earlier relative to LPS-lesioned rats challenged with saline control (P<0.01). Treatment with formoterol lowered the intra-nigral LPS-induced increases in CD68 expression within the substantia nigra, albeit these results were not deemed statistically significant. Treatment with formoterol completely alleviated the exacerbations in intra-nigral LPS-mediated increases in nigral CD68 expression in response to a subsequent peripheral immune challenge with bacterial LPS (P<0.001).



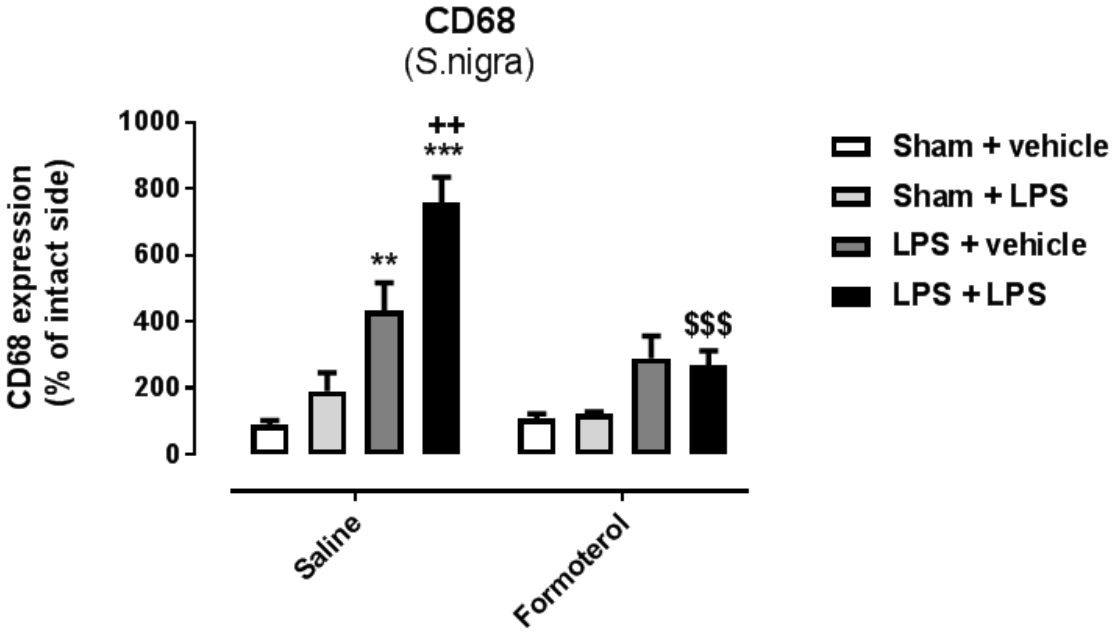
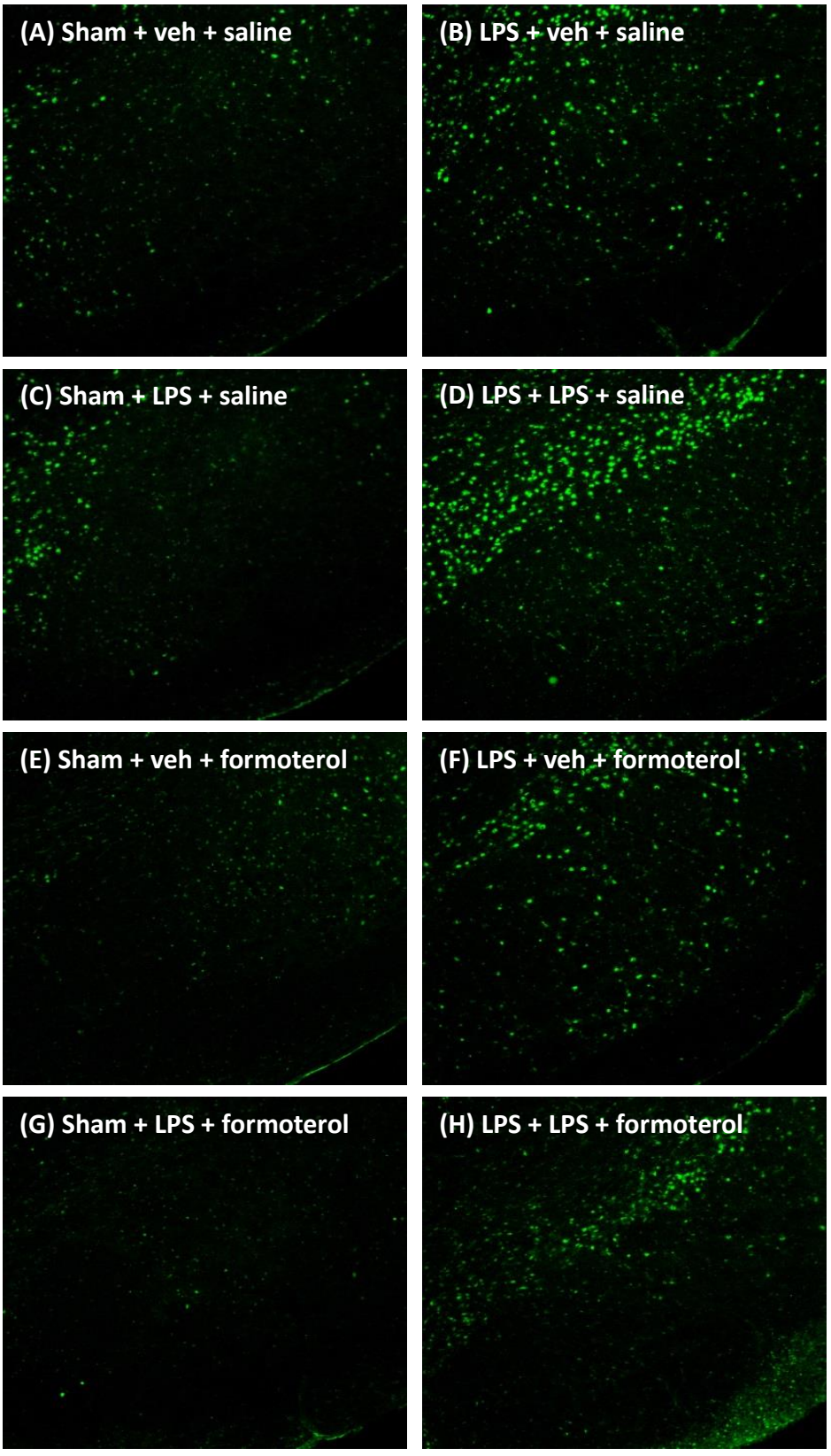


Figure 6.10 Formoterol attenuates exacerbations in intra-nigral LPS-driven increases in nigral CD68 expression in response to peripheral immune challenge. Intra-nigral injection of LPS increases CD68 immunoreactivity within the SNpc. Subsequent systemic LPS exposure exacerbates prior intra-nigral LPS-induced increases in nigral CD68 expression. Treatment with formoterol curtails increases in nigral CD68 immunoreactivity & abolishes exacerbations in intra-nigral LPS-induced increases in CD68 expression in response to subsequent exposure to a peripheral immune stressor. Data expressed as mean ± S.E.M. (n=5) ** ($P<0.01$), *** ($P<0.001$) vs. Sham-lesioned animals with systemic vehicle challenge treated with saline, ++ ($P<0.01$) vs. LPS-lesioned rats with systemic vehicle challenge treated with saline, $$$ ($P<0.001$) vs. LPS-lesioned rats with systemic LPS challenge treated with saline by Three-way ANOVA with *post hoc* Bonferroni.

