

The Neuroprotective and Behavioural Effects of Group III Metabotropic Glutamate Receptor Ligands in Rodent Models of Parkinson's Disease

Claire J. Williams

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Wolfson Neuroscience Laboratories
Division of Brain Sciences
Imperial College Faculty of Medicine
Hammersmith Hospital Campus
Burlington Danes Building
Du Cane Road
London W12 0NN

Declaration of originality

I declare that this thesis is my own work and has not been submitted in any form for another qualification at any university or other institution of tertiary education. Information derived from published or unpublished work of others has been acknowledged in this thesis and a list of references is included.

Claire J. Williams

20/07/2015

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Abstract

Current therapies for Parkinson's disease are able to ameliorate the symptoms in the early stages, however as the disease progresses, they become less effective and patients often develop debilitating side effects. There is currently a significant unmet need for disease modifying or neuroprotective drugs to slow the rate of disease progression and provide long-term symptomatic relief. Novel therapeutics that can provide symptomatic relief whilst attenuating the ongoing neurodegeneration are therefore sought. The targeting of metabotropic glutamate (mGlu) receptors has become a therapeutic focus in recent years. The group III mGlu receptors are the focus of this thesis as they currently hold the most therapeutic promise, with evidence suggesting activation of these receptors not only modulates aberrant neurotransmission in the basal ganglia to provide symptom relief, but also provides neuroprotective effects in the nigrostriatal system through a variety of mechanisms. Recent advances in the development of subtype-selective ligands that cross the blood-brain barrier provides the ability to explore the variety of functions associated with targeting individual group III mGlu receptor subtypes in a clinically relevant manner. In order to determine the potential of these receptors as suitable targets for novel therapies, the antiparkinsonian activity and neuroprotective effects of group III mGlu receptor subtypes were investigated using two novel selective ligands in the lactacystin model, which features altered protein accumulation, progressive neuronal death and development of motor deficits. Neither ligand reduced motor symptoms effectively, and no robust neuroprotection was detected. Potential anti-inflammatory mechanisms of one compound were then investigated *in vivo* and *in vitro* but showed no clear effect. Selectively targeting group III mGlu receptors did not hold the neuroprotective or antiparkinsonian potential that was initially predicted. Collectively, these studies demonstrate the importance of testing therapies in a variety of models demonstrating different aspects of disease pathogenesis.

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Abbreviations

6-OHDA	6-hydroxydopamine
A β	Amyloid β protein
ABC	Avidin-biotin complex
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
AC	Adenylyl cyclase
ACPT-I	(1 <i>S</i> ,3 <i>R</i> ,4 <i>S</i>)-1-Aminocyclopentane-1,3,4-tricarboxylic acid
ADX88178	5-methyl-N-(4-methylpyrimidin-2-yl)-4-(1Hpyrazol-4-yl)thiazol-2-amine
AIR	Amphetamine-induced rotations
AMN082	N,N-bis(diphenylmethyl)-1,2-ethanediamine
AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate
APDC	4-amino-2,4-pyrrolidinedicarboxylic acid
asf	Area sampling fraction
AST	Adjusted stepping test
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BG	Basal ganglia
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CGA	Chromogranin A
cGMP-PDE	Cyclic guanosine monophosphate phosphodiesterase
COMT	Catecholamine o-methyl transferase
DAB	Diaminobenzidine
DAT	Dopamine transporter
DBS	Deep brain stimulation
DCPG	(<i>S</i>)-3,4-dicarboxyphenylglycine
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EAE	Autoimmune encephalomyelitis
ELISA	Enzyme linked immunosorbent assay
EPSC	Excitatory post-synaptic potential
GABA	γ -aminobutyric acid
GDNF	Glial-derived neurotrophic factor
GPe	Globus pallidus pars externa
GPI	Globus pallidus pars interna
GSH	Glutathione
HRP	Horseradish peroxidase
iCa ²	Intracellular calcium
i.c.v.	Intracerebroventricular
iGlu	Ionotropic glutamate
IL	interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
IPSC	Inhibitory post-synaptic potential
L-AP4	L-(+)-2-Amino-4-phosphonobutyric acid
LB	Lewy body
L-DOPA	L-3,4-dihydroxyphenylalanine
LID	L-DOPA-induced dyskinesia
LRRK2	Leucine-rich repeat kinase 2
L-SOP	L-Serine-O-phosphate

LPS	Lipopolysaccharide
LSP1-2111	[[[(3S)-3-amino-3-carboxy]propyl][[(4-hydroxy-5-methoxy-3-nitrophenyl)hydroxymethyl]phosphinic acid
LSP4-2022	[[[(3S)-3-Amino-3-carboxy]propyl][[(4-carboxymethoxy)phenyl]hydroxymethyl]phosphinic acid
LTP	Long-term potentiation
LuAF21934	(1S,2R)-N-(3,4-dichlorophenyl) cyclohexane-1,2-dicarboxamide
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MFB	Medial forebrain bundle
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MPEP	2-methyl-6-(phenylethynyl)pyridine
MPP+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
MSA	Multiple system atrophy
mGlu	Metabotropic glutamate
NAM	Negative allosteric modulator
NET	Noradrenaline transporter
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthetase
NSAID	Non-steroidal anti-inflammatory
OS	Oxidative stress
PAM	Positive allosteric modulator
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Triton X
PD	Parkinson's disease
PHCCC	(-)-N-phenyl-7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxamide
PI-3-K	Phosphatidyl inositol-3-kinase
PINK-1	Phosphatase and tensin homolog-inducible kinase 1
PKC	Protein kinase C
PLC	Phospholipase C
PSI	Proteasome inhibitor 1
P7	Post-natal day 7
ROI	Region of interest
ROS	Reactive oxygen species
RS-PPG	(RS)-4-Phosphonophenylglycine
RT	Room temperature
s.c.	Subcutaneous
SERT	Serotonin transporter
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
ssf	Serial sampling fraction
STN	Subthalamic nucleus
TGF-β	Transforming growth factor-β
TH	Tyrosine hydroxylase
TLR-4	Toll-like receptor 4
UCH-L1	Ubiquitin C-terminal hydrolase
UPS	Ubiquitin proteasome system
VeFP	Vibrissae-evoked forelimb placement
VU0364770	N-(3-chlorophenyl)picolinamide
VU0155041	cis-2-(3,5-Dichlorophenylcarbamoyl)cyclohexanecarboxylic acid

Publications

Publications/Articles

WILLIAMS C. & DEXTER, D.T. 2014. Neuroprotective and Symptomatic Effects of Targeting Group III mGlu Receptors in Neurodegenerative Disease, *Journal of Neurochemistry*, 129, pages 4-20

*Parts of this thesis include sections from *Journal of Neurochemistry*, 129, pages 4-20: **Williams and Dexter (2014)** "Neuroprotective and symptomatic effects of targeting group III mGlu receptors in neurodegenerative disease" with permission from John Wiley and Sons included in Appendix 1.

Poster Presentations

WILLIAMS C. & DEXTER, D.T. 2013. Neuroprotective and Behavioural Effects of an mGluR8 agonist in the Lactacystin model of Parkinson's Disease, Poster at BNA 2013: Festival Of Neuroscience

WILLIAMS C. & DEXTER, D.T. 2012. Neuroprotective and Behavioural Effects of (S)-3,4-dicarboxy phenylglycine in the Lactacystin model of Parkinson's Disease, Poster at Parkinson's UK Research Conference 2012

Chapter 1: Introduction

1.1 Parkinson's Disease

1.1.1 Overview

With an ageing society, the incidence of neurodegenerative disease is increasing, and the societal and economic burden are a growing problem. Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting around 0.3% of the general population, and is the second most common neurodegenerative disease globally after Alzheimer's disease (de Lau & Breteler 2006). First described by James Parkinson in 1817 in his essay on 'the shaking palsy', PD is a disease with debilitating motor symptoms including resting tremor, bradykinesia (slowness of movement), muscle rigidity, postural instability and a shuffling, hypokinetic gait (Parkinson 2002, Tolosa *et al.* 2006). Non-motor symptoms are also common, with cognitive dysfunction such as depression, dementia, anxiety, apathy, psychosis, sleep disorders and other autonomic complications developing due to the loss of different neurotransmitter systems (Bernal-Pacheco *et al.* 2012). The characteristic neuropathological hallmarks of PD are the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and round intracellular protein inclusions known as Lewy bodies (LBs) found in degenerating neurons. Currently, there is no treatment that is able to halt or slow the progression of PD.

1.1.2 Epidemiology of Parkinson's disease

The prevalence of PD rises with age, with approximately 1% of those over 65 years old affected, rising to 2-4% of those over 80 years old, although 10% of PD cases have an earlier onset ranging from 20-50 years of age (de Lau & Breteler 2006, Tanner & Goldman 1996, Dexter & Jenner 2013). Familial forms of PD account for approximately 10-15% of diagnosed cases, and are sometimes considered atypical due to the often early onset of motor symptoms or dementia (de Lau and Breteler 2006). Current estimations are likely to underestimate the incidence or prevalence of PD, as studies cannot take into account undiagnosed patients (de Lau & Breteler 2006). There is conflicting evidence regarding prevalence between men and women, with some studies demonstrating a higher prevalence in men than women, indicating a male: female ratio of 1.9 (Shulman & Bhat 2006, Fall *et al.* 1996, Benito-León *et al.* 2003, Mayeux *et al.* 1995, Van Den Eeden *et al.* 2003), an effect linked to the potentially neuroprotective effects of oestrogen in women (Saunders-Pullman 2003), however other studies have found no significant difference in prevalence between men and women (de Rijk *et al.* 1997). Around 90% of PD cases are sporadic and idiopathic (de Lau & Breteler 2006), most likely caused by an interaction between susceptibility genes, aging and environmental factors.

1.1.3 Genetic and idiopathic causes

Different variants of familial PD have revealed many autosomal dominant and recessive genetic mutations, including mutations of ubiquitin, parkin, ubiquitin C-terminal hydrolase (UCH-L1), DJ-1, phosphatase and tensin homolog-inducible kinase 1 (PINK1) and leucine-rich repeat kinase 2 (LRRK2), glucocerebrosidase, as well as mutations in SNCA which encodes α -synuclein as illustrated in Table 1.1 below. The detection of α -synuclein mutations led to the discovery that α -synuclein is a key component of LBs (Spillantini *et al.* 1997). Some of these familial mutations such as PINK1 and DJ1 are associated with early onset PD (<45 years old), whereas others are associated with a later onset of disease (reviewed by Trinh and Farrer (2013)). Some genetic mutations such as LRRK2 are only partially penetrant and require other genetic or environmental triggers in order to induce PD (Shulman *et al.* 2011). Investigating these familial gene mutations has hugely contributed to revealing the molecular processes involved in sporadic PD pathogenesis, as illustrated in Figure 1.3 (see section 1.2). Furthermore, a number of 'risk gene' loci for PD have been identified in recent years, confirming the interaction between genetic risk and environmental triggers in the development of PD (Dexter & Jenner 2013).

Table 1.1: Common gene mutations associated with familial Parkinson's disease

Gene	Mode of inheritance	Risk (%)	Age of onset
SNCA	Autosomal dominant	Very rare: <1% of PD patients	20-85 years
LRRK2	Autosomal dominant	1.6% of PD patients	32-79 years
Parkin	Autosomal recessive	Most common: ~50% of young onset PD	16-72 years
PINK1	Autosomal recessive	3.7% of young onset PD	20-40 years
DJ1	Autosomal recessive	Rare: 1% of young onset PD	20-40 years
FBX07	Autosomal recessive	Very rare: <1% of young onset PD	10-19 years
NR4A2/NURR1	Unknown	~1% of PD patients	45-67 years
POLG	Unknown	Very rare: <1% of young onset PD	20-26 years

Adapted from Dexter and Jenner 2013. Risk percentages for each gene mutation are from Polymeropoulos *et al.* 1997, Gilks *et al.* 2005, Lucking *et al.* 2000, Le *et al.* 2002, Tan *et al.* 2006, Abou-Sleiman *et al.* 2003, Davidzon *et al.* 2006, Di Fonzo *et al.* 2009.

Sporadic cases of PD have been suggested to be due to interactions between environmental aspects and susceptibility genes, and recent meta analyses of genome-wide association studies have identified a large number of risk gene loci (de Lau & Breteler 2006, Consortium 2011, Consortium & 2011). It appears that aging is the strongest predisposing factor for developing PD (Reeve *et al.* 2014), and exposure to environmental toxins such as pesticides, herbicides, solvents and possibly heavy metals is thought to increase the risk of developing PD (de Lau & Breteler 2006). Indeed, epidemiological studies have demonstrated correlations between pesticide/herbicide exposure and PD risk (Lai *et al.* 2002). In 1983, several people intravenously injected drugs contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and developed severe parkinsonian-like symptoms, leading to the finding that MPTP selectively kills nigrostriatal dopaminergic neurons (Langston *et al.* 1983). This discovery supported the theory that environmental toxin exposure may be involved in the development of PD.

Many studies have associated brain inflammation with the pathogenesis of PD; early life viral infections have been linked to postencephalitic parkinsonism (Casals *et al.* 1998). Additionally, infectious microorganisms have been suggested to be a risk factor for PD (Liu *et al.* 2003), and systemic or central inflammation associated with viral or bacterial infection may contribute to the pathogenesis of the disease by triggering a self-perpetuating cycle of chronic and detrimental neuroinflammation (Tansey & Goldberg 2010). Additionally, some studies have shown an association between head trauma and later development of PD, although this link remains controversial (Lai *et al.* 2002). Interestingly, there is a significant inverse association between smoking and PD (Grandinetti *et al.* 1994, de Lau & Breteler 2006, Hernán *et al.* 2001) suggesting smoking of cigarettes can reduce the risk of developing PD, although it has been suggested that this could be due to a dopamine shortage in the brains of PD patients, perhaps reflecting changes in behaviour prior to the development of motor symptoms of the disease (Hernán *et al.* 2002). These protective effects may be due to lower levels of the enzyme monoamine oxidase B (MAO-B) activity in smokers, perhaps protecting against MPTP-like neurotoxins (Yong & Perry 1986). Additionally, up-regulation of nicotine receptors may be protective, and nicotine can stimulate striatal dopamine release as well as providing antioxidant action and the production of neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) (O'Neill *et al.* 2002, Quik *et al.* 2012). Additionally, coffee and caffeine consumption has been inversely correlated with PD risk, although the effect is stronger in men than in women, perhaps due to blocking of caffeine's neuroprotective effects by oestrogen in women (Hernán *et al.* 2002, Ascherio *et al.* 2004).

1.1.4 Clinical manifestations

1.1.4.1 Motor symptoms

The characteristic motor symptoms of PD are akinesia or bradykinesia, resting tremor, rigidity and postural instability. Other motor symptoms include freezing, changes in gait and a flexed posture leading to quick shuffling steps (festination), difficulties with speech (dysarthria), swallowing (dysphagia) and a masklike facial expression (hypomimia). Often there is an asymmetric onset of motor symptoms early in the disease, which later progresses to become bilateral (Rodriguez-Oroz *et al.* 2009, Jankovic 2008).

1.1.4.2 Non-motor features

Cognitive impairment is common in PD, and approximately 40% of patients develop dementia, demonstrating a spreading Lewy Body (LB) pathology and degeneration of the frontal cortex (Emre 2003, Aarsland & Kurz 2010). The non-motor symptoms associated with PD are often under-reported and undertreated and impact heavily on quality of life; the more affected an individual is by non-motor symptoms, the more severely their quality of life is affected (Martinez-Martin 2011). These symptoms can result in extreme disability (e.g. depression, anxiety and dementia) and can require long-term care. Non-motor symptoms in PD are common, occurring at any stage of the disease and increasing in frequency with advancing disease (Chaudhuri *et al.* 2006). On average, patients have between 9 and 12 non-motor symptoms (Martinez-Martin *et al.* 2007). A number of non-motor symptoms have been associated with PD including olfactory deficit, constipation, sleep disorders, autonomic dysfunction, anxiety, depression, apathy, fatigue, pain, cognitive changes and visual disturbances (Bonnet *et al.* 2012). Some non-motor symptoms such as hyposmia/olfactory dysfunction, mood disorders, dysfunction of the autonomic nervous system and sleep disorders are present in the early stages of the disease, perhaps years prior to clinical diagnosis (Chaudhuri *et al.* 2006, Lang 2011). Degeneration of non-dopaminergic systems outside of the SNc are thought to contribute to or be responsible for the non-motor features of the disease, and therapies targeting noradrenergic, serotonergic and cholinergic transmission are often used to treat the non-motor symptoms of PD (Bernal-Pacheco *et al.* 2012). The motor and non-motor features of PD are summarised in Table 1.2.

Table 1.2 Motor and non-motor symptoms of Parkinson's disease

Motor symptoms	Non-motor symptoms
<p>Bradykinesia Akinesia Muscle rigidity Tremor Postural instability Shuffling gait and festination Dystonia Striatal deformities of the hands and feet Masked facies Dysarthria</p>	<p>Neuropsychiatric Cognitive dysfunction, dementia, depression, apathy, anxiety, anhedonia, attention deficit, hallucinations and delusions</p> <p>Autonomic Orthostatic hypotension, sensory symptoms, bladder disturbances such as urgency and nocturia, anosmia, sexual dysfunction, abnormal sweating, weight loss</p> <p>Gastrointestinal Dysphagia, reflux and vomiting, constipation and bowel dysfunction, hypersalivation</p> <p>Sleep disorders Excessive daytime sleepiness, restless legs syndrome, REM behaviour disorder, insomnia, vivid dreams.</p> <p>Other Pain, paresthesia, fatigue</p>

1.1.4.3 Clinical diagnosis of Parkinson's disease

There is no definitive test for PD, and as yet, no diagnostic biomarker is available for diagnosis of PD at the pre-motor stage. A diagnosis of PD is therefore based on clinical features fulfilling specific diagnostic criteria. The well-established and validated Unified Parkinson's Disease Rating Scale (UPDRS) is used to assess impairment and the degree of disability, as well as monitoring the burden of the disease over time (Goetz *et al.* 2008). Diagnosis of PD requires the presence of at least two of the characteristic motor symptoms; resting tremor, bradykinesia, postural instability and rigidity (Rao *et al.* 2003). Motor symptoms are progressive, often have an asymmetric onset and are also responsive to L-3,4-dihydroxyphenylalanine (L-DOPA) treatment (Litvan *et al.* 2003). There can be difficulties in differentiating PD from other types of parkinsonism such as multiple system atrophy (MSA), as symptoms overlap (Tolosa *et al.* 2006). Neuroimaging techniques using tracers to measure dopamine transporter uptake (DAT scans) can measure the integrity of dopaminergic function in the brain, further supporting clinical diagnosis and helping to differentiate PD from other forms of parkinsonism (Brooks & Piccini 2006). Confirmation of the PD diagnosis can only take place post-mortem, with neuropathological examination demonstrating 80-90% of cases are correctly clinically diagnosed (Litvan *et al.* 2003). The prognosis for PD is poor, reducing life expectancy and increasing mortality risk, with longer disease duration and the presence of dementia increasing mortality risk (de Lau *et al.* 2005).

1.1.5 Neuropathological features

PD pathology is characterised by the selective loss of dopaminergic cells from the substantia nigra pars compacta (SNc) and the appearance of cytoplasmic protein inclusions within surviving neurons called Lewy bodies (LBs) as well as aggregates within processes, forming Lewy neurites (Lees *et al.* 2009). In PD brains, transverse sections through the SNc show a loss of the characteristic black neuromelanin-containing dopaminergic neurons. The loss of these dopaminergic neurons results in a loss of dopamine in the striatum, a subcortical nucleus comprised of the caudate and putamen. This leads to dysfunctional signalling in the basal ganglia (BG), a collection of nuclei involved in motor control (Rodriguez-Oroz *et al.* 2009), producing the characteristic motor symptoms of PD (Parkinson, 2002, Tolosa *et al.*, 2006). Reactive gliosis is also present in the SNc of PD brains, with microglia/macrophages containing scavenged neuromelanin in areas of neuronal loss.

First described by Friedrich Lewy in 1912, spherical shaped LBs accumulate within vulnerable neurons and are made of dense granular and filamentous material comprising intracytoplasmic aggregations of proteins and lipids (Spillantini *et al.* 1997, Lewy 1912) as illustrated in Figure 1.1. LBs and Lewy neurites can be found in both central and peripheral autonomic neurons

as well as the cerebral cortex (Hawkes *et al.* 2009, Braak *et al.* 2003), and inclusions can be degraded to form extracellular LBs. For PD to be neuropathologically diagnosed, LB pathology is usually observed in surviving neurons, however LB presence is not exclusive to PD; it is present in Lewy body dementia and MSA as well as other rarer disorders. Nor is LB pathology present in all cases of PD, as some genetic cases do not present with this pathology (Halliday *et al.* 2011). An aggregated, hyperphosphorylated form of the presynaptic terminal protein, α -synuclein, is one of the main constituents of LBs and Lewy neurites, alongside the presence of other ubiquitinated and phosphorylated proteins, including neurofilament proteins (Spillantini *et al.* 1998, Betarbet *et al.* 2005b). It is unknown what factors lead to the abnormal conformation of α -synuclein, and whether formation of these inclusion bodies is an adaptive process, or is more directly involved in neurodegeneration is yet to be elucidated fully.

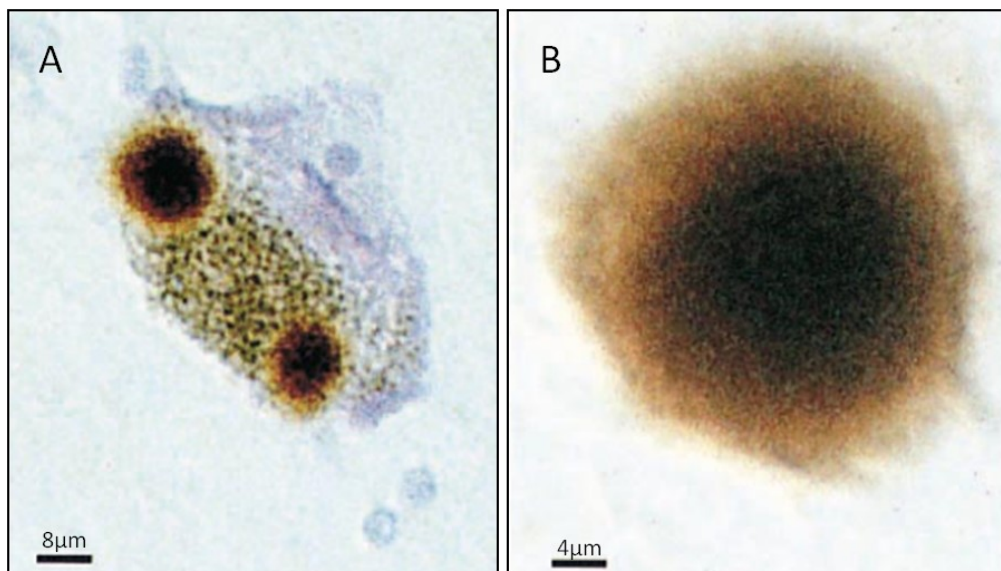


Figure 1.1: Lewy bodies in nigral dopaminergic neurons from patients with Parkinson's disease. Lewy bodies are comprised of dense granular and filamentous material, particularly the hyperphosphorylated form of α -synuclein. A) A nigral dopaminergic neuron containing two α -synuclein positive lewy bodies. (brown staining). B) An extracellular α -synuclein positive lewy body. Images adapted from Spillantini *et al.* (1997).

1.1.5.1 Neuropathological staging

Degeneration throughout the central and peripheral nervous system occurs in PD, and many of the non-motor features of the disease are attributed to the loss of non-dopaminergic neuronal types including noradrenergic, γ -aminobutyric acid (GABA)ergic, cholinergic and serotonergic neurons situated in many regions of the brain including the hypothalamus, amygdala, dorsal motor nucleus of the vagus, nucleus basalis of Meynert, locus coeruleus and other cortical and limbic regions (Chaudhuri et al. 2006). These affected regions all demonstrate LB pathology, indicating a common multifocal pathogenic process affecting more than the SNc alone. In fact, non-motor symptoms often precede the appearance of motor symptoms in PD, and the progression of LB pathology closely correlates with these symptoms (Braak et al. 2003). For example, impaired olfaction is believed to be one of the earliest symptoms of PD, and is thought to be associated with LB pathology in the olfactory bulb and other centres involved in olfaction such as the anterior olfactory nucleus (Wattendorf *et al.* 2009, Pearce *et al.* 1995). A sequential staging process of LB and α -synuclein pathology has been hypothesised based on topographical lesions observed in PD brains. Braak and colleagues (2003) proposed that pathology originates in the dorsal motor nucleus of the vagus and the anterior olfactory nucleus in the olfactory bulb (stage 1), spreading upwards via the pons (stage 2) to reach the midbrain (stage 3), when motor symptoms become apparent due to extensive dopaminergic neurodegeneration. It then continues to spread to the basal forebrain and mesocortex (stage 4) and eventually reaches the neocortex (stages 5 and 6), when late stage symptoms of dementia and neuropsychiatric disturbances are common (Braak et al. 2003). This caudorostral spreading of pathology is consistent with progressing symptoms, although not all cases of PD demonstrate the same sequential pattern of spreading pathology, suggesting the staging system should be interpreted cautiously (Kalaitzakis *et al.* 2008, Beach *et al.* 2009). More recent proposals have developed the Braak staging scheme and suggested that the autonomic neurons in the peripheral nervous system are affected prior to the involvement of the CNS (Minguez-Castellanos *et al.* 2007, Del Tredici & Braak 2012).

1.1.6 The Basal Ganglia: Pathophysiology of Parkinson's disease

The BG comprise a number of interconnected subcortical nuclei which have a key involvement in the regulation of motor control. Organisationally, the BG form a network of parallel loops that connect and integrate different cerebral regions, BG nuclei and the thalamus (Alexander *et al.* 1986, Alexander *et al.* 1989, Parent & Hazrati 1995). In the normal BG, glutamatergic neurons from the cortical motor regions project to the striatum, which consists of the caudate, putamen and nucleus accumbens and is considered the main input region of the BG. Striatal projections then form two distinct pathways,

known as the 'direct' and 'indirect' pathways, to the output nuclei of the BG: the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr). In the direct pathway, neurons project straight from the striatum to the output nuclei, providing a strong GABAergic inhibition to the GPi and SNr (Obeso *et al.* 2000). In contrast, neurons in the indirect pathway project from the striatum, inhibiting the external segment of the globus pallidus (GPe), which in turn disinhibits the subthalamic nucleus (STN) and subsequently provides excitatory glutamatergic input to the GPe/SNr (Obeso *et al.* 2000). Striatal activity is modulated by dopaminergic efferents from the SNc. Dopamine acts on both excitatory D1 receptors and inhibitory D2 receptors, both of which are expressed in the striatum. Neurons expressing the excitatory D1 receptors project via the direct pathway to the GPi and SNr, whereas neurons expressing the inhibitory D2 receptors project to the GPe in the indirect pathway (Obeso *et al.* 2000). Dopamine released in the striatum from SNc projections therefore activates the direct pathway whilst inhibiting the indirect pathway, thus reducing the activity of the output nuclei. Neurons projecting from the output nuclei have GABAergic inhibitory synapses within the ventrolateral thalamus, which itself provides excitatory control of cortical motor regions (Parent & Hazrati 1995). Initiation of movement requires the activation of the direct pathway, whereas cessation of movement requires indirect pathway activation (Obeso *et al.* 2000). The balance between direct and indirect pathway activity therefore enhances and suppresses motor activity respectively. A simplified schematic of the normal and Parkinsonian BG motor circuit is illustrated in Figure 1.2.

In the Parkinsonian brain, the loss of dopaminergic modulation from the SNc results in a deficiency in striatal dopamine, causing significant signalling alterations in downstream BG pathways and excessive inhibition of the thalamocortical pathways, producing the characteristic motor symptoms of PD (Obeso *et al.* 2000, Rodriguez-Oroz *et al.* 2009). Additionally, excess glutamatergic signalling in projections from the STN are thought to generate further neuronal cell loss via excitotoxic damage to the SNc, potentiating the neurodegeneration (Blandini *et al.* 2001). Nigrostriatal dopaminergic neurons are believed to be particularly vulnerable to glutamate toxicity due to impaired mitochondrial activity and high levels of oxidative stress (discussed below in section 1.2). Interestingly, in the early stages of PD, increased glutamatergic stimulation from the STN may enhance the activity of dopaminergic neurons in the SNc (Shimo and Wichmann, 2009), but as the disease progresses and the gradual functional alterations in BG activity develop, the chronic and increasing excess glutamatergic activity in the SNc may become neurotoxic, sustaining the progression of the neurodegenerative process (Blandini *et al.* 2001). By the time motor symptoms are apparent, a significant proportion (60-70%) of nigral dopaminergic neurons have degenerated and approximately 80% of striatal dopamine has been depleted (Riederer & Wuketich 1976). It is likely that compensatory

mechanisms such as upregulation of dopamine receptors and increased dopamine release are responsible for this delay in the appearance of motor symptoms.

The BG organisation discussed here is expected to be in reality more complex in structure and function than established models suggest. Indeed, a number of observations do not completely support this perhaps oversimplified model of organisation of the BG pathway. The direct and indirect pathways may not be as functionally distinct as originally suggested; anatomical tracing studies in primates and rodents have demonstrated that single striatal projections can innervate both the GPi and GPe, therefore signalling through both the direct and indirect pathways (Lévesque & Parent 2005, Castle *et al.* 2005). Additionally, a number of studies have demonstrated colocalisation of both D1 and D2 receptors on a subset of striatal neurons in rodents and primates, rather than single receptor subtypes as suggested in the classical model, although the size of the population coexpressing these receptors varies in estimation between studies, perhaps due to the technical difficulties in measuring protein or mRNA coexpression (Surmeier *et al.* 1996, Aubert *et al.* 2000, Lester *et al.* 1993). Furthermore, cortical neurons can also directly innervate the STN with strong excitatory input, therefore bypassing striatal control of the indirect pathway completely (Nambu 2008). It has been suggested that this pathway has functional significance and is involved in inhibiting and/or changing motor plans, although its exact contribution to movement still remains to be elucidated (Nambu *et al.* 2002). Therefore, BG circuitry is likely to be more complex than current models suggest, although the intricacies of the BG in both health and disease are still being investigated.