6.3.9 Treatment with formoterol restrains systemic LPS-mediated increases in IL-1β expression in the primed SNpc

Three-way ANOVA demonstrated an effect of intra-nigral LPS ($F_{(1, 32)}$ = 45.32, P<0.0001) systemic LPS ($F_{(1, 32)}$ = 7.015, P=0.0124) and formoterol ($F_{(1, 32)}$ = 9.704, P=0.0039) on nigral IL-1β expression. There was an interaction effect between formoterol x intra-nigral LPS ($F_{(1, 32)}$ = 15.46, P=0.0004) and intra-nigral LPS x systemic LPS ($F_{(1, 32)}$ = 4.156, P=0.0498). Bonferroni *post hoc* analysis revealed that intra-nigral LPS injection increased IL-1β expression in the SNpc relative to sham-lesioned controls (P<0.05). Intra-nigral LPS injection in combination with a subsequent systemic LPS administration 4 weeks later also elevated nigral IL-1β expression relative to Sham-lesioned controls (P<0.001). Systemic administration of LPS alone had no effect on nigral IL-1β expression. Systemic LPS challenge exaggerated nigral IL-1β levels in animals previously lesioned with intra-nigral LPS (P<0.05). Nigral IL-1β levels were reduced in LPS-lesioned rats treated with formoterol relative LPS-lesioned animals treated with saline control, albeit these differences were not deemed statistically significant. Treatment with formoterol curtailed systemic LPS-induced exacerbations in nigral IL-1β expression in rats previously lesioned centrally with bacterial LPS (P<0.001).


(A) Sham + veh + saline
(B) LPS + veh + saline
(C) Sham + LPS + saline
(D) LPS + LPS + saline
(E) Sham + veh + formoterol
(F) LPS + veh + formoterol
(G) Sham + LPS + formoterol
(H) LPS + LPS + formoterol

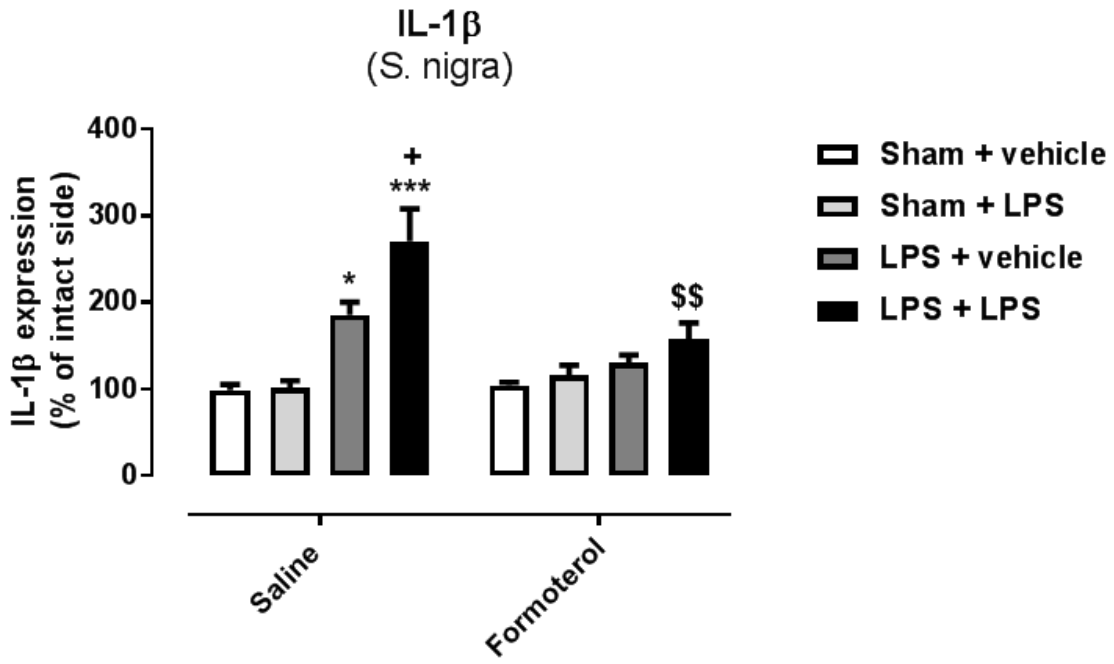


Figure 6.11 Formoterol alleviates systemic LPS-mediated increases in IL-1β production in the inflamed substantia nigra. Intra-nigral LPS increases IL-1β expression in the SNpc. Subsequent exposure to systemic LPS 4 weeks later exacerbates IL-1β production in animals previously lesioned with intra-nigral LPS. Treatment with formoterol reduced nigral IL-1β expression levels and attenuated the exaggerated IL-1β response to subsequent peripheral immune challenge with bacterial LPS. Data expressed as mean ± S.E.M. (n=5) * ($P<0.05$), *** ($P<0.001$) vs. Sham-lesioned animals challenged with vehicle & treated with saline, + ($P<0.05$) vs. LPS-lesioned animals challenged with vehicle & treated with saline, $$ ($P<0.01$) vs. LPS-lesioned animals challenged with LPS & treated with saline by Three-way ANOVA with *post hoc* Bonferroni.

6.4 Discussion

Dysregulated immune functioning is a natural process of aging whereby the trigger of ample innate immune activation to relegate a pathogenic infection is compromised, rendering elderly individuals more susceptible to the detrimental effects of a bacterial infection (Dorshkind et al., 2009). The extensive neuro-immune interaction between the CNS and the periphery raises the importance of sufficient immune competency in preserving neuronal populations against inflammation-mediated cytotoxicity in response to bacterial infections (Becher et al., 2000). In patients with Parkinson's disease, in which an underlying chronic inflammatory component is known to contribute to dopaminergic neurodegeneration, this could have potentially calamitous effects on the development of Parkinsonism, whereas the timely elimination of bacterial toxaemia could provide neuroprotection against advancements in disease progression. Here we demonstrate that an acute episode of bacterial sepsis can exacerbate microglial activation, ongoing dopaminergic neuronal loss & motor dysfunction, whereas pharmacologically stimulating β_2-AR's directly with formoterol could halt accelerations in Parkinson's disease progression.

Our data shows that a prior LPS-induced dopaminergic neuropathology within the substantia nigra predisposed to a heightened degree of $Iba1^+$ reactive microgliosis, TH^+ dopamine cell loss in the SNpc, dopaminergic nerve terminal degeneration in the striatum and associated motor deficits upon subsequent peripheral exposure to a low dose of bacterial LPS (250 μg/kg i.p.). Systemic immune challenge with bacterial LPS aggravated the loss of TH^+ dopamine neurons in the SNpc and propelled dopaminergic nerve terminal degeneration forward in the ipsilateral striatum in animals previously lesioned with an intra-nigral injection of LPS 6 weeks earlier. Similar findings were reported in (Cunningham et al., 2009) demonstrating that the superimposition of a transient episode of systemic inflammation (bacterial LPS; 100 μg/kg i.p.) in pre-existing neurodegenerative disease (ME7 prion disease mice) acutely exacerbates indices of sickness behaviour (impairments in burrowing, locomotor activity, hypothermia) elevates microglial IL-1β production and exaggerates cognitive decline. In the PD field, studies by (Ling et al., 2006) have shown that prenatal exposure to bacterial LPS (10,000 endotoxin units (EU)/kg body weight at gestation day 10.5) prolongs the neuro-inflammatory profile *in vivo* and drives progressive dopaminergic neuronal loss within the SNpc in response to a subsequent supra-nigral infusion of LPS (0.02 μg/h for 14 days). The authors show that supra-nigral LPS infusion exacerbated nigral OX-6-ir cell counts in 7 month old animals exposed prenatally to LPS (of the same serotype), and augmented TH-ir cell loss in the SNpc and deficits in striatal DA content, findings which were accompanied by depletions in nigrostriatal antioxidant reserve (GSH levels) and prolonged elevations in the pro-inflammatory cytokines TNF-α and IL-1β.