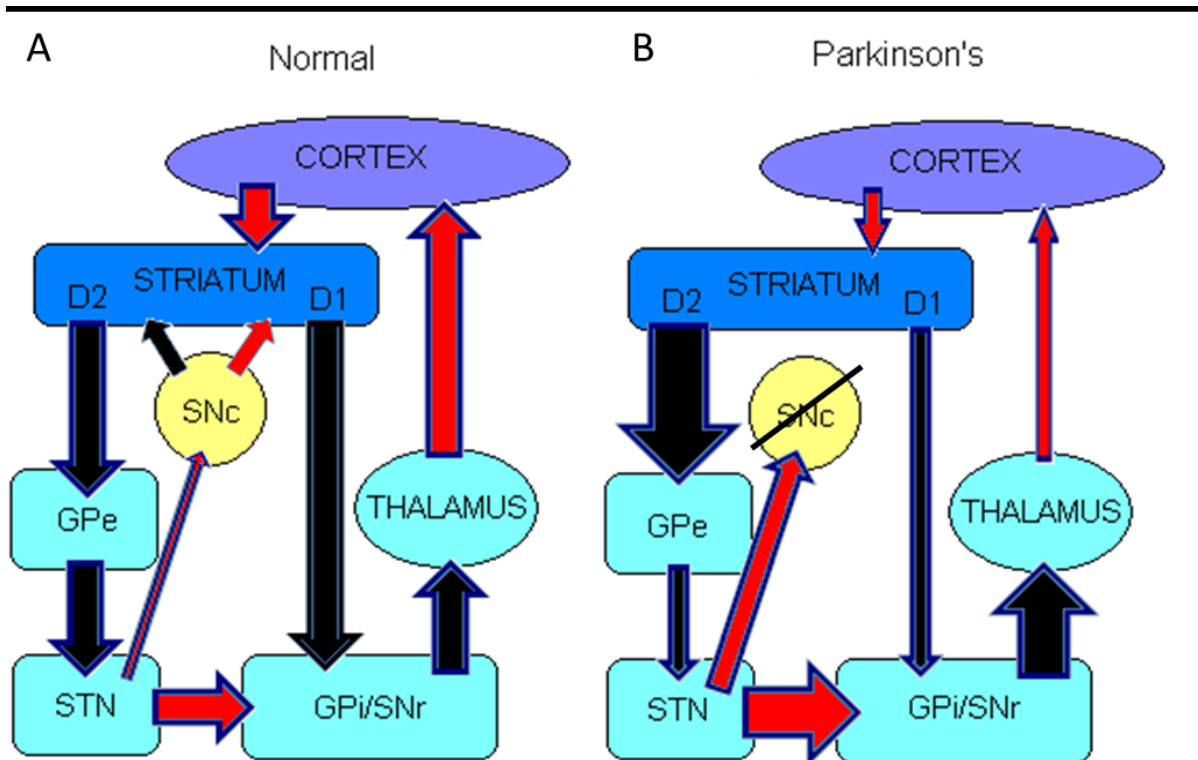


Figure 1.2: Simplified schematic of the Basal Ganglia. Red arrows are excitatory projections; black arrows are inhibitory projections. A: In the normal brain, dopaminergic signalling from the SNc regulates the downstream pathways of the BG, and the direct and indirect pathways of the BG are balanced. Dopaminergic signalling from the SNc activates both excitatory D1 striatal receptors (red arrow) which project via the direct pathway, and inhibitory D2 striatal receptors (black arrow) which project via the indirect pathway. B: In PD, the loss of dopaminergic signalling from the SNc to the striatum causes overactivity in the indirect pathway. Overactivity of glutamatergic projections from the STN to the output nuclei (the GPI/SNr) are one of the main causes of PD symptoms, and this excess signalling in projections to the SNc is also thought to generate further neuronal cell loss via excitotoxicity (Rodriguez et al., 1998). Additionally, striatal inhibition of the output nuclei is reduced via the direct pathway. The output nuclei then increase inhibitory activity to the thalamus and reduce cortical activation, leading to the clinical manifestations of PD. Thickness of the projection arrows indicates activity level; in B, thicker arrows indicate overactive pathways and thinner arrows indicate underactive pathways. Image adapted from Wichmann and DeLong (1996), Niswender and Conn (2010).

1.2 Pathogenic mechanisms

The underlying causes of neuronal cell loss in PD seem to be multifactorial and complex, with interacting pathological mechanisms implicated in disease progression such as protein aggregation and abnormal protein handling, inflammation, mitochondrial dysfunction, oxidative stress and excitotoxicity contributing to neurodegeneration. Cases of rare genetic mutations in familial PD have helped to reveal common molecular events that lead to neurodegeneration. Although it is still unknown what the initial triggers for the development of PD are, multiple pathogenic mechanisms are believed to result in final common pathways which lead to cell death, as discussed below and illustrated in Figure 1.3.

1.2.1 Mitochondrial dysfunction and oxidative stress

Oxidative stress (OS) is a common feature of neurodegenerative disease and results from an imbalance between the levels of reactive oxidative species (ROS) and antioxidants. ROS such as hydrogen peroxide, nitric oxide (NO), superoxide and hydroxyl radicals can lead to oxidative damage and induce cell death (Barnham *et al.* 2004). NO can react with superoxide to form peroxynitrite, which is an extremely reactive, potent and damaging oxidant (Beckman & Koppenol 1996). OS has been linked to many processes that occur in neurodegeneration, and oxidative injury to lipids, proteins and DNA have all been detected in patients with neurodegenerative disease (Andersen 2004, Ischiropoulos & Beckman 2003), although its role in the initiation or progression of neurodegeneration is still unclear due to difficulties in detecting ROS directly. Additionally, decreased antioxidant levels such as glutathione (GSH) and decreased activity of the cytosolic enzyme glutathione peroxidase which detoxify peroxynitrite have been reported in the SNc of PD brains (Sian *et al.* 1994, Kish *et al.* 1985), reducing the ability of neurons to protect themselves against oxidative damage. The oxidative metabolism of dopamine has been proposed to be a source of ROS (Tse *et al.* 1976, Graham 1978) and this, combined with reduced antioxidant levels, may account for their selective vulnerability to damage in PD. In LBs, α -synuclein is found in a nitrated form which is consistent with oxidative and nitrative damage and may encourage aggregation (Giasson *et al.* 2000), although it is unknown whether this is an early or late event in disease pathogenesis (Goedert 2001).

The inhibition of complex I of the mitochondrial electron transport chain leads to an increased production of excess ROS, which leads to oxidative damage and mitochondrial dysfunction in both neurons and microglia (Schapira *et al.* 1989, Cleeter *et al.* 1992, Eckmann *et al.* 2013). The discovery of the neurotoxicity of MPTP via its active metabolite, the 1-methyl-4-phenylpyridinium ion (MPP⁺), highlighted the key role of complex I inhibition in PD pathogenesis (Ramsay *et al.* 1986). Additionally, other compounds that are toxic to dopaminergic neurons similarly inhibit complex I, such

as rotenone and annonacin (Lannuzel *et al.* 2003). Mitochondrial DNA mutations have been detected in PD patients with LBs (Betts-Henderson *et al.* 2009), and a significant reduction in complex I activity has been detected in the SNc of patients with PD (Schapira *et al.* 1990a, Schapira *et al.* 1990b). Inhibition of, or deficits in complex I activity have also been detected in the muscle tissue and platelets of a proportion of PD patients (Mann *et al.* 1992, Parker *et al.* 1989). Complex I deficiency may be a key source of OS and subsequent α -synuclein aggregation (Giasson *et al.* 2000). Additionally, overexpression of α -synuclein induces mitochondrial abnormalities and degeneration (Martin *et al.* 2006) and increases sensitivity to mitochondrial toxins (Norris *et al.* 2007). Furthermore, dopaminergic neurons in an α -synuclein knockout model are resistant to the mitochondrial toxin MPTP (Klivenyi *et al.* 2006). There therefore seems to be a clear interaction between α -synuclein and mitochondrial function; mitochondrial dysfunction causes α -synuclein aggregation, and α -synuclein can in turn damage the mitochondria, creating a progressive cycle of impairment. Genetic investigations of familial PD have identified that mutations in α -synuclein, PINK1, parkin, DJ-1 and potentially LRRK2 proteins are associated with mitochondrial dysfunction (Schapira 2011, Schapira 2008), leading to altered mitochondrial structure and function and reduced complex I activity/assembly. Additionally, DJ-1, parkin and PINK1 loss-of-function mutations decrease mitochondrial protection against OS which further exacerbates mitochondrial dysfunction (Dexter & Jenner 2013).

1.2.2 Glutamate dysregulation and excitotoxicity

Glutamate is the principal excitatory neurotransmitter in the brain and activates fast acting ionotropic glutamate (iGlu) receptors such as alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors, as well as metabotropic glutamate receptors (mGlu receptors, see 1.4) which couple to G-proteins and have a modulatory role (Conn & Pin 1997, Nicoletti *et al.* 1996). Excessive activation of iGlu receptors contributes to cell death in acute pathological conditions such as ischaemia or trauma; however it is also likely to play a central role in more chronic neurodegenerative conditions such as PD (Mehta *et al.* 2013). When glutamate release is excessive, it stimulates extrasynaptic (rather than synaptic) NMDA receptors which lead to suppression of pro-survival pathways resulting in neuronal degeneration (Hardingham *et al.* 2002). Excitotoxicity may also occur in vulnerable neuronal types indirectly; this is triggered by impaired energy metabolism (e.g. through mitochondrial dysfunction) and the resulting ATP depletion decreases cell membrane potential (Schulz *et al.* 1997). This partial depolarisation of the neuronal cell membrane results in secondary activation of voltage-dependent NMDA receptors and subsequently, neurotoxic increases in Ca^{2+} influx under low or physiological levels of glutamate, rendering neurons more vulnerable to excitotoxic damage (Novelli *et al.* 1988, Zeevalk & Nicklas 1992). Mitochondrial

dysfunction can lead to reduced cellular ATP levels, impairing the cell's ability to maintain membrane polarity and leading to activation of NMDA receptors, influx of excess Ca^{2+} and subsequent excitotoxicity (Greenamyre 2001). Additionally, the influx of Ca^{2+} activates nitric oxide synthetase (NOS) leading to NO production and other ROS which can react with superoxide to cause oxidative damage (Gunasekar *et al.* 1995). Mitochondrial function is therefore impaired by the damaging conditions triggered by NO, further increasing neuronal vulnerability to excitotoxic damage and resulting in a vicious cycle of toxicity (Szatkowski & Attwell 1994, Wang & Qin 2010). Glutamate is also produced and released by activated microglia, raising the extracellular concentrations of glutamate and increasing excitotoxic damage to neurons via NMDA receptors (Barger & Basile 2001, Takeuchi *et al.* 2005).

Overactivity of glutamatergic projections from the STN to the output nuclei (GPi and SNr) of the BG is one of the main causes of PD symptoms, and there is also evidence that glutamatergic activity is altered in the corticostriatal pathway (Obeso *et al.* 2008). Additionally, excess signalling from the STN occurs in projections to the SNc, which is thought to generate further neuronal cell loss via glutamate-induced excitotoxicity (Rodriguez 1998). Targeting the STN with deep brain stimulation (DBS) modulates symptomatology and some evidence suggests it might also limit disease progression in PD (Tagliati *et al.* 2010), although others have found little effect (Hilker *et al.* 2005). Furthermore, stimulation or lesioning of the STN can reduce the loss of nigral dopaminergic neurons in animal models of PD (Maesawa *et al.* 2004, Breit *et al.* 2001, Wallace *et al.* 2007), supporting the role of excitotoxicity in neuronal cell death. Weak NMDA receptor antagonists such as amantadine are also neuroprotective against nigrostriatal damage in PD models, providing further evidence that excess glutamate is neurotoxic and involved in nigrostriatal degeneration (Lange & Riederer 1994, Turski *et al.* 1991). Pharmacological modulation of excess glutamatergic signalling from the STN could therefore provide an effect comparable to DBS in those patients unsuitable for this procedure, and furthermore, may potentially protect against further nigrostriatal degeneration.

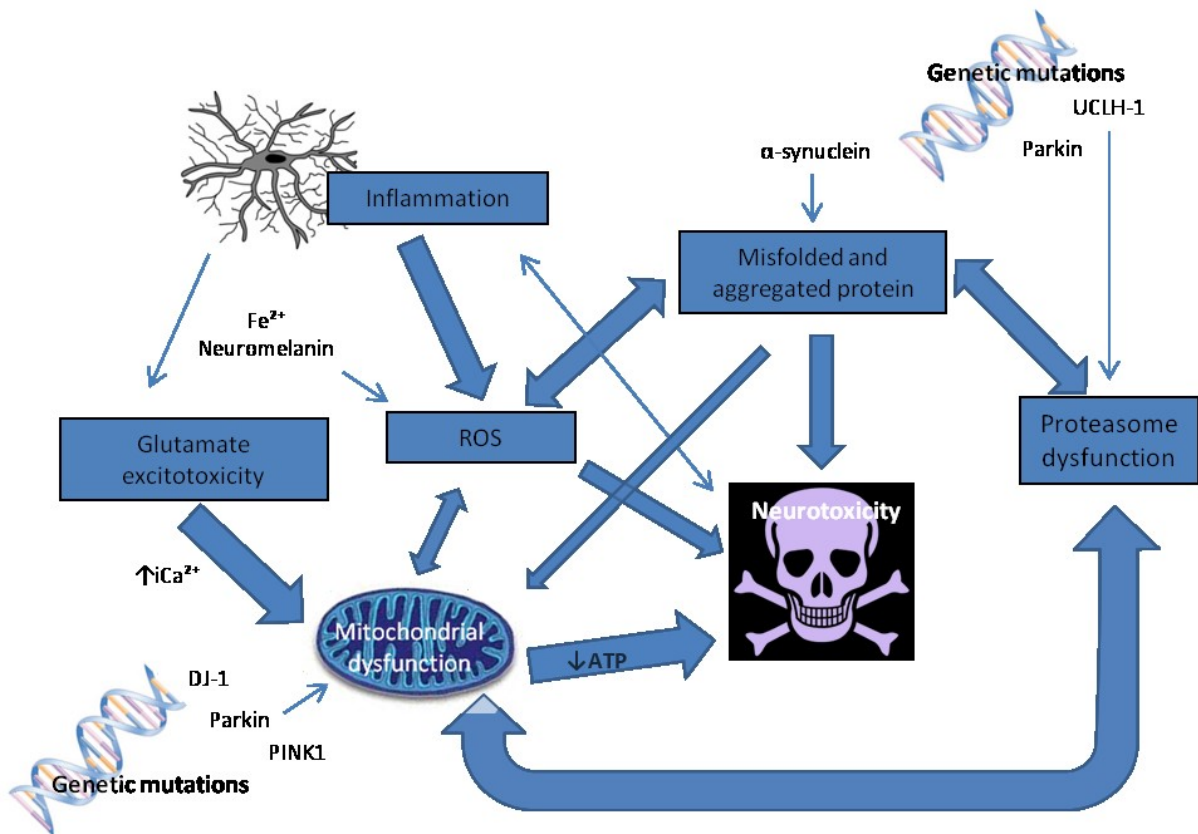


Figure 1.3: Key pathogenic mechanisms contributing to neurodegeneration of dopaminergic neurons in the SNc in Parkinson's disease. A number of interacting mechanisms are involved in the neurodegenerative process. Blue arrows demonstrate interactions between these molecular mechanisms. Additionally, common familial genetic mutations and their effects on specific molecular mechanisms are indicated. Abbreviations: ROS: reactive oxygen species, ATP: adenosine triphosphate, iCa^{2+} : intracellular calcium.

1.2.3 Proteasome dysfunction

Aggregation of abnormal proteins is a common feature of many neurodegenerative diseases and is likely to be a main contributor to cell death. The factors involved in the initiation of abnormal protein accumulation and aggregation are complex, although protein coding mutations, abnormal protein handling and proteasome dysfunction all play a role (Ross & Poirier 2004). Proteasome activity decreases with age reducing a cell's ability to clear unwanted proteins which may explain why aging is a risk factor for developing neurodegenerative disease (Keller *et al.* 2002). In PD, a dysfunction of the ubiquitin-proteasome system (UPS) is thought to lead to the toxic accumulation of modified proteins which form LBs within the cytoplasm (Giasson & Lee 2003). The ubiquitin-proteasome system (UPS) is responsible for the breakdown of normal and misfolded, oxidatively damaged or mutant cellular proteins. Such proteins are polyubiquitinated and subsequently degraded by the 26/20S proteasome complex (Ciechanover 2005), as illustrated in Figure 1.4 below. Impairment of the UPS is thought to play a role in the pathogenesis of PD due to its vital role in protein degradation within cells (McNaught *et al.* 2001). Proteasome inhibition is common to the pathogenesis of both familial and idiopathic PD (Betarbet *et al.* 2005a, McNaught & Olanow 2006). Indeed, in some forms of familial PD, mutations in genes encoding enzymes of the UPS, parkin (an E3 ubiquitin-ligase) and UCH-L1, a de-ubiquitinating enzyme, support the central role of UPS dysfunction in the pathogenesis of PD, as illustrated in Figure 1.3 above (Kitada *et al.* 1998, Leroy *et al.* 1998). A decline in the catalytic functions of the 20S proteasome and reduced expression of α -subunits of the 26S proteasome have been detected in the SNc of sporadic PD patients, but not in other regions with no neuronal loss (McNaught *et al.* 2003, McNaught *et al.* 2002a, McNaught & Jenner 2001, Furukawa *et al.* 2002, Tofaris *et al.* 2003). It is therefore apparent that failure of the UPS leads to protein aggregation and the formation of LBs within neurons, although it is still unclear whether LBs are toxic themselves, or are a form of protective response to the accumulation of misfolded toxic proteins and may be an attempt to slow neuronal death (McNaught & Olanow 2006).