Moreover, prior intra-nigral infusion of bacterial LPS (0.1 μg/2μl) primes $CD11b^+$ nigral microglia to respond more vigorously to a subsequent paraquat regimen commencing 2 days later (3 x weekly 10 mg/kg; i.p doses for 3 weeks) and bolsters the blood & brain pro-

inflammatory milieu, augmenting TH^+ dopamine cell loss in the SNpc, and exacerbating measures of behavioural impairment in gait, posture & muscle tone (Mangano and Hayley, 2009). Thus, prior LPS-induced neuro-immune sensitisation of dopamine neurons predisposes to a heightened degree of neurodegeneration in response to subsequent pesticide exposure. In line with this, inflammatory priming of the substantia nigra with 20µg of the viral mimetic and TLR3 agonist Poly(I:C) sensitises midbrain dopamine neurons and their affiliated nerve fibers in the striatum to oxidative stress-mediated neurotoxicity in response to a subsequent low dose of intra-striatal 6-OHDA (5 µg/3.5 µl) 12 days later, findings which were ameliorated by systemic administration of an IL-1 receptor antagonist (Deleidi et al., 2010), highlighting a role for IL-1β in exacerbating neuronal loss in the primed midbrain in response to a subsequent oxidative stressor. Indeed, a sub-toxic, intra-nigral LPS lesion (0.09 µg) shifts primed microglia to a pro-inflammatory state, elevates nigral IL-1β protein expression and exacerbates prior ongoing intra-striatal 6-OHDA (20 µg)-mediated dopaminergic neurodegeneration, accelerating the pathogenesis, and increasing the severity, of motor deficits in the stepping test of forelimb akinesia (Pott Godoy et al., 2008). More importantly however, the author's further show that chronic systemic (i.v.) adenoviral-mediated human IL-1β expression commencing 7 days post intra-striatal 6-OHDA lesioning promotes end-stage $MHCII^+$ microglial activation and exacerbates dopaminergic neuronal loss in the SN both via its direct activity and via downstream NO production, findings which were reversed upon administration of IL-1ra. More recently it has been shown that systemic LPS (750 µg/kg i.p.) increases blood & hippocampal IL-1β expression and exacerbates $TUNEL^+$ apoptosis in the CA1 region of the hippocampus in ME7 prion diseased mice in a partially IL-1RI-dependent manner, elucidating a direct mechanism by which IL-1β production mediates neuronal death, independent of circulating IL-1β levels (Murray et al., 2011; Skelly et al., 2018). Thus, in our current study, in a dissociable IL-1-dependent mechanism from elevated circulating IL-1β levels, midbrain microglia are likely to be a major cellular source of overt IL-1β production in the inflamed Parkinsonian brain in response to systemic LPS challenge, which may also contribute to the death of proximal dopaminergic neurons directly in a partially IL-1RI-dependent fashion.

In line with the reports above, in our current study, we propose that a prior intra-nigral LPS-mediated inflammatory priming of the substantia nigra augments the ongoing microgliopathy and the midbrain pro-inflammatory milieu in response to a subsequent acute episode of bacterial sepsis 4 weeks later. The expansion of nigral $Iba1^+$ microgliosis in response to peripheral immune challenge in animals previously lesioned centrally with LPS was paralleled by concomitant increases in nigral CD68 expression and elevations in IL-1β production. Thus, primed microglia are possibly tilting towards a highly reactive, pro-inflammatory phenotype in response to systemic LPS challenge, exacerbating IL-1β expression and propelling dopaminergic neurodegeneration forward in the inflamed SN. Moreover, the proportion of $Iba1^+$ amoeboid cells per SN increased dramatically, indicating an end-stage morphological activation state, and/or possibly increased immune cell

infiltration of peripheral macrophages (macrophages are immuno-positive for both Iba1 & CD68). This exaggerated immune response to subsequent exposure of a peripheral immune stressor lies at the fulcrum of overt exacerbations in TH^+ dopamine neuronal loss in the SNpc, and the affiliated, tangibly augmented deterioration of TH^+ nerve fibres in the ipsilateral striatum, culminating in accelerated, and indeed amplified degeneration of the nigrostriatal dopaminergic system and worsening motor deficits in these animals.

Keeping with the capacity for secondary insults to propagate ongoing neurodegeneration, chronic exposure to variate stressors (e.g. forced swimming, restraint, isolation food/water deprivation) enhances prior LPS-induced increases in the number of activated microglia in the SN, elevates nigral TNF-α, IL-1β, IL-6, iNOS, CD200, CX3CR1 & MCP-1 mRNA expression, and exacerbates the loss of dopamine neurons in the SNpc (de Pablos et al., 2014). A minimally toxic intra-SNpc injection of bacterial LPS (0.2 μg/μl) in combination with α-synuclein oligomers (0.0125 μg/μl) induces synergistic microglial activation & selective, progressive dopaminergic neurodegeneration with concomitant striatal dopamine depletion in a manner dependent on microglial NOX2 activation and superoxide production, findings which were associated with an increased mRNA & protein expression of PKC-δ, phosphorylated-p38, P-ERK1/2, P-JNK, $NF\kappa B_{P50}$ in primary rat microglial cultures treated with LPS (0.5 ng/ml) and α-Syn (20 nmol/L) in combination (Zhang et al., 2018). Our results, in conjunction with those reported above, support the ''multiple hit'' hypothesis of Parkinsonism, whereby a combination of factors, environmental, inflammatory and/or genetic, precipitate the degeneration of the nigrostriatal dopaminergic system. Epigenetic changes within microglia, including histone modifications & DNA methylation are bespoke regulators in the acquisition of microglial phenotypes in response to various stimuli in the healthy and pathologic CNS (Cheray and Joseph, 2018). Indeed, corresponding reports have demonstrated a role for methylation of histone 3 on lysine 4 (H3K4me2) in the priming of microglia (Keren-Shaul et al., 2017), and a role for microglial sirtuin 1 (SIRT1) deficiency in enhanced IL-1β expression and age-related or tau-mediated cognitive decline via hypo-methylation of specific CpG (cytosine-guanine linked by a phosphodiester bond) sites of the IL-1β gene (Cho et al., 2015). Moreover, genetic deletion of microglial histone deacetylases ($Hdac1^{-/-}$ & $Hdac2^{-/-}$) reduces cortical and hippocampal amyloid plaque burden and improves spatial learning & memory via modulating the Aβ phagocytic capabilities of microglia in 5xFAD mice (a transgenic AD mouse model that develops severe amyloid pathology) (Datta et al., 2018). Thus, tinkering with the transcriptional & epigenetic clockwork of CNS microglia modifies their gene expression profiles and regulates their activation state phenotype & cellular reprogramming in a context-dependent manner, and is therefore an intrinsic mechanism involved in regulating the plasticity of microglia in order for glia to perform their pleiotropic effector functions at the interface of neurodegenerative disease.

Studies by (Wendeln et al., 2018) have shown that peripheral immune stimulation with bacterial LPS enhances cerebral β-amyloidosis in 3-month old APP23 mice when assessed 6 months later for cortical Aβ plaque load & total Aβ levels, findings which were in accordance

with heightened neuritic damage. Prior to this finding, the authors demonstrated that 2 x systemic LPS doses in wildtype mice (one consecutive dose per day) induced brain-specific immune training in CNS macrophages (microglia) leading to elevations in IL-1β production & TNF release, highlighting a role for peripheral immune stimulation in evoking innate immune memory in brain-resident microglia. Moreover, the authors found that systemic LPS administration induces persistent epigenetic reprogramming of CNS microglia in AP223 mice presumably due to the provision of a secondary inflammatory stimulus from amyloid-β, and alters their transcriptional activity, raising the pro-inflammatory milieu and impairing microglial phagocytosis of β-amyloid deposits. In our current study we have also shown that a systemic LPS challenge augments nigral microglial activation and IL-1β production and propagates ongoing dopaminergic neurodegeneration in the Parkinsonian brain when assessed 6 weeks post intra-nigral lesioning with bacterial LPS. Here we propose that subsequent exposure to a peripheral immune challenge induces immune training in primed microglia previously imprinted with immunological memory, altering their transcriptional activity to produce exorbitant levels of IL-1β with detrimental consequences for the nigrostriatal dopaminergic system. Thus, in line with the report above, innate immune training of nigral microglia may also be a mechanistic blueprint behind shaping neuropathological hallmarks of Parkinson's disease progression.

This becomes increasingly plausible considering the immunohistochemical evidence demonstrating complement protein expression and interferon-induced MxA in association with Lewy bodies and swollen neuites in humans and preclinical experimental models of PD, thus supporting a role for neurovirulent influenza A virus in spreading to the substantia nigra and the postulated viral aetiology for Parkinson's disease (Takahashi and Yamada, 1999). Moreover, earlier epidemiological studies have shown that individuals born in the years close to that of the influenza pandemics of 1890-1930 have an increased risk of developing idiopathic Parkinson's disease later in life, fuelling the hypothesis that Parkinson's disease could follow intra-uterine influenza on the basis that it may be cytotoxic to the developing foetal substantia nigra, leading to decreased nigral cell counts and limited striatal dopamine reserves which can become exacerbated upon ageing or exposure to environmental stressors to an extent whereby clinical manifestations of PD become apparent (Mattock et al., 1988).

The most promising finding from our current investigation however is that β_2-AR stimulation with formoterol curtailed exacerbations in nigral Iba1$^+$ microgliosis, restrained overt IL-1β production and prevented further degeneration of the nigrostriatal dopaminergic system in response to peripheral immune challenge with bacterial LPS. Nullifying exacerbations in these neuropathological indices lead to improvements in motor function, and raised our interest in how treatment with formoterol may be halting such exaggerated immune responses and mitigating enhancements in dopaminergic neurodegeneration in these animals. Formoterol may be circumventing the process of innate immune training in nigral microglia, undercutting their epigenetic reprogramming in response to a secondary

inflammatory stimulus, downgrading the ominous tier of microgliotic activity and mitigating the midbrain of its threateningly inauspicious pro-inflammatory milieu. Indeed, treatment with formoterol, by nature of intervening with activated microglia previously imprinted with immunological memory due to a prior intra-nigral LPS lesion, could pacify the nigral expansion in reactive microgliosis and IL-1β production in response to subsequent LPS exposure, and such potent immunomodulation may very well be the modus operandi as it were, underlying the relatively astute halt in the progression of dopaminergic neurodegeneration along the nigrostriatal tract observed in these animals. An anticipated, yet justifiably intriguing finding of our current study is that treatment with formoterol is more judiciously protective against progressive dopaminergic neuronal death when LPS-mediated neurotoxicity is derived from a systemic origin. Just in the way that intra-nigral LPS-induced microglial activation, IL-1β production and dopaminergic neurodegeneration more extensively pervades throughout the SN in response to superimposition of a peripheral immune challenge, the nigrostriatal tract is remarkably imbued with an intervening prophylaxis against these indices of exacerbation in disease progression and heightened motor impairments when therapeutically buttressed by a 7-day treatment regimen with formoterol.

We have shown previously that a 7-day treatment with the NRI atomoxetine (3 mg/kg i.p.) alone or in combination with the α_2-AR antagonist idazoxan (1 mg/kg i.p.) commencing 4 hours post intra-nigral lesioning with bacterial LPS inhibits midbrain microglial activation and TNF-α, IL-6 & IL-1β mRNA expression whilst upregulating BDNF, CDNF & GDNF transcripts, and attenuates the loss of TH^+ dopamine neurons in the SNpc, preserving nerve terminal degeneration in the striatum and abrogating nigrostriatal dopamine depletion, leading to partial improvements in motor function (Yssel et al., 2018). Moreover, noradrenaline diminishes LPS-induced increases in iNOS activity and NO generation in a β_2-AR-dependent manner & potently suppresses IL-1β production from LPS-activated primary rat cortical microglia (Dello Russo et al., 2004a). Other groups have demonstrated that treatment with the long acting β_2-AR agonist salmeterol (1-10 µg/kg/day doses by continuous infusion for 2 weeks) commencing 2 days prior to lesioning, protects against MPTP (15 mg/kg for 6 days s.c.)-induced dopaminergic neurotoxicity (Qian et al., 2011). The same authors show that commencing the same salmeterol treatment regimen 3 months post LPS (5 mg/kg i.p.) administration inhibits microglial activation and dispels LPS-induced increases in TNF-α, superoxide and NO production, attenuates the loss of dopamine neurons in the SNpc and alleviates motor impairments in the rotarod test when assessed 8 and 10 months later. Moreover, seminal work by (Mittal et al., 2017) has shown that the β_2-AR agonists metaproterenol, clenbuterol and salbutamol reduces *SNCA* mRNA expression levels and relative α-synuclein protein abundance in rat primary cortical neurons and that administration of clenbuterol decreased nigral α-synuclein mRNA expression and protein levels and attenuated the MPTP-induced loss of TH^+ dopamine neurons in the SNpc. Moreover, the authors further reveal that the β_2-AR is a regulator of α-synuclein gene

transcription via H3K27 acetylation across promoter & enhancer regions of the human *SNCA* locus and is linked to an individual's risk of developing Parkinson's disease.

Taken the reports above as an ensemble with the results of our current investigation, noradrenaline augmentation strategies and indeed, pharmacologically targeting β_2-AR's directly furnishes a tripartite index for neuroprotection against the development & advancement of Parkinsonism via (a) inhibiting microglial activation & pro-inflammatory mediator production, (b) stimulating neurotrophic factor synthesis & release from astrocytes and (c) downregulating α-synuclein gene expression and relative protein abundance. In summary, our findings demonstrate that an acute episode of bacterial endo-toxaemia grossly exacerbates the midbrain neuro-inflammatory phenotype and ongoing degeneration of the nigrostriatal dopaminergic system, precipitating exaggerated motor impairments in animals previously lesioned with an intra-nigral injection of bacterial LPS. Treatment with the long-acting, lipophilic and highly selective β_2-AR agonist formoterol curtails intra-nigral LPS-induced microglial activation and halts exacerbations in nigral IL-1β production and dopaminergic neuronal loss in response to superimposition of a peripheral immune stressor, findings which underpinned overt protection against aggravations in motor deficits. Our data presented herein demonstrates a clear role for β_2-AR activation as a pharmacological measure to combat the neuro-inflammatory component of PD in order to slow/halt the progression of Parkinson's disease neuropathology & motor dysfunction.

Chapter 7

Concluding remarks

7.1 Intra-nigral injection of LPS establishes an inflammatory-based *in vivo* rat model of Parkinson's disease.

Inflammation is a major contributor to the pathogenesis of Parkinson's disease. Indeed, the chronic inflammatory component of PD may underlie the progressive nature of the disease. Irrespective of PD aetiology, when dopamine neurons die within the SNpc, they release numerous immunogenic factors (e.g. α-synuclein, neuromelanin, MMP-9) which activate proximal microglia. This sustained microglial activation subsequently leads to overt pro-inflammatory mediator production (e.g. TNF-α, IL-1β, IL-6) and ensuing dopaminergic neurotoxicity which in turn amplifies microglial activation and PD-related neuropathology. Thus, a self-perpetuating cycle of reactive microgliosis and dopamine cell loss occurs within the substantia nigra which is likely to accelerate neurodegenerative disease progression.

Here we have established an inflammation-mediated rodent model of experimental PD using a local, unilateral intra-nigral injection of the bacterial endotoxin LPS (10 µg/2µl). We have characterised the impact of a single intra-nigral LPS injection at a behavioural, cellular & biochemical level via motor function tests, immunohistochemical analysis of glial cell activation & nigrostriatal integrity and HPLC-ECD analysis of nigrostriatal dopamine concentrations respectively. Our data indicated that intra-nigral injection of LPS induced a robust $Iba1^+$ microgliosis and TH^+ dopamine cell loss within the SNpc, leading to dopaminergic nerve terminal degeneration in the ipsilateral striatum, nigrostriatal dopamine loss and lateralised motor deficits. Here we show that an LPS-mediated microgliopathy within the substantia nigra promotes the degeneration of the nigrostriatal dopaminergic tract and ensuing motor dysfunction in the staircase test of skilled motor function, the stepping test of forelimb akinesia and the cylinder test of asymmetric limb use. Thus, a single unilateral injection of LPS highlights the impact of an inflammatory stimulus on PD-related neuropathology and motor dysfunction, and creates an experimental model of hemi-Parkinsonism in which the therapeutic efficacy of anti-inflammatory & potentially neuroprotective pharmacological strategies can subsequently be tested.