Inhibition of the UPS with proteasome inhibitors such as lactacystin or proteasome inhibitor 1 (PSI) results in an accumulation of misfolded ubiquitinated proteins within the cytosol, eventually leading to cell dysfunction and death (Betarbet *et al.* 2005b, McNaught & Olanow 2006). Nigrostriatal dopaminergic neurons seem to be preferentially sensitive to damage by proteasome inhibitors. This selective vulnerability has been suggested to be due to the increased levels of OS in nigrostriatal neurons leading to increased oxidative damage of proteins and therefore a higher requirement for oxidised and cytotoxic protein degradation (McNaught *et al.* 2002b). It can therefore be surmised that altered protein handling is responsible for the formation of LBs and possibly dopaminergic neuronal

degeneration in both familial and idiopathic PD, and likely plays a key role in the initiation and/or progression of PD pathology.

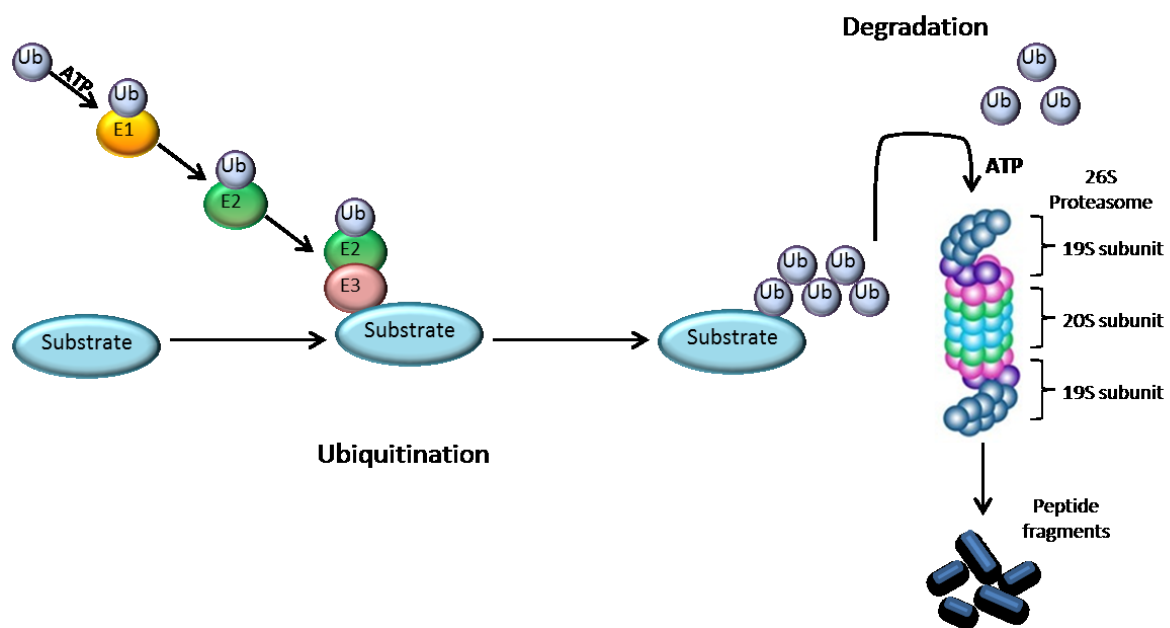


Figure 1.4: The ubiquitin-proteasome system. Targeted ubiquitination of substrate proteins begins with a series of coordinated reactions by three enzymes: ubiquitin is attached to an E1 enzyme, the ubiquitin-activating enzyme, in an ATP-dependent manner. Ubiquitin is then transferred to a second enzyme, E2, the ubiquitin conjugating enzyme. E2 then interacts with E3, a ubiquitin ligase enzyme, which ligates ubiquitin to the substrate protein. Ligation of ubiquitin is repeated to form a polyubiquitin chain on the target protein, tagging it for proteasomal degradation. The 26S proteasome is comprised of the 20S core where degradation occurs and 19S regulatory subunits. In an ATP-dependent reaction, ubiquitin is removed and recycled, and the protein enters the core of the proteasome and is digested to peptides (Lecker et al. 2006). Impaired UPS function has been demonstrated in the SN of idiopathic PD (McNaught & Jenner 2001). Ub, ubiquitin.

1.2.4 Inflammation

Microglia are the resident macrophages found throughout the CNS and their activation is vital for defence against invading microorganisms and responding to dying or damaged cells (Block *et al.* 2007, Kreutzberg 1996). In the normal brain, microglia are found in a resting state with a ramified morphology, but when activated in response to a change in the environment they display an amoeboid morphology (Kreutzberg 1996, Nimmerjahn *et al.* 2005). Microglia are vital for the removal of dead or dying cells and debris in the brain, although in some cases their activation can have detrimental effects. Activated microglia adopting a pro-inflammatory M1-like phenotype can release glutamate and neurotoxic pro-inflammatory molecules such as cytokines including interleukins 1 α and 1 β (IL-1 α , IL-1 β), IL-6 and tumour necrosis factor- α (TNF- α), and cytotoxic factors including free radicals and NO, amplifying excitotoxic damage and exacerbating neurodegeneration (Barger & Basile 2001, Barger *et al.* 2007, Cunningham *et al.* 2005, Hald & Lotharius 2005, Kreutzberg 1996, Parker *et al.* 2002). Additionally, potentially viable cells can be phagocytosed prematurely by activated microglia, contributing to the degenerative process (Marinova-Mutafchieva *et al.* 2009, Streit 2005). Furthermore, prolonged activation of microglia will prevent them from carrying out their neurosupportive functions such as the release of key growth factors like GDNF, which are vital for cell survival (Benoit *et al.* 2008, Dexter & Jenner 2013).

Normal ageing is associated with a progressive increase in numbers of activated microglia including an increase in those with phagocytic morphology (Vaughan & Peters 1974, Sheng *et al.* 1998). This may lead to an amplified inflammatory response to stimuli such as infection or protein aggregation, explaining why age is a key risk factor for developing neurodegenerative disease (Block *et al.* 2007). Notably, recent findings indicate that microglia can be primed by chronic CNS disease or inflammation, then subsequently display an exaggerated response to later systemic or central inflammation, exacerbating the degenerative process (reviewed in Cunningham (2013)). It has been postulated that both the initiation and progression of PD may be triggered by neuroinflammation (Qin *et al.* 2007) and there is a growing body of evidence that in some cases, PD is linked to head trauma, viruses and infections which subsequently trigger microglial activation and neurodegeneration (Herrera *et al.* 2005, Liu *et al.* 2003). Systemic inflammation or infection seems to exacerbate symptoms in neurodegenerative disease, and infiltration of peripheral inflammatory molecules into the CNS is thought to occur due to increased BBB permeability (Kortekaas *et al.* 2005, Collins *et al.* 2012).

In PD patient brains, activated microglia and astrocytes are found to cluster in areas of degeneration (Glass *et al.* 2010, McGeer *et al.* 1988). Additionally, raised levels of pro-inflammatory cytokines including IL-1 α , IL-1 β , and TNF- α have been found in the brain and CSF of PD patients (Hirsch

et al. 2003). Microglial toxicity to dopaminergic neurons is well documented in a number of cell culture studies and animal models of PD (Gao *et al.* 2002b, Gao *et al.* 2003, Liu & Hong 2003). The role of reactive astrocytosis in PD is less clear, but it may also have a central role in the neurodegenerative process, as activated astrocytes secrete pro-inflammatory molecules such as IL-6 and prostaglandins, and anti-inflammatory molecules such as neurotrophic factors like BDNF or GDNF (McGeer & McGeer 2008). These pro-inflammatory cytokines can be directly toxic, but also induce indirect cytotoxic effects through the upregulation of microglial inducible nitric oxide synthase (iNOS) which can catalyse the production of NO (Chao *et al.* 1992, Hunot *et al.* 1996). Activated microglia have also been shown to express the ROS-generating enzyme NADPH oxidase, producing superoxide (Shimohama *et al.* 2000, Wu *et al.* 2005), which can then react with NO to form damaging peroxynitrite (Hirsch *et al.* 2003). Microglial produced ROS are therefore thought to contribute to oxidative damage in PD.

It has been proposed that a self-sustaining cycle of neuroinflammation and neurodegeneration occurs in PD (Tansey & Goldberg 2010). Supporting this are findings that activated microglia are detected in human and non-human primate brains many years after MPTP exposure (McGeer *et al.* 2003, Langston *et al.* 1999). Neuromelanin released from degenerating dopaminergic neurons is usually phagocytosed by microglia, but it also has the potential to directly activate microglia and induce production of cytotoxic factors and ROS, leading to further neurodegeneration of the SNc (Zhang *et al.* 2011). Therefore, gliosis and inflammation are becoming increasingly accepted as central aspects of PD pathology (Whitton 2009), and therapeutically reducing inflammation may provide neuroprotection. Microglia can adopt an anti-inflammatory M2-like phenotype with beneficial properties such as clearing pathogens, debris and toxins, releasing neurotrophic factors and playing a role in repair and neurogenesis (Kreutzberg 1996, Nimmerjahn *et al.* 2005, Takeuchi & Suzumura 2014). Additionally, blocking microglial activation has been demonstrated to be neuroprotective in a mouse model of PD (Wu *et al.* 2002), indicating that reducing or altering microglial activation in PD is likely to have multiple beneficial consequences on the progression of disease pathology. It is still under debate however, whether activated microglia are responsible for the initial degeneration of neurons in PD, or are simply responding to cellular distress and α -synuclein aggregation (Graeber *et al.* 2011, Liu & Hong 2003, McGeer & McGeer 2008, Rodriguez-Pallares *et al.* 2007).

1.3 Modelling Parkinson's disease in animals

Many animal models of PD have been developed over the years, and are essential for developing suitable therapeutic treatments, as well as discovering more about the mechanisms underlying PD pathogenesis. Rare familial cases of PD have provided insights into the underlying molecular mechanisms involved in the pathogenesis of PD and demonstrated that genetic and sporadic forms of

PD are likely to share similar pathogenic mechanisms, aiding the identification of possible therapeutic targets. However, PD is a multi-faceted disease, and no single model has yet replicated all of its hallmarks.

1.3.1 Genetic models

Only around 10% of PD cases are linked to known genetic mutations (Dauer & Przedborski 2003), and genetic models are relatively new compared to toxin-based models of PD. The key mutations that have been developed in animal models are α -synuclein, LRRK2, PINK1, Parkin, DJ-1 and UCH-L1. Identifying the effects of these gene mutations has demonstrated common disease mechanisms shared with sporadic PD, including mitochondrial dysfunction (α -synuclein, PINK1, DJ-1, LRRK2) and altered protein handling (α -synuclein, parkin, UCH-L1) (Duty & Jenner 2011), as illustrated in Figure 1.3 previously. It is hoped that detection of the early disease processes that occur in these genetic models could reveal biomarkers of disease progression, as well as providing information about potential therapeutic targets and disease pathogenesis.

Three point mutations in the α -synuclein (SNCA) gene have been found to be responsible for familial PD (A53T, A30P, and E46K of the SNCA gene), and duplication or triplication also causes PD (Singleton *et al.* 2003). However, single mutations in α -synuclein have failed to translate well into transgenic models of PD (Chesselet 2008). Although models with knockout and overexpression of α -synuclein have demonstrated pathological changes including α -synuclein positive protein inclusions, mitochondrial abnormalities, reactive gliosis, loss of motor neurons and functional abnormalities in the nigrostriatal system, none have consistently demonstrated a loss of nigral dopaminergic neurons, and hence, do not replicate this key aspect of PD pathology (Dawson *et al.* 2010, Giasson *et al.* 2002, Matsuoka *et al.* 2001, Chesselet 2008). Transgenic models carrying LRRK2 mutations similarly develop protein inclusions, dopaminergic dysfunction and behavioural abnormalities, but again fail to demonstrate nigrostriatal neurodegeneration (Li *et al.* 2009, Li *et al.* 2010). Furthermore, knockout of the autosomal-recessive genes PINK1, parkin or DJ-1 in models have demonstrated mitochondrial dysfunction and nigrostriatal signalling abnormalities, but display no nigral dopaminergic neuronal degeneration (Goldberg *et al.* 2005, Chen & Feany 2005, Kitada *et al.* 2007, Li *et al.* 2009, Itier *et al.* 2003). Even triple knockout mice with silencing of parkin, DJ-1 and PINK1 do not demonstrate dopaminergic neuronal loss (Kitada *et al.* 2009), suggesting that an additional impact of epigenetic factors such as environmental triggers or ageing contribute to nigrostriatal degeneration in PD. Indeed, mitochondrial toxins can exacerbate neurodegeneration in *Drosophila* and *C. elegans* models with LRRK2 mutations (Ng *et al.* 2009, Saha *et al.* 2009). Additionally, it has been suggested that compensatory mechanisms may mask the effects of knocked-out or over-expressed genes in the

developing embryo (Dawson et al. 2010, Jackson-Lewis *et al.* 2012). These genetic models likely represent only the earliest pathological changes in PD that might occur, and since nigral neurodegeneration is yet to be demonstrated, they are not useful for testing potential neuroprotective therapies at present. The only genetic model available that does demonstrate degeneration at present is the MitoPark mouse model, in which the selective removal of the mitochondrial transcription factor Tfam causes severe mitochondrial dysfunction; the model demonstrates a progressive loss of midbrain dopaminergic neurons and behavioural deficits that respond to L-DOPA treatment (Galter *et al.* 2010).

The use of viral vectors to deliver transgenes that drive overexpression of α -synuclein (wild-type and mutant) in rodents and non-human primates has been explored in recent years, and moderate degeneration of the nigrostriatal pathway has been demonstrated alongside behavioural motor impairments (Oliveras-Salv   *et al.* 2013, Chung *et al.* 2009, Decressac *et al.* 2012, Yamada *et al.* 2004, Kirik *et al.* 2003). However, this has not been a consistent finding and further optimisation of the types of viral vectors and methods used are likely needed before they can be used to reliably test therapies.

1.3.2 Toxin models

Toxin-based models have been used to induce specific dopaminergic neurodegeneration alongside motor behavioural changes, providing valuable models of disease pathology for the testing of potentially neuroprotective and antiparkinsonian therapies. Neurotoxin-based models of PD have also been invaluable for investigating the treatment of motor symptoms and complications such as LIDs that arise from long-term dopamine replacement therapy and disease progression. These toxin-based models are also helpful in identifying the molecular and cellular processes leading to dopaminergic cell death, and the actions of drugs on these processes. A number of different toxins have been utilised to induce nigrostriatal degeneration via various pathogenic mechanisms associated with PD (see Table 1.3). Toxins such as 6-hydroxydopamine (6-OHDA) and paraquat can produce OS, MPTP, 6-OHDA and rotenone can induce mitochondrial dysfunction, lipopolysaccharide (LPS) generates neuroinflammation and oxidative stress, glutamate, quinolinic acid and ibotenic acid all cause excitotoxicity, and proteasomal inhibition can be induced by compounds such as lactacystin, PSI and epoxomicin (Dexter & Jenner 2013). Acute pharmacological models such as reserpine and haloperidol-treated rodents have been used to transiently model the motor symptoms of akinesia and catalepsy respectively, and can be reversed by treatment with L-DOPA (Carlsson *et al.* 1957, Duty & Jenner 2011). However, these pharmacological models are limited to the testing of symptomatic therapies as no degeneration of the nigrostriatal pathway occurs.