There are however, numerous caveats in using an intra-nigral LPS injection to create an experimental model of PD. Our model evokes a ''one hit wonder'' in the sense that a single intra-nigral injection of LPS induces localised microglial activation, nigrostriatal neurodegeneration, dopamine loss and motor deficits within a two-week period. The spontaneity in the induction, and indeed time-course of progression of motor abnormalities

in our model does not match the clinical condition, in which a progressive degeneration of the nigrostriatal dopaminergic system and loss of striatal dopamine content over decades slowly but surely culminates in phenotypical advancements in motor dysfunction in human PD patients. This limitation is corroborated in our studies demonstrating an instantaneous effect of LPS on microglial activation and dopamine cell loss (occurring as early as 48 hours post lesioning) leading to detectable motor deficits in the staircase test, the stepping test and the cylinder test at 7 days post lesioning which remain static at 14 days post lesioning, thus indicating that in our current model, the extent of gliosis and PD-related neuropathology occurs prior to 7 days and does not worsen thereafter. As of such, we would redefine the intra-nigral LPS model of Parkinson's disease as a mode to recapitulate the potency & enormity of glial-derived neuro-inflammatory events in contributing towards dopaminergic neurotoxicity and motor deficits at the peak of neurodegeneration as opposed to an experimental model of PD itself. Moreover, the aetiology of PD is still relatively unknown, and henceforth, despite the current general consensus that microglial activation and neuro-inflammation are salient features of the disease, the inflammatory component of PD may be a by-product / cellular & molecular construct invoked by virtue of dopaminergic neuronal loss due to an unknown trigger(s) as opposed to an instigator of this pathology itself. To this end, the well-documented and widely used intra-nigral LPS ''model'' of Parkinson's disease is indeed a misnomer, and therefore, it is seemingly more meticulously appropriate to redefine the use of this endotoxin in modelling PD as a means to modelling the pathophysiology of inflammatory-derived Parkinsonism instead.

Intra-nigral LPS induces a pocket of dopaminergic neurodegeneration in parallel with the extent of microgliosis at the focal point of the injection site whilst leaving anterior (in front of) and posterior (behind) nigral regions surrounding the lesion core relatively unscathed by immunohistochemical comparison of dopamine neurons and nigral microglia respectively (as verified by anti-TH & anti-Iba1 immunolabelling). Thus, a single intra-nigral LPS injection induces a partial lesion of the SNpc, which instigates a dopaminergic neuropathology that is profound, albeit not uniform throughout the nigrostriatal tract and is thus admittedly less ubiquitous in its nature by comparison to that which is seen clinically in the post mortem brains of PD patients. Nevertheless, despite the fact that an intra-nigral LPS lesion does not encompass true Parkinsonism as we are familiar with clinically in humans, this lack in pathologic homogeneity may underpin our observations of more modest nigrostriatal DA losses and leaves room for expansion of the lesion in order to more accurately map the neuropathological & behavioural features of ''true'' Parkinsonism. Perhaps the experimental addition of an environmental toxin or aging itself as a pathologic conjunct to neuro-inflammation would aid in the construct of a more clinically relevant animal model of Parkinson's disease.

Moreover, despite our characterisation study of the impact of an intra-nigral LPS injection acting as a testimony to the potency of an inflammatory stimulus (and indeed the sensitivity of dopamine neurons) in driving dopaminergic neuropathology, ensuing nigrostriatal DA loss

& associated motor deficits, we cannot solicit that the overt levels of microglial activation observed within the nigra in response to an LPS lesion are likely to be representative of that which is seen in the human condition. Indeed, even if nigral microglial activation and intrinsic neuro-inflammatory events were at the root of degeneration of the nigrostriatal dopaminergic system in Parkinson's disease, these cellular and molecular signalling processes would not occur as spontaneously and rigorously as they are induced in our current model, they would more likely initiate at a more inauspicious degree and then gradually progress in tandem with the level of neurodegeneration invoked within the midbrain. Moreover, the contraction of a bacterial / viral infection with the prospect of fabricating a pro-inflammatory environment within the midbrain via neuro-immune signalling is more likely to be of systemic rather than central origin, particularly in elderly individuals where age-related immunosenescence gives rise to a dysregulated immune system and an increased susceptibility to contracting an infection (Helle and Klarlund, 2000). Henceforth, perhaps changing the route of LPS administration from central to systemic (via the intraperitoneal route) would provide a better *in vivo* representation of this clinical scenario. As described previously, other research groups have demonstrated that higher doses of LPS (5 mg/kg i.p.) activate nigral microglia and induce the progressive loss of dopamine neurons over longer periods of time (23% loss of TH-immunopositive DA neurons within the SNpc after 7 months advancing to a 47% loss after 10 months), findings which were associated with increases in brain TNF-α, IL-1β, MCP-1 expression and elevations in NfκB activation (Liya et al., 2007).

Despite these caveats, the intra-nigral LPS model of PD promotes itself as a valuable *in vivo* approach to model how immune-mediated glial-derived neuropathology can impact dopamine loss and motor dysfunction. Given the prominent role of inflammation in the pathogenesis of PD and spotlighting activated microglia specifically as key cellular effectors of this process, our model can subsequently be used to verifiably assess the anti-inflammatory and neuroprotective potential of drug candidates as prospective PD pharmacotherapies, the significance of which, in spite of our models outlined limitations, could be deemed unequivocal.

7.2 Reactive astrocytes contribute to LPS-induced dopaminergic neurodegeneration in the Parkinsonian brain

As discussed previously, the midbrain contains the highest density of microglial cells in the entire rodent brain, albeit a relatively low density of astroglial cells in the SNpc, where the vast majority of dopamine cell bodies reside. Given the role of astrocytes in detoxifying oxygen free radicals and providing neurotrophic support via the secretion of growth factors such as GDNF in particular, a numerical paucity in midbrain-resident astrocytes may render nigral dopaminergic neurons more vulnerable to an inflammatory / cytotoxic insult. Thus, the glial cyto-architecture of the midbrain promotes itself as an indelible hotspot of

dopaminergic neurodegeneration in response to an inflammatory stimulus. Indeed, we have previously shown that an intra-nigral LPS lesion induces robust microgliosis and severe degeneration of the nigrostriatal dopaminergic system, leading to dopamine loss & associated motor impairments. Our primary focus on microglia being the key cellular culprits of this process however, detracted from our suspicion that reactive astrocytes may also play a direct role in protecting against / contributing towards this process of inflammation-mediated neurodegeneration and motor dysfunction. To this end, we devised an experimental setting in which the neuroprotective / neurotoxic role of reactive astrocytes in response to an inflammatory stimulus could be investigated within the Parkinsonian brain.

Functionally disabling astrocytes with L-alpha-aminoadipic acid (L-AAA) facilitated an *in vivo* scenario within the rodent midbrain in which we could assess the impact of acute astrocytic dysfunction on glial cell activation and nigrostriatal integrity in response to an intra-nigral LPS lesion, and therefore allow us to evaluate whether midbrain astrocytes are neuroprotective / neurotoxic under overt pro-inflammatory conditions in the Parkinsonian brain. Our results demonstrated that concurrent L-AAA-induced acute astrocytic dysfunction transiently mitigated LPS-induced dopaminergic neuronal loss within the SNpc, preserved dopaminergic nerve terminals in the ipsilateral striatum, suppressed nigrostriatal DA loss and provided partial protection from motor deficits. The transient nature of neuroprotection afforded by L-AAA is likely to be inexorably linked to the acute effects of the glio-toxin *in vivo* which permit a delayed process of dopaminergic neurodegeneration in the LPS model. For example, our data indicated that in the presence of L-AAA, intra-nigral LPS induced significant deficits in TH-immunoreactivity in the nigra & striatum at 14 days only, a time-point at which there was no direct evidence of astroglial dysfunction, at least in terms of nigral GFAP & S100β expression. In the absence of L-AAA however, LPS-induced deficits in TH-immunoreactivity in the SNpc were evident as early as 48 hours p.i. and motor abnormalities across the board were as prominent at 7 days post-lesioning as they were after 2 weeks. These findings highlight that reactive astrocytes must therefore be contributing to the LPS lesion and are active cellular culprits of inflammation-mediated neurodegeneration of the nigrostriatal dopaminergic tract, and the road to motor dysfunction.

The dichotomy between the pharmacological profile of these two agents provide an interesting mode to gauge the astrocytic input in protecting against or contributing towards an inflammatory lesion of the nigrostriatal dopaminergic tract. Here we have a transient astrocytic toxin (L-AAA) with acute affects interacting with and contorting the pathological processes of an instantaneous inflammagen (LPS) with chronic / permanent effects on the nigrostriatal dopaminergic system. Given the transient mechanism of action of L-AAA, you could argue that continuously infusing the toxin via an osmotic mini-pump over the 2 week period may provide a better experimental setting to investigate the role of midbrain astrocytes in the process of LPS-induced dopaminergic neurodegeneration albeit the merit of this argument is flawed; The LPS lesion is so instantaneous and effective from the get go,

with robust glial cell activation and ensuing dopaminergic neuropathology in effect immediately post-lesion. Therefore, with peak LPS-mediated pro-inflammatory and pathological events occurring within hours to days post lesioning of the SNpc, a single, concurrent high dose of L-AAA is necessary to render midbrain astrocytes grossly dysfunctional in order to accurately model the astrocytic deduction or contribution against / towards an inflammatory-based lesion respectively. Moreover, the continuous infusion of a lower dose of L-AAA over the two-week period would also likely induce some level of constant nigral gliosis (involving both microglia & astrocytes) by virtue of the implanted osmotic mini-pump itself, which would be an unwanted infringement upon the validity of the experiment. Taken together, the single cocktail administration of L-AAA alone and LPS+L-AAA in combination is the most experimentally dexterous and discrete approach under the consignment of that which was possible in our laboratory to shed light on the role of astrocytes in the inflamed PD brain.

Any initial governing expectations of the neuroprotective potential of midbrain astrocytes under the premise of an intra-nigral LPS-mediated pro-inflammatory environment are far too sanguine and impractical; these glial cells are responsive to TLR4 stimulation directly, they outweigh microglia by approximately 10:1 in the brain, and are known cellular effectors of inflammation-mediated neurodegeneration by virtue of releasing soluble factors or via bidirectional crosstalk with nascent microglia. Thus, based on the data gathered herein, our studies are refractory to the relatively naïve consensus that midbrain astrocytes are interminably protective against dopaminergic neurotoxicity and as of such, supports an unwitting dissension from their functional renown as consummately protective cells in response to CNS injury. Admittedly, we must also acknowledge astrocytes as a vital cellular source of antioxidants, as adroit architects of trophic support in the brain, and as synthesizers of anti-inflammatory mediators. As of such, their acclaimed clinical relevance as pharmacological targets for neuroprotection must not be vacated. The point is however, that they must first be polarised to perform such actions, either by environmental circumstance within the brain (e.g. in response to pathologic insult), or by pharmacological intervention (e.g. β_2-AR stimulation), and even then the measure of protection afforded is context-dependent (i.e. the potency of direct neurotoxic insult, the level of neuro-inflammation and the evident influence of glial crosstalk at the interface of CNS pathology etc. are all factors that can restrict the margins of neuroprotection).

Here, we identify reactive astrocytes as glial usurpers to the reigns of the midbrain inflammatory axis in a multi-modal manner which is likely to be intrinsically linked to the dictative inflammatory influence of the release of soluble factors such as S100β, and to crosstalk with LPS-activated microglia, thus highlighting these astroglia as dark horses of neurodegeneration in the inflamed Parkinsonian brain. In synopsis, reactive astrocytes are over-looked adept cellular effectors of inflammation-mediated neurodegeneration of the nigrostriatal dopaminergic system in the Parkinsonian brain, and promote themselves as

ripe targets for immunomodulatory strategies aimed at alleviating the burden of inflammatory-derived neuropathology in the PD brain.

7.3 Enhancing noradrenergic tone inhibits microglial activation in the substantia nigra and protects against LPS-induced degeneration of the nigrostriatal dopaminergic system

The extensive degeneration of the locus coeruleus noradrenergic system is thought to induce a marked impact on Parkinson's disease progression. Forfeiting the innate immunomodulatory and neurotrophic potential of midbrain & striatal noradrenergic afferents is likely to curtail NA-mediated glial-derived anti-inflammatory mediator production & forestall trophic support to vulnerable nigrostriatal dopamine neurons and is therefore likely to heavily impact on the susceptibility of these neurons to neurotoxic stimuli to an extent that makes it pragmatically inevitable to negatively impact on Parkinson's disease progression. Under this pathological presupposition, pharmacological methods aimed at enhancing CNS noradrenergic tone to circumvent the detrimental effect(s) of a nigrostriatal noradrenergic deficiency may provide palpable neuroprotection and thus slow / halt further dopaminergic neurodegeneration and progressively worsening motor abnormalities over time.

Bearing in mind the majorly overlooked noradrenergic deficiency in PD brains, perhaps preclinical animal models of Parkinson's disease should incorporate a degenerating LC-noradrenergic system as well as the classical dopaminergic pathology along the nigrostriatal tract. Prior DSP4-induced LC-degeneration & noradrenergic depletion in combination with a subsequent intra-nigral LPS lesion (or a high dose of systemically delivered LPS) would serve to recreate an ample experimental scenario *in vivo* that more closely matches the clinical manifestations of Parkinsonian neuropathology. By doing so, we could assess the impact of the loss of noradrenergic cell bodies in the locus coeruleus on the survival rate of dopamine neurons in the SNpc over time, and any potential knock-on effects in the striatum in terms of nerve terminal loss and reductions in nigrostriatal dopamine content could also be assessed. This in turn would more accurately mimic the clinical representation of human PD patients and would further advertise a premise for targeting the brains noradrenergic system to combat PD should the results garnered from such experiments demonstrate a role for the LC-noradrenergic system in protecting against neuro-inflammation, dopaminergic neurodegeneration, striatal DA loss and motor dysfunction.

Nevertheless our data demonstrated that treatment with the NRI atomoxetine alone or in combination with the α_2-AR antagonist idazoxan restrained intra-nigral LPS-induced Iba1$^+$ microglial activation & pro-inflammatory gene expression, attenuated the loss of TH$^+$ dopaminergic neurons in the SNpc and repressed striatal denervation, findings which permitted alleviated LPS-induced reductions in nigrostriatal dopamine content and provided partial protection against motor dysfunction (Yssel et al., 2018). Midbrain glial cells are

caught up in a causal nexus of neuroinflammation, PD-related neuropathology and motor dysfunction in response to an intra-nigral LPS lesion, and are therefore, crucial targets for immunomodulation & neuroprotection afforded by enhancing CNS noradrenergic tone; Indeed, densely populated nigral microglia are goaded cellular effectors of LPS-mediated dopaminergic neurotoxicity and thus, by curtailing their pronounced reactivity in response to an immune stimulus, we can pre-emptively arrest the expansion of microgliosis in place, quell dysregulated pro-inflammatory mediator production and preserve the nigrostriatal dopaminergic system from inflammation-mediated neurodegeneration.

As with microglia, midbrain astrocytes are mutual glial contributors towards dopaminergic neurodegeneration, but also in protecting the nigrostriatal dopaminergic system from inflammatory-derived neuropathology. Here we have shown that elevated CNS noradrenaline levels induced a presumably astrocytic neurotrophic response in the guise of boosted growth factor production (e.g. BDNF & GDNF in particular) in the midbrain, which possibly occurred in an adrenergic receptor-dependent manner, and is likely to contribute to the protective effects afforded by treatment with the NRI atomoxetine alone or in combination with the α_2-AR antagonist idazoxan in the inflamed Parkinsonian brain. Therefore, it would appear that NA exerts a bi-modal neuroprotective role in the brain by inhibiting microglial activation & downregulating pro-inflammatory gene expression, and also by stimulating growth factor production from midbrain astrocytes and promoting a neurotrophic environment in which dopaminergic neurons can thrive even in the face of an LPS lesion. The extensive preservation of dopamine neurons and their affiliated nerve terminals in the striatum of LPS-lesioned rodents treated with atomoxetine +/- idazoxan highlights the therapeutic impact of pharmacologically raising CNS noradrenergic tone to protect against degeneration of the nigrostriatal tract in the inflamed Parkinsonian brain. These findings bear propitious clinical relevance considering that these agents are currently used to treat unrelated illnesses such as ADHD, and are safe to use for prolonged periods of time (Zhou, 2004).