The classical MPTP model in non-human primates and the 6-OHDA model in rodents are currently used as the 'gold standard' for testing symptomatic therapies due to their quantifiable motor deficits. This makes them useful for pharmacological screening of compounds that can act on the dopaminergic system, and they have had relative success in predicting clinical efficacy. The MPTP model is able to replicate many of the pathological and behavioural hallmarks of PD, particularly in monkeys (Jackson-Lewis *et al.* 1995, Langston *et al.* 1983). Mice are also susceptible to the toxin, although they don't develop the progressive motor symptoms that monkeys do (Przedborski *et al.* 2001). MPTP can be administered systemically as it crosses the blood-brain barrier (BBB), and it is a rapidly acting and selective toxin when administered acutely, causing dopaminergic neurons to degenerate from 12 hours post-exposure (Jackson-Lewis *et al.* 1995). In the brain, MPTP is metabolised by astrocytes to its active metabolite, MPP+, by the enzyme MAO-B. MPP+ then acts as a substrate for the dopamine transporter (DAT), accumulating inside dopaminergic neurons and causing mitochondrial complex I defects, resulting in mitochondrial dysfunction, OS, microglial activation and selective death (Javitch *et al.* 1985, Kurkowska-Jastrzębska *et al.* 1999). 6-OHDA is selective for catecholaminergic neurons and, in contrast to MPTP, is administered locally as it is unable to cross the BBB. 6-OHDA enters the cell via the DAT, where it can auto-oxidise in the cytosol, producing intracellular OS to induce damage (Graham 1978, Sachs & Jonsson 1975). First demonstrated by Ungerstedt (1968), locally administered 6-OHDA to the SNc, medial forebrain bundle (MFB) or striatum induces a significant and selective loss of nigral dopaminergic neurons and a subsequent loss of striatal dopaminergic nerve terminals. 6-OHDA induces rapid cell death, with extensive cell loss seen by 24h post-exposure (Jeon *et al.* 1995). The 6-OHDA model induces damage primarily via OS, but it also demonstrates other features of PD pathology such as iron accumulation, mitochondrial dysfunction, and microglial activation (Sachs & Jonsson 1975, Akiyama & McGeer 1989). However, both models fail to reproduce all pathological aspects of PD, particularly LB-like cytoplasmic protein inclusions or aggregations (Halliday *et al.* 2009, Schober 2004). Importantly, there has been a lack of translation of neuroprotective and restorative therapies that have proved effective in these classical models of PD into the clinic, likely due to their failure to accurately replicate some of the key features of the disease.

Models using toxins such as 6-OHDA and MPTP that destroy nigrostriatal dopaminergic neurons also induce microglial activation, which exacerbates neuronal damage. The role of inflammation in PD has been further investigated using LPS, a potent inducer of inflammation in the brain (discussed later in methods 2.1). Studies have demonstrated that although LPS is not directly toxic to neurons, it induces an innate inflammatory reaction associated with proinflammatory cytokine formation and increased ROS and nitrative stress, leading to neuronal death (Herrera *et al.*

2000). Intranigral injection of LPS induces microglial activation and degeneration of the nigrostriatal dopaminergic system (Castaño *et al.* 1998, Herrera *et al.* 2000, Machado *et al.* 2011) and can cause measurable motor deficits (Tanaka *et al.* 2013, Zhou *et al.* 2012). Although the LPS model is controversial as it only reproduces one aspect of PD pathology which may not be the primary mediator of neurodegeneration, it does reproduce the neuroinflammation and degeneration associated with the disease and so provides a platform for the testing of anti-inflammatory compounds. LPS can rapidly and robustly activate microglia when given intranigally, however the large degree of astrogliosis that also occurs in response to LPS is not seen as strongly in human PD, suggesting the inflammatory reaction in the LPS model is much more rapid and complete than that seen in post-mortem PD brains (Duty and Jenner, 2010).

Table 1.3: Toxins used in animal models and primary pathogenic mechanisms induced

Pathogenic mechanism induced	Toxin used
Oxidative and nitrative stress	6-OHDA, paraquat, LPS
Neuroinflammation	LPS
Mitochondrial dysfunction	Rotenone, 6-OHDA, MPTP/MPP+
Excitotoxicity	glutamate, ibotenic acid
Proteasome dysfunction	PSI, lactacystin, epoxomycin

Adapted from Dexter and Jenner, 2013.

Pesticides such as paraquat and rotenone are examples of environmental toxins that have been used to model PD in rodents, and exposure to these compounds is a risk factor for PD (Tanner *et al.* 2011, Di Monte *et al.* 2002). Pesticides can be administered peripherally as they easily cross the BBB, and mediate their toxicity through OS and mitochondrial dysfunction. Paraquat and rotenone induce both fibrillation and aggregation of α -synuclein and induce nigrostriatal damage in rodents (Tanner *et al.* 2011, Betarbet *et al.* 2000, Manning-Bog *et al.* 2002). Rotenone toxicity has also demonstrated other hallmarks of PD including inflammation, LB-like cytoplasmic inclusions in the SN and motor deficits (Betarbet *et al.* 2000, Greenamyre *et al.* 2010). However, there are some key limitations of using pesticides for modelling PD as inconsistencies in lesion production have been reported in these models, with some studies demonstrating a failure to induce substantial dopaminergic neurodegeneration, non-specific lesions outside of the nigrostriatal system and strong systemic toxicity at higher doses that can cause death (Wu & Johnson 2011, Blandini & Armentero 2012, McCormack *et al.* 2002, Thiruchelvam *et al.* 2000).

Due to the involvement of UPS impairment in PD (see section 1.2.3), proteasomal inhibitors such as lactacystin (discussed later in 2.2) and PSI have been used to provide progressive and reproducible models of PD in rodents. These models have been shown to recapitulate many key pathological features of PD when administered directly into the SNc or MFB in rodents, with proteasomal dysfunction leading to phosphorylated α -synuclein immunopositive protein aggregates/inclusions, localised glial activation, iron accumulation, nigral dopaminergic neurodegeneration and motor and non-motor deficits (Xie *et al.* 2010, Bentea *et al.* 2015, Zhu *et al.* 2007, Vernon *et al.* 2010, Vernon *et al.* 2011, McNaught *et al.* 2002b). Additionally, the slower progression of neurodegeneration in these models means that pharmacological treatments that can potentially reverse or reduce dopaminergic neuronal cell death are more likely to succeed (McNaught *et al.*, 2002). Systemic administration of proteasome inhibitors such as PSI has been demonstrated to induce dopaminergic cell death too (McNaught 2004), although there is some controversy

surrounding this model as others have been unable to reproduce it (Bové *et al.* 2006, Kordower *et al.* 2006, Manning-Bog *et al.* 2002); this could relate to the fact that PSI is unstable in solution and hence may have degraded prior to systemic administration.

For toxins such as 6-OHDA and lactacystin which are injected at their site of action, the location of toxin administration has an impact on the speed and degree of neurodegeneration, and can be adapted to model different stages of PD neuropathology and symptomatology. These toxins generally have to be introduced directly into the SNc, striatum or medial forebrain bundle (MFB) in order to induce localised degeneration mirroring that seen in PD. Lesioning can be unilateral or bilateral, depending on the types of behavioural deficits required. Lesions of the MFB can produce extensive dopaminergic neuronal loss in the SNc depending on the dose of toxin used due to the simultaneous spreading of the toxin towards both the striatum and SNc, as well as a substantial loss of neurons in the ventral tegmental area (VTA) (Deumens *et al.* 2002). This degree of nigrostriatal lesion is almost complete; perhaps best modelling the advanced stages of PD, so more selective lesions have been used targeting the SNc or striatum. Lesions of the SNc lead to a pattern of cell loss reflecting that seen in human PD, with a more gradual loss of striatal dopamine. The degree of cell loss in the SNc is dependent on the type and concentration of toxin used, modelling mid-stage to advanced disease (Deumens *et al.* 2002). Additionally, due to the more selective destruction of nigral dopaminergic neurons, measurable parkinsonian behavioural deficits often develop (Carman *et al.* 1991). Cell death following 6-OHDA injection into the MFB or SNc is rapid, with neurons beginning to actively degenerate within 12 hours of exposure and producing a 60% reduction in striatal dopamine by day 3, a similar rate to that seen in the acute MPTP model (Faull & Laverty 1969, Schober 2004, Jackson-Lewis *et al.* 1995). In contrast, intrastriatal administration of toxin generally produces a more gradual retrograde induced degeneration and a partial lesion over a number of weeks, mirroring the earlier stages of PD (Deumens *et al.* 2002, Lee *et al.* 1996), although the concentration given and the location of administration in the striatum all contribute to the degree of lesion that develops.

Importantly, although no toxin model replicates Parkinson's pathology perfectly, individually they all mirror certain aspects of the disease. This makes toxin-based models ideal for testing therapies and helps to reveal the possible effects of drugs on aspects of disease pathogenesis, but highlights the importance of confirming the effects of therapies in more than one model of the disease. The lactacystin model was chosen for the testing of potentially antiparkinsonian and neuroprotective therapies in this study due to its ability to replicate the key pathogenic features of PD: protein accumulation and aggregation, localised inflammation, progressive dopaminergic neuronal cell death and the development of motor deficits. Additionally, the LPS model was used as a model of

neuroinflammatory-induced neurodegeneration to provide a platform to test the possible anti-inflammatory mechanisms of neuroprotection by compounds.

1.4 Current therapies for Parkinson's Disease

Current PD therapies mainly focus on motor symptom control through the replacement of lost striatal dopamine. Treatment with L-DOPA, the dopamine precursor, has become the "gold-standard" therapy for PD since its introduction in the 1960's (Katzenschlager & Lees 2002, Poewe *et al.* 2010). Unlike dopamine itself, L-DOPA can cross the BBB and once in the brain, it is converted by the enzyme DOPA decarboxylase into dopamine by remaining dopaminergic neurons (Whitfield *et al.* 2014). However, L-DOPA can also be converted to dopamine in the peripheral nervous system, so co-treatment with a peripherally-acting DOPA decarboxylase inhibitor such as carbidopa can increase the amount of L-DOPA entering the brain, making more available to the remaining neurons for conversion to dopamine (Whitfield *et al.* 2014). Many patients experience side effects such as L-DOPA-induced dyskinesias (LIDs), fluctuations in response to medications ("on-off" symptoms) and psychological or behavioural disturbances such as hallucinations and impulse control disorders (Obeso *et al.* 2000, Stacy 2008). Treatment with dopamine receptor agonists such as pramipexole and ropinirole have also demonstrated antiparkinsonian efficacy and have longer half-lives than L-DOPA, helping to limit the development of dyskinesias (Poewe *et al.* 2010, Buck & Ferger 2010, Blandini & Armentero 2014). The additional use of enzyme inhibitors such as catecholamine o-methyl transferase (COMT) inhibitors which inhibit L-DOPA metabolism, and MAO-B inhibitors which prevent the degradation of dopamine, have been added in more recent years to augment the remaining dopaminergic neurotransmission and reduce the required doses of L-DOPA (Dexter & Jenner 2013). Early on, these treatments can provide a substantial relief of PD motor symptoms, however, due to the continuing degeneration of dopaminergic neurons, long-term (>5 years) administration can result in adverse motor effects associated with a loss of response and escalating drug doses (Müller 2002). Importantly, dopamine-replacement therapies fail to treat many of the non-motor symptoms of PD, as the degeneration of non-dopaminergic systems are also thought to be involved. Treatment of non-motor symptoms is limited, but has included the use of anticholinergic drugs such as Benztropine to treat autonomic dysfunction as well as tremor, and serotonergic drugs such as the serotonin reuptake inhibitor Fluoxetine to treat depression, anxiety and apathy (Rascol *et al.* 2002, Seppi *et al.* 2011). Additionally, treatment with the NMDA receptor antagonist memantine has been used in both Alzheimer's and PD as a treatment for dementia (Buck & Ferger 2010, Aarsland *et al.* 2009), and the weak NMDA receptor antagonist amantadine has demonstrated efficacy in treating LIDs (Wolf *et al.* 2010).

DBS can be used to treat patients who suffer with complications such as LIDs and on-off effects due to the medical management of their motor symptoms, and has mainly supplanted surgical ablation of nuclei such as the STN, which can result in permanent neurological deficits (Guridi & Obeso 2001, Alvarez *et al.* 2009). DBS uses implanted electrodes to stimulate regions of the BG, particularly the STN or GPi, in order to directly modulate abnormal activity and improve symptomatology, although the exact mechanism of DBS is still being investigated. DBS can improve the motor features and some non-motor features of PD, partly due to the effects of stimulation, but also due to the reduction in drug treatment and the associated adverse effects (Fasano *et al.* 2012). Although it is effective in many PD patients, a large number of those with PD are not eligible for DBS depending on symptoms, age and stage of disease, and the invasive nature and expense of the surgery means there is a clear need to find drugs that can mimic the effects of DBS in the BG without the necessary surgical intervention.

Currently, there is a mainly symptomatic approach to treating motor symptoms (with little effect on non-motor features), and although dopamine replacement therapies are effective at reversing motor symptoms for a number of years, their long-term use is associated with adverse and debilitating side-effects, and there is a clear unmet need for agents that can both relieve symptoms and slow or halt the progression of dopaminergic neuronal death in PD. There is obviously a need for sensitive biomarkers of PD in order to have the possibility of diagnosis at an early stage in the disease course, when neuroprotective therapies are likely to have most efficacy as many functional neurons still remain and neurodegenerative mechanisms could be attenuated before they cause extensive damage. Given the limitations of dopamine replacement therapies in PD, a key focus has been to investigate alternative non-dopaminergic drugs that are effective at novel targets, with the potential to provide both symptomatic relief and neuroprotection.

1.5 Metabotropic glutamate receptors

1.5.1 Introduction

In PD, deficits in levels of striatal dopamine cause a signalling imbalance between direct and indirect pathways of the BG, and in particular, an overactivity of glutamatergic STN outputs. Given the central role of glutamate in both the pathogenesis and pathophysiology of PD (as discussed in section 1.2.2), attenuation of excess glutamatergic activity represents a possible pharmacotherapeutic target to provide both antiparkinsonian effects via normalisation of BG signalling and neuroprotection via a reduction in excitotoxic synaptic levels of glutamate. Inhibiting iGlu receptors, particularly NMDA receptors, with antagonists to treat neurodegeneration was previously an attractive target due to the

involvement of glutamate excitotoxicity in many diseases (Lau & Tymianski 2010). However, this approach mainly failed due to narrow therapeutic windows and marked CNS side effects of iGlu receptor antagonists such as sedation and psychotomimetic effects in both animal models and humans, likely due to the widespread non-selective blockade of NMDA receptors (Montastruc *et al.* 1992, Muir 2006, O'Neill & Siemers 2002). To avoid this broad antagonism of NMDA receptors, selective targeting of NMDA receptor subtypes has been explored and been shown to have antiparkinsonian efficacy in animal models of PD (Nash *et al.* 1999, Steece-Collier *et al.* 2000, Nash *et al.* 2000), although no efficacy was shown in a clinical trial with MK-0657, a selective NR2B-subunit targeting NMDA receptor antagonist (Addy *et al.* 2009). In more recent years, the targeting of mGlu receptors, particularly those in the group III subclass, has become a therapeutic focus. Functionally, mGlu receptors exhibit subtler effects than iGlu receptors, allowing modulation of neuronal excitability via second messenger signalling pathways. Additionally, the regional distribution of mGlu receptors in the brain allows a more targeted modulation of glutamatergic activity. By virtue of these receptors' distribution and function, compounds targeting mGlu receptors are predicted to provide more favourable safety and tolerability profiles, yielding symptomatic improvements combined with neuroprotective properties through the modulation of multiple neurodegenerative mechanisms (Hovelsø *et al.* 2012).

1.5.2 Metabotropic glutamate receptor subtypes and their signalling transduction

There are eight mGlu receptor subtypes divided into 3 groups based on their sequence homology, signal transduction pathways and ligand binding patterns (Pin & Acher 2002). Group I mGlu receptors (mGlu1 and 5) are expressed postsynaptically, couple to Gαq and positively modulate neuronal excitability through activation of phospholipase C (PLC) leading to an increase in intracellular calcium (iCa^{2+}). Group II receptor subtypes (mGlu2 and 3) and group III receptor subtypes (mGlu4, 6, 7 and 8) are mainly localised pre-synaptically, classically couple to Gαi/o and negatively modulate neuronal excitability (Conn & Pin 1997). Activation of group I mGlu receptors can therefore enhance postsynaptic excitability, exacerbating neuronal damage. In contrast, group II and III activation can reduce glutamatergic signalling and dampen neuronal excitability, giving them potential neuroprotective properties (Nicoletti *et al.* 1996). Splice variants exist for many of the mGlu receptors and may lead to differences in their protein interaction characteristics and receptor function.

Both group II and III mGlu receptor stimulation leads to an inhibition of adenylyl cyclase (AC), activation of K⁺ channels and inhibition of presynaptic voltage-gated calcium channels by the βγ subunits of G-proteins, thus reducing Ca²⁺ entry into the cell and modulating neurotransmitter release from the synapse (Benarroch 2008, Hovelsø *et al.* 2012, Niswender & Conn 2010). Group II and III

subtypes have also been shown to couple to other signalling pathways such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI-3-K) pathways to provide neuroprotection via suppression of pro-apoptotic pathways (Iacovelli *et al.* 2002). Activation of group III subtypes typically reduces cyclic AMP (cAMP) levels; however, mGlu7 activation also inhibits Ca^{2+} channels through stimulation of PLC or protein kinase C (PKC) activity (Pelkey *et al.* 2005, Perroy *et al.* 2000). Group III mGlu receptors are distributed throughout different regions of the CNS, and in particular the BG (see Table 1.4), with the exception of mGlu6 which is limited to the retina and is therefore not predicted to be a therapeutic target in neurodegenerative disease (Nakajima *et al.* 1993). Group III mGlu receptors are predominantly located in axonal active zones and inhibit the release of neurotransmitters, likely acting as autoreceptors on glutamatergic terminals and heteroreceptors on GABAergic and other neurotransmitter terminals (Cartmell & Schoepp 2000, Ferraguti & Shigemoto 2006). At the subcellular level, mGlu4 and 8 are mainly found perisynaptically (commonly on presynaptic terminal), whilst mGlu7 is found clustered centrally on the presynaptic terminal where synaptic vesicles fuse with the membrane (Palucha & Pilc 2007, Shigemoto *et al.* 1997). The central localisation of mGlu7 at the synapse and its low affinity for glutamate is indicative of its direct involvement in regulating neurotransmitter release, acting as a low pass filter inhibiting synapses that fire above certain frequencies (Shigemoto *et al.* 1996, Schoepp 2001). The group III mGlu receptor subtypes present in the brain (mGlu4, 7 and 8) are in a prime synaptic location to modulate neuronal excitability, and recent advances in the compounds available that selectively target these receptor subtypes makes them an attractive therapeutic focus for PD.

Table 1.4: Classification and key features of mGlu receptors

Group	Receptor subtype and splice variants	CNS cellular expression	Synaptic localisation	Signalling pathways
I	mGlu1 receptor a-f, taste mGlu1 mGlu5 receptor a, b	Neurons, taste buds (taste mGlu1) Neurons, astrocytes	Mainly postsynaptic	PLC stimulation, AC stimulation, MAPK phosphorylation
II	mGlu2 receptor mGlu3 receptor, variants GRM3A2, A4, A2A3	Neurons Neurons, astrocytes	Pre- and postsynaptic	AC inhibition, K ⁺ channel activation, Ca ²⁺ channel inhibition
III	mGlu6 receptor a-c	Retina (ON-bipolar retinal cells)	Postsynaptic	cGMP PDE stimulation
	mGlu4 receptor, taste mGlu4	Neurons (widespread expression), astrocytes, microglia. Taste buds (taste mGlu4)	Mainly presynaptic	AC inhibition, K ⁺ channel activation, Ca ²⁺ channel inhibition
	mGlu7 receptor a-e	Neurons (widespread expression), astrocytes.	Presynaptic (active zone)	
	mGlu8 receptor a-c	Neurons (more restricted and lower expression than mGlu4/7), astrocytes, microglia	Mainly presynaptic	

Table includes both rodent and human mGlu receptor splice variants and rodent cellular expression. PLC: phospholipase C, AC: adenylyl cyclase, MAPK: mitogen-activated protein kinase, cGMP PDE: cyclic guanosine monophosphate phosphodiesterase. Table adapted from Niswender and Conn (2010).

1.5.3 Targeting mGlu receptors

Group II and III agonists and group I antagonists can reduce glutamatergic signalling and dampen neuronal excitability giving them potential neuroprotective and antiparkinsonian properties (Nicoletti *et al.* 1996, Schoepp *et al.* 1999). However, specific subtypes seem to be more effective than others at mediating this effect. Much research has helped determine specific receptor subtypes as potential therapeutic targets in cognitive disturbances such as anxiety and psychosis (Palucha *et al.* 2004, Galici *et al.* 2006, Stachowicz *et al.* 2004). With regards to PD, antagonists or negative allosteric modulators targeting group I mGlu receptors, particularly the mGlu5 subtype, have demonstrated promise for the treatment of LIDs in animal models, as well as a small degree of nigrostriatal neuroprotection (Rylander *et al.* 2010, Dekundy *et al.* 2006, Vernon *et al.* 2007a, Yamamoto & Soghomonian 2009). However, group I mGlu receptors are thought to play a modulatory role in long-term potentiation (LTP), with mGlu1 receptor deficient mice demonstrating reduced LTP and impaired learning (Aiba *et al.* 1994), suggesting that inhibiting these receptors may have unwanted effects on memory. A selection of ligands targeting group II mGlu receptors have also been investigated, but none have yet been developed that are specific for the mGlu3 receptor subtype alone. These would be particularly interesting to study as ligands that activate group II receptors non-selectively are found to be neuroprotective, whereas ligands selective for the mGlu2 subtype may actually be neurotoxic, suggesting that it is the mGlu3 subtype that has neuroprotective properties (Corti *et al.* 2007). Unfortunately however, mGlu3 receptors appear to desensitise rapidly in response to prolonged or repeated agonist exposure, potentially limiting the long-term therapeutic efficacy of mGlu3 receptor agonists/enhancers (Iacovelli *et al.* 2013, Iacovelli *et al.* 2009). An abundance of research over the past 19 years indicates that activation of group III mGlu receptors in the CNS is neuroprotective in a variety of cell types and models of neurodegeneration. Activation of group III mGlu receptors with subtype-selective ligands have been suggested to have efficacy in a broad range of conditions including epilepsy, anxiety, depression, addiction, schizophrenia, pain, stroke, neuroinflammation and neurodegeneration (Hovelsø *et al.* 2012). There are a number of subtype-selective ligands available for group III at present, making the effects of activating these subtypes far easier to study pharmacologically, and is the focus of this thesis.

1.5.4 Expression and distribution of group III mGlu receptors

The distribution and function of group III mGlu receptors in key areas of the BG make them promising candidates to target in PD for treating motor symptoms. Additionally, targeting specific mGlu receptor subtypes on selected cell populations (see Table 1.4 above) in the BG has the potential to provide therapeutic benefit. Expression of the group III mGlu receptors has been less extensively studied than groups I and II and still requires detailed mapping in health and disease states. However, *in-situ*

hybridisation studies have demonstrated that group III mGlu receptors (excluding the mGlu6 receptor) are expressed throughout different regions of the rodent BG (Messenger *et al.* 2002). Group III mGlu receptor localisation in the BG is illustrated in Figure 1.5. In the rodent BG and regions associated with PD, mGlu4 receptor mRNA is strongly expressed in the ventral thalamus, nucleus accumbens and striatum, with lower expression in the SNc and SNr (Messenger *et al.* 2002). Positive immunohistochemical staining for mGlu4 has been demonstrated strongly in striatopallidal projections, as well as in the SNr and striatum (Bradley *et al.* 1999, Corti *et al.* 2002). Outside of the BG, mGlu4 receptors have been found to be strongly expressed in the cerebellum, hippocampus (pre- and post-synaptically) and olfactory bulb (Corti *et al.* 2002, Messenger *et al.* 2002, Testa *et al.* 1994).

In the rodent BG, mGlu7 receptor protein immunoreactivity is detected presynaptically on corticostriatal, striatonigral, striatopallidal and subthalamonigral projections (Kosinski *et al.* 1999). In addition, very low levels of mGlu7 receptors have been detected postsynaptically in the GP and striatum (Bradley *et al.* 1999, Kosinski *et al.* 1999), although electrophysiological evidence suggests no functional post-synaptic action occurs here (Valenti *et al.* 2003). Moderate to high mRNA expression and immunopositive staining has been demonstrated in the striatum, GP and SNr, and lower levels are detected in the SNc and STN (Kosinski *et al.* 1999). Moderate mRNA expression has also been demonstrated in the nucleus accumbens and premotor cortex (Messenger *et al.* 2002). Outside of the BG, mGlu7 receptors are also found presynaptically distributed throughout the hippocampal formation (Bradley *et al.* 1996). In comparison, mGlu8 receptor expression is less well characterised, with mRNA found to be widely expressed throughout the CNS in rodents and humans (Robbins *et al.* 2007) but at lower levels than mGlu4 or 7 receptors. Moderate mRNA expression has been detected in the striatum, nucleus accumbens and premotor cortex, the SNc and STN (Messenger *et al.* 2002). Additionally, moderate to high levels of mGlu8 immunoreactivity have been detected in the striatum and SNr (Austin *et al.* 2010, Duty 2010). This indicates that mGlu8 receptors may be present presynaptically on corticostriatal, striatonigral and subthalamonigral projections (see Figure 1.5). In mice, mGlu8 receptor activation has anticonvulsant/anti-epileptic properties (Folbergrová *et al.* 2008), as well as anxiolytic properties (Duvoisin *et al.* 2010).

It is likely that there are compensatory or contributory changes in mGlu receptor expression in different neurodegenerative pathologies due to altered glutamate handling, for example, pathological changes in PD models affect the expression of different mGlu receptor subtypes in different regions of the BG, possibly worsening the pathophysiology. Indeed, MFB 6-OHDA lesioned rats demonstrate a reduction in mGlu4 receptor expression in the striatum (by 13.8%) and the premotor cortex (by 15.8%), likely due to the increase in extracellular glutamate levels following lesioning (Messenger *et al.* 2002). The varying expression of mGlu receptor subtypes on different

neuronal populations is likely to have both functional and pathological implications, so targeting specific receptor subtypes on selected neuronal populations has the potential to be utilised therapeutically. However, the functionality of these receptor subtypes at their different regional locations will play a key role in the efficacy of therapeutics specifically targeting them. Given its glutamatergic nature and the efficacy of STN-DBS for the treatment of PD (see sections 1.1.6 and 1.4), the use of drugs targeting excess glutamatergic activity from the STN may provide the possibility of mimicking the effects of DBS without the need for invasive surgical intervention. Additionally, as the overactivity of glutamatergic feedback projections from the STN to the SNc is thought to enhance the rate of neuronal cell loss via excitotoxicity, normalising this pathway has the potential to be neuroprotective in PD (Rodriguez 1998). Ligands selective for group III mGlu receptors have the potential to normalise activity within the BG by modulating activity in the indirect pathway, and are therefore predicted to alleviate clinical symptoms of PD (Conn *et al.* 2005, Rouse *et al.* 2000). Additionally, targeting overactivity in the indirect pathway has the potential to reduce excitotoxicity, providing neuroprotection against further SNc degeneration. An important aspect to consider is the counteractive presence of group III mGlu receptors on GABAergic striatonigral terminals in the direct pathway, which if activated have the potential to exacerbate the imbalance in signalling in the BG, potentially reversing the benefits of inhibiting glutamate release from STN projection terminals and theoretically worsening symptoms. Indeed, electrophysiological studies have shown that the broad group III mGlu receptor agonist L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4) can inhibit striatal-evoked inhibitory post-synaptic potentials (IPSCs) in the SNr (Wittmann *et al.* 2001). However, the evidence to date suggests no detrimental effect of activating these receptors therapeutically in PD, as in conditions of dopamine depletion L-AP4 no longer inhibits striatal-evoked IPSCs in the SNr, but still maintains its inhibitory effect on STN-evoked excitatory post-synaptic potentials (EPSCs) in the SNr (Wittmann *et al.* 2001). Additionally, many models of PD have demonstrated symptomatic improvements following treatment with group III mGlu receptors (discussed in section 1.6). This evidence suggests that in the parkinsonian state, the beneficial effects of group III mGlu receptors on subthalamonigral terminals may outweigh the potentially detrimental effects on striatonigral terminals.

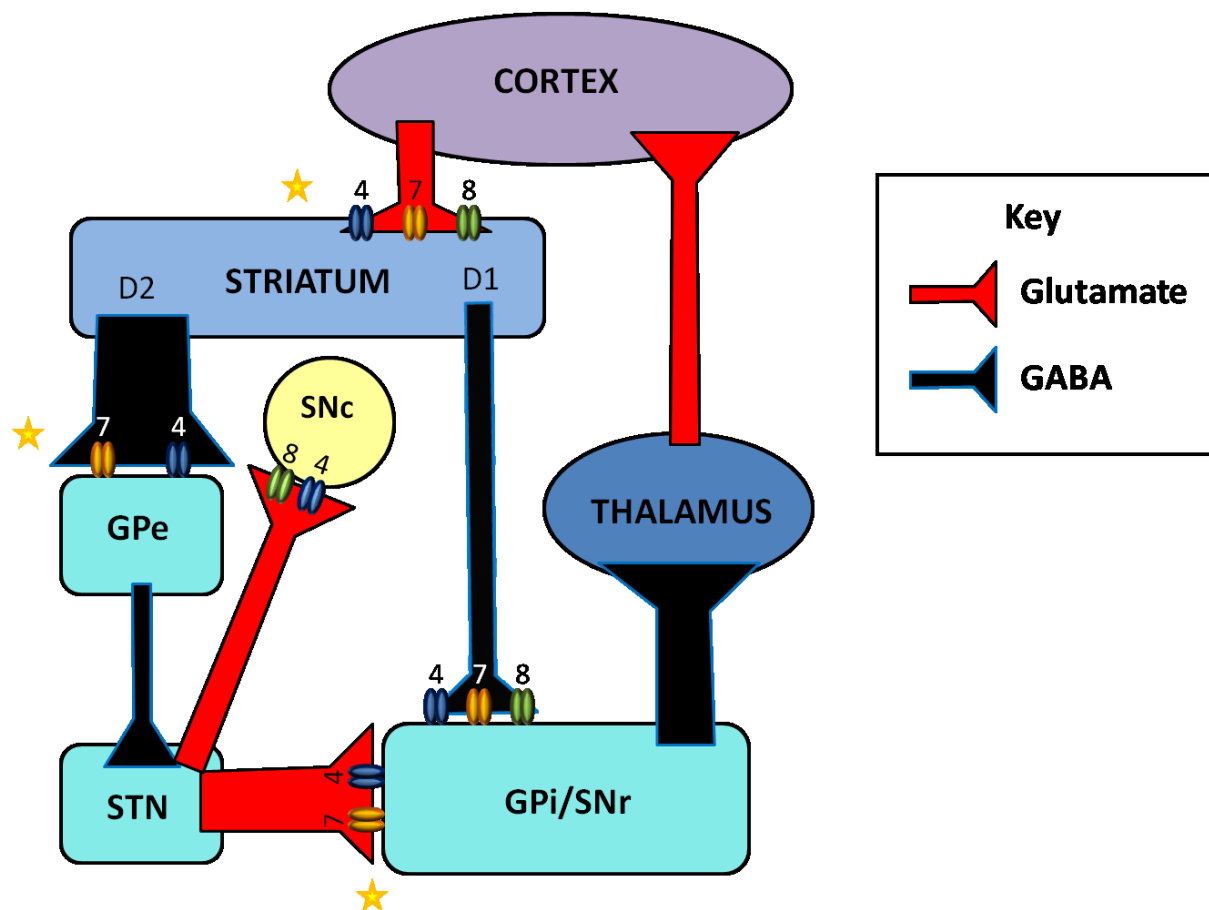


Figure 1.5: The group III mGlu receptor subtypes and their neuronal localisation in the BG: potential therapeutic targets in Parkinson's disease. The receptor subtypes mGlu4, 7 and 8 are all present presynaptically on corticostriatal neurons. In the indirect pathway, mGlu4 and 7 receptors are present presynaptically on overactive striatopallidal and subthalamonigral (SNr) projections. Additionally, mGlu4 and 8 are present on overactive terminals at the subthalamonigral (SNc) synapse. In the direct pathway, mGlu4, 7 and 8 are all present presynaptically on striatonigral projections (Amalric *et al.* 2013, Duty 2010). Wider connections indicate increased activity in PD, whereas a narrower width indicates reduced activity. Yellow stars indicate key synapses for targeting group III mGlu receptors to modulate overactive signalling.