There is however, one major affiliated caveat from this treatment study that warrants stringent acknowledgement. Commencing a treatment regimen with atomoxetine and idazoxan alone or in combination as early as 4 hours post intra-nigral lesioning with LPS is likely to suppress an LPS effect before it has been given sufficient time to culminate in peak neuro-inflammatory events and ensuing dopaminergic neurotoxicity, nigrostriatal dopamine loss and associated motor deficits. This is a crucial point that largely dispels the clinical relevance of our findings. For example, when human PD patients are diagnosed clinically, their condition has already progressed to an advanced stage of the disease where motor deficits are already apparent due to the extensive death of dopaminergic cell bodies in the SNpc and severe striatal dopamine loss. Thus, bearing our current experimental setting in mind, despite commencing our treatment regimen 4 hours post intra-nigral LPS injection, our data provided herein demonstrating an anti-inflammatory & neuroprotective effect of enhancing noradrenergic tone is arguably more prophylactic in nature instead of neuro-

restorative, and the question as to whether treatment with an NRI such as atomoxetine would prove efficacious in PD patients remains to be answered. Indeed, the therapeutic potential of pharmacologically elevating extra-synaptic noradrenergic bioavailability to ameliorate the cardinal pathological hallmarks in the Parkinsonian brain (e.g. neuroinflammation, dopaminergic neurodegeneration, dopamine loss & motor deficits) will need to be established in future studies where these pathologic features are already actively progressing *in vivo* prior to commencing a treatment regimen in order to bear more clinical relevance to promoting the use of atomoxetine in particular as a potentially disease modifying therapy to treat the human condition.

In spite of this, our data gathered herein demonstrates the therapeutic efficacy of pre-emptive NA augmentation strategies in alleviating the Parkinsonian brain from the neurotoxic burden of robust microglial activation within the SNpc, and overtly dysregulated, uncontrolled glial-derived pro-inflammatory mediator production, a calamitous process which if left uninhibited, would lead to strident reductions in nigrostriatal dopamine content and austere motor dysfunction. In conjunction with other preliminary datasets generated from our laboratory demonstrating a similar therapeutic effect of treatment with the β_2-AR agonists clenbuterol or formoterol on intra-nigral LPS-induced microglial activation, dopaminergic neurodegeneration, nigrostriatal DA loss and motor deficits, we propose that the anti-inflammatory & neuroprotective role exerted via blockade of the NAT is primarily mediated in a β_2-AR-dependent manner.

7.4 β_2-adrenoceptor stimulation restrains microglial activation in the substantia nigra & halts accelerations in LPS-mediated degeneration of the nigrostriatal dopaminergic system in response to systemic inflammation.

A dysregulated immune system is a natural process of aging which is partially characterised by persistent inflammatory responses involving diverse immune cell lineages which contribute to a heightened pro-inflammatory milieu, constructing persistent inflammation which relegates an ample innate immune activation in response to a pathogenic infection(s), thus rendering individuals more susceptible to the detrimental effects of an infection, be it of viral or bacterial origin. Innate immune ageing at a cellular level shows heterogeneous, context-dependent ageing phenotypes depending on their developmental state, tissue context & activation profile, and occurs in multiple tissues & organs with potential implications for age-related neurodegenerative diseases such as Parkinson's disease. Due to the extensive neuro-immune interactions between the CNS and periphery, it is possible that the systemic inflammation that occurs in response to a peripheral immune stressor via the contraction of a bacterial infection shall we say, can signal to the brain and influence the neuro-inflammatory environment that midbrain dopamine neurons are already residing in due to coexisting and ongoing dopaminergic pathology. Hence, bearing in mind the dysregulated immune system of the elderly population, ongoing pro-inflammatory &

neurodegenerative processes and also the possibility of a leaky BBB to facilitate immune cell infiltration, this systemic inflammatory component could grossly accelerate neurodegenerative disease progression and drastically worsen motor abnormalities in patients, leading to a more strenuous quality of life.

Indeed, our data demonstrated that prior intra-nigral LPS-induced dopaminergic neuropathology along the nigrostriatal tract predisposed to a heightened degree of neuro-inflammation, neurodegeneration and motor deficits upon subsequent systemic exposure to a low dose of bacterial LPS. It is likely that a prior intra-nigral LPS-induced inflammatory priming of the substantia nigra may very well have sensitized dopamine neurons to a greater degree of inflammation-mediated cytotoxicity in response to a subsequent peripheral immune stimulus at least in part, via immunomodulation of midbrain glial cell activity. There are multiple reasons as to why this may have occurred, one probable explanation is a cellular phenomenon known as innate ‘’immune training’’.

The original dogma stapled the adaptive arm of the immune system (primarily B and T lymphocytes) exclusively in building immunological memory to prevent the detrimental effects of microbes upon re-exposure to the same pathogen. The current consensus is that infection or indeed vaccination can stimulate innate immune cells such as monocytes, macrophages and natural killer cells to develop increased pro-inflammatory response upon subsequent microbial exposure, lending to a vigorous capacity to eliminate infection, a process termed innate immune training (Netea et al., 2016). Despite lacking the same level of antigen specificity, clonality and longevity of an adaptive immune response to a pathogen, these innate immune cells have the ability to remember too, upon reencountering the same pathogen (Yoshida and Ishii, 2015). This immunological memory is shorter lived and less specific to that of the adaptive arm of immunity yet can provide the host a leg up in immune defence in against an infectious agent and improve survival of the host. Trained immunity is broadly orchestrated by epigenetic reprogramming such as DNA methylation and histone modifications which induces transcriptional and cell physiology alterations without permanently inducing genetic changes, such as mutations or recombination (Foster et al., 2007).

As previously conceptualised by (Wendeln et al., 2018), brain-resident microglia are imprinted with innate immune memory due to previous exposure(s) to a certain microbe, for example, and can augment an immune response to subsequent microbial exposure to prevent reinfection. This can be beneficial to the host in the sense that peripheral immune training can improve the immune system’s ability to fight against & eliminate reinfection(s), but can also be particularly dangerous in individuals with ongoing inflammatory conditions or in those whom are suffering from a neurological disease harbouring an inflammatory component such as in Parkinson’s disease patients. The authors above demonstrated that a single low dose of bacterial LPS in an APP23 Alzheimer’s disease mouse model was sufficient to induce microglial immune training, likely due to the provision of a secondary pro-

inflammatory stimulus afforded by amyloid-β accumulation. Interestingly, 4 consecutive daily doses of LPS in APP23 mice conversely lead to immune tolerance (an alternate form of immunological memory whereby the immune response is dampened). The authors show that immune training exacerbated the burden of amyloid-β accumulation whereas immune tolerance decreased Aβ plaque load. Immune memory in microglia persisted in the brains of these APP23 mice for at least 6 months, possibly on one hand due to the very long lifespan of microglial cells and on the other hand immunological memory being well maintained due to persistent Aβ-mediated brain inflammation facilitating its endurance (Füger et al., 2017).

Thus, in our current investigation, immunological memory is possibly shaping Parkinson's disease hallmarks in the rat nigrostriatal tract due to immune-training of midbrain microglia in response to a subsequent peripheral immune stimulus, augmenting nigral microgliosis and the pro-inflammatory milieu, and hence driving forth exacerbations in dopaminergic neuropathology and motor dysfunction. This partial explanation becomes even more conceivable given the previously mentioned long lifespan & cellular density of midbrain microglia, in which epigenetic reprogramming of midbrain microglia engrains alterations in their molecular profile, altering their transcriptional activity in response to a peripheral immune challenge with severe pathological & functional consequences due to innate immune training. The inaccessibility of midbrain tissue in living patients with PD however, stunts the clinical prospect of demonstrating its existence in humans, and indeed delays notions of exploiting immunological microglial memory therefore, as a pharmaceutical target to alleviate PD-related neuropathology and motor dysfunction. Perhaps the analysis of the CSF of Parkinson's disease patients for inflammatory signalling molecules could be performed as an indirect proxy for microglial immunological memory?

Immune cells, and indeed inflammatory molecules, are transported through the bloodstream into sites of CNS pathology. Perhaps peripheral immune cells are infiltrating the inflamed midbrain and further activating nigral microglia, aggressively expanding microgliosis in the proximity of dopamine neurons. Or perhaps peripheral nerves such as the vagus nerve as an example, is signalling that inflammation has occurred. Indeed, one major possible signalling pathway is via the gut-brain axis whereby host microbiota can modulate the maturation and function of CNS microglia (Erny et al., 2015). Bearing the above factors in mind, one raises the question as to whether inadvertent targeted disruption of immunological training underlies the immunomodulatory and neuroprotective effects afforded by glial β_2-AR stimulation. Indeed, treatment with formoterol may very well be circumventing the immune training of LPS-activated nigral microglia in response to a subsequent peripheral immune stimulus, and by doing so, preventing exacerbations in microgliosis, nigrostriatal neurodegeneration and associated motor deficits. This of course raises another important question as to how it may be doing so. One explanation is that formoterol may be alleviating the blood quanta of LPS-mediated increases in circulating immune cells and inflammatory mediators capable of crossing the BBB and reaching sites of CNS dopaminergic neuropathology. Restricting immune cell infiltration (e.g. peripheral

macrophages & leukocytes) & the invasion of soluble inflammatory mediators would alleviate the pro-inflammatory milieu within the SNpc and certainly contribute, at least in part, towards dispelling exacerbations in dopaminergic neurodegeneration in response to a subsequent systemic immune challenge with bacterial LPS.