1.5.5 Group III mGlu receptor selective ligand discovery and development

The availability of subtype-selective group III mGlu receptor compounds has dramatically progressed in recent years, allowing more in-depth research into the properties of these receptors and their roles in health and disease. Classical orthosteric agonists have often suffered from a lack of BBB penetration or subtype-selectivity (Amalric *et al.* 2013) so ligands targeting the same receptors with novel mechanisms of action have been pursued in recent years. Positive and negative allosteric modulators (PAMs and NAMs respectively) are named so because they bind to an alternative 'allosteric' binding site to that of the endogenous orthosteric ligand-binding site (Wang *et al.* 2009). It has been nearly a decade since the discovery of the first group III subtype-selective PAM, (-)-*N*-phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxamide (PHCCC), which binds to mGlu4 (Maj *et al.* 2003, Marino *et al.* 2003), and many more selective compounds have emerged since (see Table 1.5). Recently, several subtype-selective agonists and PAMs of group III mGlu receptors have been developed and further investigated, particularly by Jeffrey Conn's research group at Vanderbilt University via high-throughput screening (Bennouar *et al.* 2013, Betts *et al.* 2012, East *et al.* 2010, Engers *et al.* 2010, Engers *et al.* 2009, Jimenez *et al.* 2012, Jones *et al.* 2011, Jones *et al.* 2012, Le Poul *et al.* 2012, Niswender *et al.* 2008a, Niswender *et al.* 2008b, Williams *et al.* 2009a, Williams *et al.* 2009b).

As PAMs have greater lipophilicity, unlike many orthosteric agonists (which are mainly charged amino acids) they can more easily cross the BBB (Ritzén *et al.* 2005). Recently however, a number of agonists that do cross the BBB have been developed such as LSP1-2111 and LSP4-2022 (Beurrier *et al.* 2009, Goudet *et al.* 2012). These mGlu4 subtype-selective agonists could be more clinically viable as the hydrophobic nature of PAMs can reduce their solubility and bioavailability, potentially resulting in more off-target effects (Goudet *et al.* 2012). PAMs are thought to have greater subtype selectivity due to the sequence divergence in allosteric sites across receptor subtypes compared to orthosteric sites (Engers & Lindsley 2012). However, it now appears that orthosteric agonists can also be designed to have subtype-selectivity; LSP4-2022 is an elongated agonist that binds both the orthosteric binding site and a less conserved binding area on the mGlu4 receptor (Goudet *et al.* 2012). Agonists at mGlu receptors can cause desensitization or internalisation of receptors following direct tonic activation, although it has been shown that some mGlu receptor subtypes, particularly those coupled to Gai/o as group III mGlu receptors do not desensitise or internalise in response to binding by agonists (Conn *et al.* 2009, Flor & Acher 2012). As mGlu receptor PAMs are not in competition with glutamate for the orthosteric site, they are predicted to be effective at relatively low concentrations. True PAMs only exert their effect in the presence of endogenous orthosteric ligands, potentiating the natural ligand's effects and maintaining physiological patterns of activation

and should theoretically avoid desensitisation (Conn et al. 2009). PAMs are therefore expected to have a better safety profile as they prevent constant receptor activation which may be responsible for adverse side effects (Célanire & Campo 2012). Allosteric modulation of glutamate receptors in regions where excessive glutamate levels are present, such as in the overactive BG pathways in PD, makes these compounds particularly attractive as novel targeted therapeutics. Nonetheless, some PAMs have been shown to exert multiple effects on different receptor subtypes, for example PHCCC is a PAM at mGlu4 and a partial antagonist at mGlu1, whilst other compounds are mixed allosteric agonist/PAMs, for example VU0155041 at mGlu4, complicating evaluation of their physiological effects (Maj et al. 2003, Niswender et al. 2008a, Marino et al. 2003, Mathiesen *et al.* 2003). The numerous subtype-selective ligands generated in recent years for mGlu4 have stimulated further research into the functions of this receptor, and although few exist for mGlu7 and 8 at present, some subtype-selective PAMs and NAMs have become available; the development of selective mGlu7 NAMs such as ADX71743 and MMPIP (Kalinichev *et al.* 2013, Suzuki *et al.* 2007) alongside selective PAMs and agonists such as AMN082 (an mGlu7 receptor agonist) (Mitsukawa *et al.* 2005), (S)-3,4-dicarboxyphenylglycine (DCPG, an mGlu8 agonist) (Thomas *et al.* 2001) and AZ12216052 (an mGlu8 PAM) (Duvoisin et al. 2010) provides useful pharmacological tools for resolving the functions of different mGlu receptor subtypes in future research (see Table 1.5 for key PAMs and agonists).

Table 1.5: Key ligands targeting group III mGlu receptors that may possess neuroprotective properties

Targeted group III subtype	Compound abbreviation	Action on receptor	BBB permeability/ CNS exposure	Neuro-protective data published?	Off-target effects?	References
Broad group III	L-AP4	Agonist	Poor	✓		Bruno <i>et al.</i> (1996), Austin <i>et al.</i> (2010), Gasparini <i>et al.</i> (1999), Jiang <i>et al.</i> (2006), Schoepp <i>et al.</i> (1999), Vernon <i>et al.</i> (2007b), Vincent and Maiese (2000), Vernon <i>et al.</i> (2005)
	L-SOP	Agonist	Poor	✓		Bruno <i>et al.</i> (1996), MacInnes <i>et al.</i> (2004), Gasparini <i>et al.</i> (1999)
	RS-PPG	Agonist	Poor	✓		Bruno <i>et al.</i> (2000), Sabelhaus <i>et al.</i> (2000), Gasparini <i>et al.</i> (1999)
	ACPT-I	Agonist	Good			Lopez <i>et al.</i> (2012), Lopez <i>et al.</i> (2008), Palucha <i>et al.</i> (2004), Palucha-Poniewiera <i>et al.</i> (2008)
mGlu4 receptor	PHCCC	PAM	Poor	✓	Partial mGlu1 antagonist	Annoura <i>et al.</i> (1996), Battaglia <i>et al.</i> (2006), Maj <i>et al.</i> (2003), Marino <i>et al.</i> (2003)
	VU0155041	Mixed allosteric agonist/ PAM	Poor	✓		Betts <i>et al.</i> (2012), Jones <i>et al.</i> (2011), Niswender <i>et al.</i> (2008a), Williams <i>et al.</i> (2009a)
	VU0400195 (ML182)	PAM	Excellent		Partial PAM activity at other mGlu receptors- mGlu5, 6 and 7	Jones <i>et al.</i> (2011)
	ADX88178	PAM	Excellent		Synergistic effects with L-DOPA in PD models	Le Poul <i>et al.</i> (2012), Reynolds (2011)
	LuAF21934	PAM	Good		Synergistic effects with L-DOPA in PD model	Bennouar <i>et al.</i> (2013)
	LSP4-2022	Agonist	Excellent		High affinity for mGlu7 also	Goudet <i>et al.</i> (2012)
	LSP1-2111	Agonist	Excellent	✓		Amalric <i>et al.</i> (2013), Beurrier <i>et al.</i> (2009)
	VU0364770 (ML292)	PAM	Excellent		Weak antagonist against mGlu5, PAM at mGlu6	Jones <i>et al.</i> (2012)
mGlu7 receptor	AMN082	Allosteric agonist	Good	✓ (in immature rats)	Major metabolite Met-1 has affinity for SERT, DAT and NET. Complex activity <i>in vivo</i>	Palucha-Poniewiera <i>et al.</i> (2010), Greco <i>et al.</i> (2010), Sukoff Rizzo <i>et al.</i> (2011), Wang <i>et al.</i> (2012)
mGlu8 receptor	DCPG	Agonist	Good	✓ (in immature rats)	100-fold selectivity for mGlu8 over mGlu4. AMPA-receptor antagonist	Beurrier <i>et al.</i> (2009), Johnson <i>et al.</i> (2013), Moldrich <i>et al.</i> (2001), Thomas <i>et al.</i> (2001), Lopez <i>et al.</i> (2007)
	AZ12216052	PAM	Good		Additional pharmacological activities likely involving other receptors	Duvoisin <i>et al.</i> (2010), Duvoisin <i>et al.</i> (2011)

Chemical names: L-AP4: L-(+)-2-Amino-4-phosphonobutyric acid, L-SOP: L-Serine-O-phosphate, RS-PPG: (RS)-4-Phosphonophenylglycine, PHCCC: (-)-N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide, ACPT-I: (1S,3R,4S)-1-Aminocyclopentane-1,3,4-tricarboxylic acid, VU0155041: cis-2-(3,5-Dichlorophenylcarbamoyl)cyclohexanecarboxylic acid, VU0400195: N-(3-Chloro-4-((1R,2S,3R,4S-bicyclo[2.2.1]hept-5-ene-1,3-dioxo-1H-isoindol-1-yl)phenyl)picolinamide, ADX88178: 5-methyl-N-(4-methylpyrimidin-2-yl)-4-(1Hpyrazol-4-yl)thiazol-2-amine, LuAF21934: (1S,2R)-N-(3,4-dichlorophenyl)cyclohexane-1,2-dicarboxamide, LSP4-2022: (((3S)-3-Amino-3-carboxypropyl)[(4-(carboxymethoxy)phenyl)hydroxymethyl]phosphinic acid, LSP1-2111: (((3S)-3-amino-3-carboxypropyl)[(4-hydroxy-5-methoxy-3-nitrophenyl)hydroxymethyl]phosphinic acid, VU0364770: N-(3-chlorophenyl)picolinamide, AMN082: N,N-bis(diphenylmethyl)-1,2-ethanediamine, DCPG: 3,4-dicarboxyphenylglycine, AZ12216052: 2-(4-bromobenzylthio)-N-(4-sec-butylphenyl)acetamide

1.6 Therapeutic potential of group III mGlu receptor ligands in Parkinson's Disease

1.6.1 Symptomatic relief

1.6.1.1 Broad spectrum group III mGlu receptor agonists

Reserpine or haloperidol administration in animals results in a reversible depletion of monoamines including dopamine, temporarily modelling akinetic or cataleptic symptoms respectively (Carlsson et al. 1957, Valenti et al. 2003). Catalepsy is an inability of the animal to respond to stimuli or correct abnormal posture, and although it is not directly associated with human symptoms of PD, it has been likened to both rigidity and movement initiation deficits, and is responsive to L-DOPA treatment (Elliot et al., 1990, Duty and Jenner, 2011). L-Serine-O-phosphate (L-SOP) and L-AP4, both broad spectrum classical orthosteric agonists, can reverse reserpine-induced akinesia following direct injection into the SNr (Austin et al. 2010, MacInnes et al. 2004). L-SOP, when administered supranigally, was shown to reduce glutamate release in the SNr by 48% (Austin et al. 2010), indicating that a reduction in glutamate signalling from subthalamonigral projections is responsible for behavioural alteration. L-AP4 also relieves behavioural deficits in the reserpine, haloperidol and 6-OHDA models of PD following injection into the GPe, reducing GABA release here (MacInnes & Duty 2008, Marino et al. 2003, Valenti et al. 2003) and likely normalising downstream activity in the indirect pathway (Beurrier et al. 2009, Lopez et al. 2007, MacInnes et al. 2004). (1S,3R,4S)-1-Aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I) was recently demonstrated to reverse haloperidol-induced catalepsy in rats, as well as alleviating akinesia in bilaterally 6-OHDA-lesioned animals after 2 weeks of chronic ACPT-I peripheral administration (Lopez et al. 2012). Collectively, these studies indicate that broadly activating group III mGlu receptors in the indirect pathway of the BG in a variety of PD models provides symptomatic relief.

1.6.1.2 Subtype specific group III mGlu receptor agonists and PAMs

Many of the compounds targeting mGlu4 have demonstrated symptomatic improvements in animal models of PD by reducing activity at striatopallidal and subthalamonigral synapses, normalising signalling in the indirect pathway of the BG (Marino et al. 2003, Valenti *et al.* 2005, Valenti et al. 2003). Administration of the mGlu4 selective PAM PHCCC reverses reserpine-induced akinesia in rats when given intracerebroventricularly (i.c.v.), intranigally (into SNr) and systemically (Battaglia et al. 2006, Broadstock *et al.* 2012, Marino et al. 2003) indicating that the mGlu4 subtype is involved in inhibition of excess glutamate release at SNr. Similarly, the mGlu4 mixed allosteric agonist/PAM VU0155041 dose-dependently reversed reserpine-induced akinesia and haloperidol-induced catalepsy in rats (Niswender et al. 2008a) and significantly reduced motor dysfunction in the unilateral 6-OHDA-lesioned rat when injected supra-nigally (Betts et al. 2012). However, both of these compounds have

weak potencies and are limited by their poor BBB permeability making them less useful clinically (Célanire & Campo 2012). More recently, several mGlu4-selective PAMs and agonists with improved properties have been developed. The mGlu4-selective orthosteric agonists LSP1-2111 and LSP4-2022 and mGlu4 PAMs VU0364770, Lu AF21934 and ADX88178 are all effective antiparkinsonian agents when systemically administered in animal models of PD (Bennouar et al. 2013, Beurrier et al. 2009, Goudet et al. 2012, Jones et al. 2012, Le Poul et al. 2012).

The mGlu7 receptor is found presynaptically at corticostriatal, striatopallidal and subthalamonigral (SNr) synapses (Kosinski et al. 1999), all of which are overactive in PD, providing multiple therapeutic targets for activation by selective compounds. Activation of mGlu7 with the selective and brain-penetrant mGlu7 allosteric agonist AMN082 has been shown to reverse motor deficits in rat models of PD (Broadstock et al. 2012, Konieczny & Lenda 2013, Mitsukawa et al. 2005, Greco et al. 2010). However, the actions of AMN082 should be interpreted with caution; it is a complex agonist with many off-target effects and rapid metabolism to Met-1, a mixed monoamine transporter inhibitor (SERT, DAT and NET), perhaps explaining its actions as an antidepressant and anxiolytic-like agent (Flor & Acher 2012, Pałucha-Poniewiera et al. 2010, Sukoff Rizzo et al. 2011, Ugolini *et al.* 2008). Additionally, AMN082 can induce internalisation of mGlu7 receptors in hippocampal neurons (Pelkey *et al.* 2007) potentially limiting its usefulness. There are also contradictory findings regarding the efficacy of the mGlu8 receptor agonist DCPG for reversing motor symptoms in PD models, although this is likely to be due to differences between models and treatment regimes (Beurrier et al. 2009, Johnson et al. 2013, Broadstock et al. 2012, Lopez et al. 2012, Ossowska 2004). Conn's group recently demonstrated that DCPG can robustly reverse reserpine-induced akinesia and haloperidol-induced catalepsy in rats, but only if prolonged pre-treatment of reserpine or haloperidol is given (several doses of either reserpine or haloperidol over 18-20h). Additionally, DCPG was able to improve forelimb-use asymmetry in unilateral 6-OHDA-lesioned rats, suggesting DCPG only provides antiparkinsonian effects in models with prolonged dopamine depletion (Johnson et al. 2013).

1.6.2 Neuroprotection by group III mGlu receptor activation

Stimulation with the broad group III agonist L-AP4 improves neuronal survival in response to a variety of challenges such as toxin and NO exposure (Vernon et al. 2005, Vernon et al. 2007b, Vincent & Maiese 2000) and provides both histological and functional neuroprotection in rodent models of PD (Austin et al. 2010, Vernon et al. 2005, Vernon et al. 2007b, Vincent & Maiese 2000). The mGlu4 receptor is thought to be the most promising of the group III mGlu receptor subtypes so far. VU0155041, an mGlu4 receptor allosteric agonist/PAM, has been shown to provide around 40% neuroprotection against dopaminergic neuronal loss in the unilateral 6-OHDA-lesioned rat, however

this compound is limited by its poor BBB permeability (Betts et al. 2012, Célanire & Campo 2012). In comparison, LSP1-2111 crosses the BBB easily and is neuroprotective in the MPTP model of PD when administered systemically 30 minutes prior to and every 12 hours after MPTP for 7 days (Amalric et al. 2013).