There is of course, a more direct route of immunomodulation underlying the therapeutic effects of formoterol treatment to be considered. Stimulation of glial β_2-AR's directly, altering their transcriptional activity and thus polarising their function towards an immunosuppressive phenotype and dampening the peripherally mediated LPS-induced expansion of microglial activation and in turn, attenuating the midbrain pro-inflammatory milieu. Thus, it is likely that formoterol treatment is combating immune -mediated exacerbations in nigrostriatal neurodegeneration via a variety of mechanisms responsible for the overt neuropathology observed. Overall, our findings fortify the use of a β_2-AR agonist (particularly formoterol) to slow/halt Parkinson's disease progression in instances where an inflammatory component is driving disease progression. What makes this potentially prospective PD therapeutic even more promising is that formoterol is a widely used FDA-approved treatment (marketed under brand name: Symbicort® as a metered dose inhaler) for asthmatics and COPD patients, and thus it is safe to use for prolonged periods of time in humans.

Indeed, the seminal work of (Mittal et al., 2017) labels the β_2-AR as a regulator of the α-synuclein gene *SNCA,* modulating its transcription rate and relative protein abundance. As a matter of fact, speaking frivolously, the presence of midbrain α-synucleinopathies are akin to the occurrence of weeds in a garden *per se.* In the healthy midbrain, dopamine neurons are devoid of Lewy body pathology, and requisite dopaminergic neurotransmission is keeping motor function tightly controlled, analogous to the way in which a well-kept lawn (midbrain) spurts homogenously dispersed blades of grass (neurons) which grow within the remit of how well the lawn is kept. Should α-synucleinopathies develop however, as in the PD brain for example, Lewy Body deposits, like unwanted weeds restricting the growth & function of other plants (e.g. dopamine neurons) in a garden (CNS) disturb the tightly controlled equilibrium of dopaminergic neurotransmission and convolute an individual's motor function. Under this pathological premise, noradrenaline, or indeed a β_2-AR agonist such as formoterol for example, may be chaliced as an organic fertilizer of the midbrain, a neuro-chemical mulch, blocking the development of α-synucleinopathies, killing weeds (Lewy body pathology) at the root, as it were. Indeed, degeneration of LC noradrenergic afferents innervating the midbrain & striatum due to the preceding calamitous loss of NAergic cell bodies in the locus coeruleus, could quite conceivably underpin the development of Lewy body formation in the PD brain by virtue of forfeiting the innate β_2-AR-dependent regulatory potential of noradrenaline on α-synuclein expression. Just in the way that weeds propagate in badly cultivated, unattended soil, the greater the extent of noradrenergic deficiency, the greater the expansion of α-synucleinopathy, a neural playground for misfolded proteins as it were. To this end, a conspicuous commonality

between Alzheimer's disease & Parkinson's disease pathology is indeed, a severe loss of noradrenergic cell bodies in the LC that exceeds that of which occurs within the nucleus basalis of meynert in the AD brain and the SNpc in the PD brain. Perhaps the burdening β-amyloid depositions in the AD brain are to some extent, cut from the same cloth of noradrenergic deficiency?

On a closing note, taken in tandem with our current observations of glial-derived immunomodulation and neurotrophism in response to β_2-AR stimulation, these findings give traction to propelling the notion forward that enhancing noradrenergic tone, and indeed a β_2-AR agonist, could sub-serve a tripartite neuroprotective role in the Parkinsonian brain by downregulating glial-derived pro-inflammatory mediator production, stimulating growth factor synthesis & release, and also, by regulating α-synuclein protein abundance.

7.5 Future directions

1. Retracing experimental steps towards modelling ''true'' Parkinsonism: The overexpression of α-synuclein in midbrain DAergic neurons using viral vectors for example, offers a more clinically relevant *in vivo* scenario to model PD-related neuropathology in rats. Using an adeno-associated viral (AAV) vector construct we can drive the overexpression of human wild-type α-synuclein in midbrain dopamine neurons to instigate the progressive loss of dopaminergic neurons in the SNpc, promoting axonal and nerve terminal degeneration in the striatum, leading to nigrostriatal DA loss and progressive motor dysfunction. An *in vivo* PD model of this nature ticks more boxes in the sense that it is a closer representative of the human condition, replicating Lewy Body pathology as well as neuro-inflammation, dopaminergic neurodegeneration, overt nigrostriatal DA loss and motor deficits, slowly but surely progressively worsening over time. Indeed, should treatment with a β_2-AR agonist such as formoterol prove efficacious in ameliorating the pathological hallmarks of the Parkinsonian brain induced by α-synuclein overexpression, it would bolster its potential as a credible disease-modifying therapy to treat the human condition.

2. Identifying cell-specific neuro-inflammatory signalling cascades leading to dopaminergic neurodegeneration: The literature is inundated to date with the prospect of stapling the glial-derived neuro-inflammatory component as a process that may very well underlie the progressive nature of Parkinson's disease in humans. Therefore, a worthwhile future objective is to identify the glial-specific soluble litany of pro-inflammatory mediator production in the inflamed Parkinsonian brain associated with dopaminergic neurodegeneration. It is highly probable that multiple factors derived from multiple cell lineages are at play here, and to explore this prospect, using cultured microglia, astrocytes or mixed glia *in vitro*, a multitude of known glial-immunogenic stressors (e.g. LPS, α-synuclein, neuromelanin etc.) previously shown to damage/kill dopamine neurons could be

assessed for their ability to produce soluble factors (such as TNF-α, IL-1β, C1q, IL-6, S100β, MMP-9, NO, H_2O_2 etc.) known to contribute towards the neurotoxicity of mesencephalic dopamine neurons. This would create a dataset to suggest which factors produced from which glia (i.e. microglia, astrocytes or both) under inflammatory conditions have the most vigorous neurotoxic potential and which therefore, are poised for immunomodulation via β_2-AR stimulation shall we say, in order to curtail neuro-inflammation and limit neurodegenerative processes contributing to Parkinson's disease progression.

3. Refining treatment regimens to match the clinical condition: Here we have shown that enhancing noradrenergic tone with the NRI atomoxetine inhibits microglial activation within the substantia nigra and attenuates dopamine cell loss in the SNpc, restrains nerve terminal degeneration in the ipsilateral striatum, suppresses nigrostriatal dopamine loss and also provides partial protection from associated motor deficits in the intra-nigral LPS model of Parkinson's disease. Moreover, treatment with formoterol, a long acting, lipophilic, highly selective β_2-AR agonist curtails intra-nigral LPS-induced microglial activation and halts exacerbations in nigral microgliosis, dopaminergic neurodegeneration and motor dysfunction in response to a subsequent peripheral immune challenge with bacterial LPS. Taken together, these findings highlight the immunomodulatory potential of targeting the brain's noradrenergic system to provide neuroprotection from inflammation-mediated neurodegeneration and halting accelerations in Parkinson's disease progression. These results are highly promising, but mainly within the context of the model from which they were garnered, however (i.e. robust LPS-induced neuro-inflammation lead to severe dopaminergic neurodegeneration and motor dysfunction, so alleviating the inflamed Parkinsonian brain of its pro-inflammatory milieu provides palpable neuroprotection and restoration in motor function). Thus, should treatment with the NRI atomoxetine or the β_2-AR agonist formoterol prove efficacious in ameliorating α-synuclein-induced neuroinflammation and dopaminergic neurodegeneration (and α-synuclein protein abundance) as well, then usage of these noradrenergic agents would truly envelop a potentially prospective PD-pharmacotherapy, as it would not only prove efficient at restraining reactive gliosis and pro-inflammatory mediator production, but also as a possible means to reduce the burden of α-synucleinopathies in the human PD brain.

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