While mGlu4 has received much interest lately, other group III mGlu receptor subtypes also have neuroprotective potential, although the contribution of these subtypes has not yet been fully investigated. Recently, it has been demonstrated that the mGlu7 allosteric agonist AMN082 is neuroprotective *in vitro* and *in vivo* against sevoflurane-induced developmental neurotoxicity of embryonic hippocampal neurons (Wang et al. 2012). However, this finding should be interpreted carefully as developmental changes in mGlu receptor expression in different cell types complicates neuroprotective findings shown in immature animals, and research into the neuroprotective potential of this receptor is limited by the lack of subtype-specific ligands (Defagot *et al.* 2002, Hubert & Smith 2004, Maiese *et al.* 2008). (RS)-4-Phosphonophenylglycine (RS-PPG), a group III agonist with a 25-fold preference for mGlu8 over other subtypes, has neuroprotective effects on cells after hypoxic or hypoglycaemic injury, although this may still be mediated through the mGlu4 receptor subtype (Bruno et al. 2000, Sabelhaus et al. 2000). Recent reports have demonstrated synergistic effects resulting from a combination of orthosteric agonists with mGlu4 PAMs (Kłak *et al.* 2007, Bennouar et al. 2013, Valenti et al. 2005). Similarly, co-administration of the group I mGlu receptor antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) with L-AP4 resulted in additive nigrostriatal neuroprotection in the 6-OHDA model of PD (Vernon *et al.* 2008). Hence, it is possible that co-administration of a different group III mGlu receptor agonist and PAM can similarly potentiate their individual neuroprotective effects. The neuroprotection afforded by group III mGlu receptors is likely to be multi-faceted due to their varied cellular localisation and tissue distribution alongside their modulatory effects on signalling. Numerous mechanisms have been proposed to explain their efficacy and are illustrated in Figure 1.6 and discussed below.

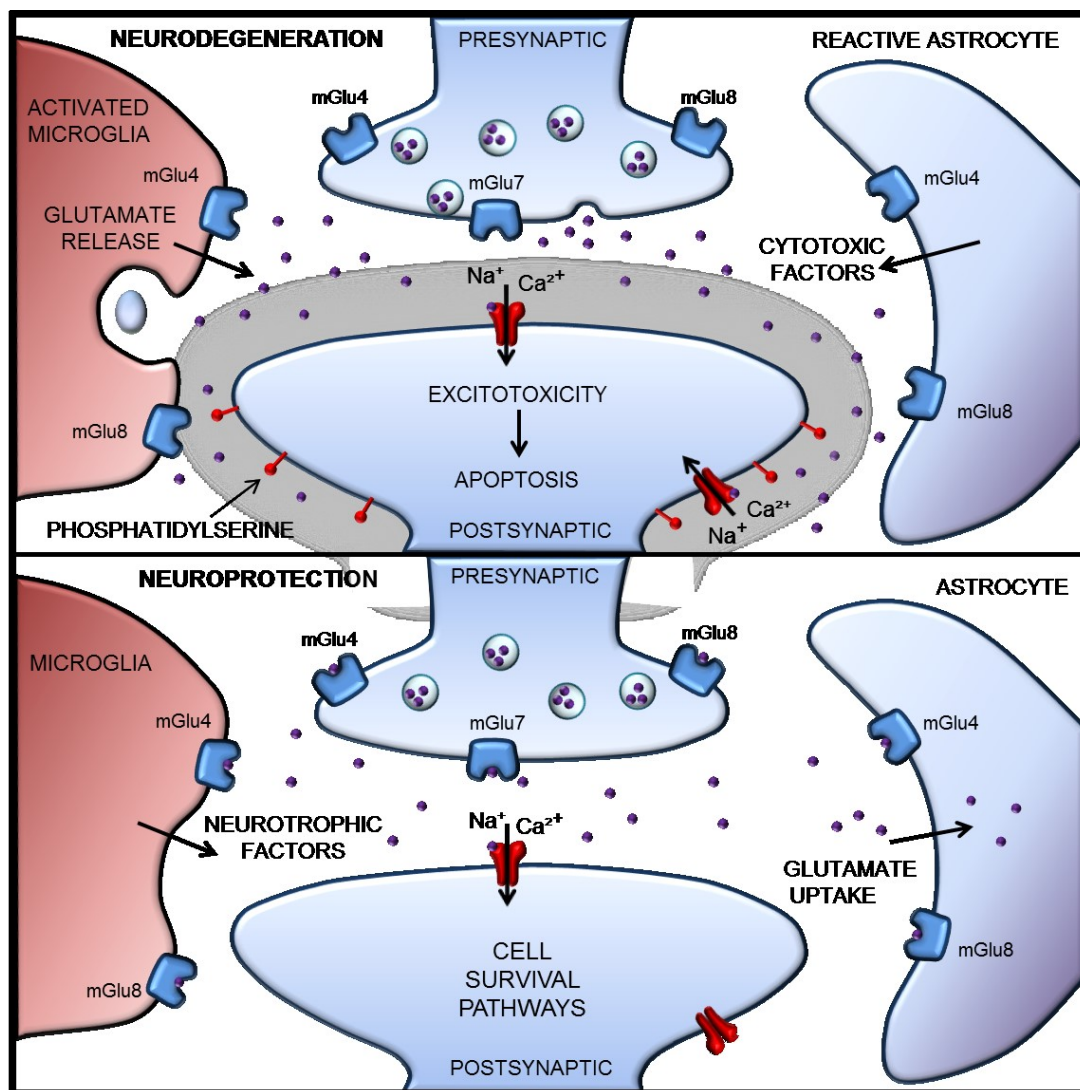


Figure 1.6: Schematic representation of neurodegeneration and neuroprotection by group III mGlu receptor activation. Top diagram: Excess extracellular glutamate causes overactivation of NMDA receptors resulting in excitotoxicity (Olney 1982). Externalisation of phosphatidylserine stimulates phagocytosis of neurons by activated microglia (Neher *et al.* 2011), which contribute to damage through glutamate and pro-inflammatory factor release (Barger *et al.* 2007, Parker *et al.* 2002). Activated astrocytes produce cytotoxic factors such as IL-6 and prostaglandins, enhancing neuronal cell death (Sofroniew 2009). Bottom diagram: Activation of group III mGlu receptors by glutamate and/or mGlu receptor ligands results in inhibition of glutamate release from presynaptic terminals and microglia, reducing excitotoxicity (McMullan *et al.* 2012). Astrocytic glutamate uptake is increased (Zhou *et al.* 2006) and microglia produce neurotrophic factors (Liang *et al.* 2010). Phosphatidylserine externalisation at the neuronal membrane surface is prevented/reversed by group III mGlu receptor activation protecting it from phagocytosis (Vincent & Maiese 2000). Figure taken from Williams and Dexter, 2013.

1.6.2.1 Modulation of neurotransmitter release

The prevention of excitotoxicity is likely to be a key factor in the neuroprotective potential of group III mGlu receptor activation. Evidence from electrophysiological and neurotransmitter-release studies suggest that targeting group III mGlu receptors in the indirect pathway of the BG results in the normalisation of glutamatergic signalling in the SNc either through direct modulation of glutamate release from subthalamonigral terminals, or indirectly through alteration of GABAergic signalling within the GP and a subsequent reduction in the downstream STN glutamatergic input to SNc. The reduction in excessive glutamatergic signalling in subthalamic projections to the SNc has been suggested to provide protection against excitotoxic damage to the dopaminergic neurons of the SNc. Indeed, this theory has been supported by a number of electrophysiological studies using brain slices where broad group III mGlu receptor agonists reduce presynaptic release of glutamate from STN projections onto SNc dopaminergic neurones, likely via mGlu4 and possibly via mGlu8 receptor subtypes (Katayama *et al.* 2003, Valenti *et al.* 2005, Wigmore & Lacey 1998).

PHCCC has been shown to protect the nigrostriatal pathway in animal models of PD when infused into the GPe and also when given systemically (Battaglia *et al.* 2006). Disinhibition of GPe projections to the STN with PHCCC reduces activity in the glutamatergic projections to SNc, protecting against nigrostriatal degeneration in MPTP-treated mice (Battaglia *et al.* 2006). This effect is thought to be mediated solely through mGlu4, as no neuroprotection occurs in mGlu4 knockout MPTP-treated mice administered PHCCC (Battaglia *et al.* 2006). PHCCC has also been shown to provide neuroprotection to mouse cortical neuronal cultures *in vitro* against NMDA toxicity (Maj *et al.* 2003). However, PHCCC's partial group I antagonistic properties complicate its mechanism of action, and its poor BBB permeability and physiochemical properties limit its potential clinical use, although it has proved a useful tool for investigating the therapeutic role of targeting mGlu4 (Broadstock *et al.* 2012, Marino *et al.* 2003).

Stimulation with the broad group III agonist L-AP4 can modulate excitatory transmission at STN-SNc synapses through the mGlu4 receptor subtype in the rat, although this finding differs between species as both mGlu4 and 8 are involved in excitatory transmission in the SNc in the mouse brain (Valenti *et al.* 2005). These species-specific differences in mGlu receptor subtype localisation may lead to inconsistencies in findings between studies and highlights the need for caution when investigating mGlu receptor subtypes in animals. Studies have shown that injection of the mGlu8 receptor agonist DCPG directly into GPe is ineffective at reducing GABA release, suggesting the mGlu8 receptor is not functional at the striatopallidal synapse (Beurrier *et al.* 2009, Lopez *et al.* 2007). However, it remains to be seen if selective activation of mGlu8 results in significant neuroprotection similar to that reported for mGlu4.

1.6.2.2 Direct neuronal protection

Group III mGlu receptor activation with L-AP4 has been shown to protect against NO-induced neuronal programmed cell death by preventing DNA fragmentation in response to NO exposure (Vincent & Maiese 2000). In response to exposure of A β [31-35] fragments of amyloid β (A β) protein, which can cause an increase in iCa²⁺ and induce apoptosis, pre-treatment of neuronal cultures with L-SOP and RS-PPG can attenuate apoptosis and lead to significantly reduced iCa²⁺ levels (Shirwany *et al.* 2007, Zhao *et al.* 2009). This effect has been suggested to be mediated via mGlu4 receptor and/or mGlu8 receptor activation (Zhao *et al.* 2009). These results indicate that the protective effects of group III mGlu receptor activation against A β -induced apoptosis are at least partially mediated through inhibition of increased iCa²⁺ levels, a function that could be extended to other neurodegenerative diseases such as PD where protein aggregation and dysregulation of iCa²⁺ occurs.

Preventing mitochondrial stress and damage is a vital neuroprotective function, and one which group III mGlu receptors are also likely to be implicated in, whether directly or indirectly. Taylor and colleagues showed that L-AP4 and RS-PPG both prevented CGA-mediated mitochondrial membrane depolarisation and subsequent cell death in microglia (Taylor *et al.* 2003). Similarly, Jiang and colleagues (2006) demonstrated that L-AP4 had directly protective effects on dopaminergic midbrain neuronal cultures when exposed to rotenone, which inhibits mitochondrial complex I and depolymerises microtubules (Jiang *et al.* 2006, Ren *et al.* 2005). They suggested L-AP4's neuroprotective activity occurred via activation of the MAPK pathway, leading to stabilisation of neuronal microtubules. Glial cells were not present in the culture, providing evidence for the direct neuroprotective action of group III mGlu receptor activation on dopaminergic neurons themselves (Jiang *et al.* 2006).

Table 1.6: Putative neuroprotective mechanisms following activation of group III mGlu receptors

Cell type	Receptor subtype expression	Putative neuroprotective mechanisms of receptor activation	References
Neurons	4, 7, 8	Inhibits neuronal excitability through ↓cAMP, inhibition of voltage-gated Ca ²⁺ channels and activation of K ⁺ channels Reversal of PS externalisation ↓intracellular Ca ²⁺ levels ↓oxidative damage ↑antioxidative capability Microtubule stabilisation	Vincent and Maiese, 2000 Gu et al., 2003 Jiang et al., 2006
Astrocytes	4, 7, 8	↑ glutamate uptake ↓inflammatory chemokine production	Zhou et al., 2006 Yao et al., 2005 Besong et al., 2002
Microglia	4, 8	↓ activation and premature phagocytosis of neurons ↓glutamate release ↑neurotrophic phenotype ↓ neurotoxic phenotype ↓mitochondrial dysfunction	Betts et al., 2012 Pinteaux-Jones et al., 2008 McMullan et al., 2012 Liang et al., 2010 Taylor et al., 2003 Byrnes et al 2009

1.6.2.3 Antioxidative properties and increased glutamate uptake by glia

As glutamate-induced depletion of the endogenous antioxidant GSH can lead to oxidative stress and subsequent cell death, a process known as oxidative glutamate toxicity (Murphy *et al.* 1989), restoration of GSH/antioxidant levels could have neuroprotective consequences. Pre-treatment of primary rat astrocyte cultures with L-AP4 attenuates LPS-induced astroglial neurotoxicity likely due to the restoration of GSH to normal levels following group III mGlu receptor activation (Zhou *et al.* 2006). GSH restoration significantly enhanced astrocytic glutamate uptake, thus reducing neuronal excitotoxicity (Zhou *et al.* 2006). L-AP4 similarly restores astrocyte-mediated glutamate uptake *in vitro* following MPTP administration and is consequently neuroprotective (Yao *et al.* 2005). In 6-OHDA unilaterally-lesioned rats, treatment with L-SOP was shown to increase serum antioxidative capability, inhibit ROS and increase GSH levels (Gu *et al.* 2003). As nigral dopaminergic neurons are more vulnerable to mitochondrial damage than other cell types due to their sensitivity to OS, the reduction in oxidative damage by group III mGlu receptor activation is likely to be indirectly protective to mitochondria. Glutamate transporters such as GLAST and GLT-1 are vital for homeostatic control of extracellular glutamate levels and normal glutamatergic neurotransmission (Anderson & Swanson 2000). Microglia have been shown to scavenge glutamate via GLT-1 (Nakajima 2011) suggesting they can uptake excess extracellular glutamate to help prevent excitotoxicity. Activation of mGlu3 and 5 subtypes can regulate glutamate transporter protein expression (Aronica *et al.* 2003) and GLAST and GLT-1 protein levels on cultures of astrocytes and neurons increase after treatment with the group II mGlu receptor agonist 4-amino-2,4-pyrrolidinedicarboxylic acid (APDC) (Beller *et al.* 2011). It is feasible that the effects of group II mGlu receptors on glutamate transporters might be extended to group III mGlu receptor activation as they similarly couple to Gi/o and stimulate MAPK and PI-3-K pathways, although there are currently no publications to our knowledge demonstrating this effect on transporters with group III mGlu receptors. Improvement in glutamate uptake might therefore be due to an increase in either expression or efficacy of astroglial glutamate transporter proteins (Aronica *et al.* 2003, Zhou *et al.* 2006) although it is not yet clear whether these effects are acute, short-term changes or more long-term modifications.

1.6.2.4 Reduction in glial activation and promotion of neurotrophic phenotype

As previously discussed, neuroinflammation is thought to play a central role in the pathogenesis of PD, and group III mGlu receptor activation has been shown to modulate this response through a variety of mechanisms. Long term use of anti-inflammatory drugs (NSAIDs) is protective against the development of Alzheimer's and PD (Chen *et al.* 2005, Vlad *et al.* 2008), and it has been suggested that they inhibit the secretion of pro-inflammatory cytokines from microglia (Combs *et al.* 2000). Targeting

activated microglia via mGlu receptors could similarly provide therapeutic benefits by reducing inflammatory damage. As illustrated in Table 1.6, microglia express both mGlu4 and 8 receptors (Byrnes et al 2009, Geurts *et al.* 2005). When stimulated with LPS, chromogranin A (CGA) or A β protein, microglial activation is attenuated by group III mGlu receptor agonists L-AP4 and RS-PPG in culture. Furthermore, L-AP4 and RS-PPG treatment can reduce LPS and CGA induced microglial neurotoxicity *in vitro* by activation of microglial group III mGlu receptors, demonstrating neuroprotective effects (Taylor et al. 2003).

Microglial phenotype is proposed to be much more highly plastic and dynamic than previously thought and group III mGlu receptor activation promotes a more neurotrophic, anti-inflammatory phenotype. Microglia can release anti-inflammatory cytokines such as IL-10, reducing the damaging effects of inflammation (Loane *et al.* 2012). Neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) are involved in the enhancement of neuronal growth and maintenance, as well as protecting against neuronal damage and promoting recovery (Connor & Dragunow 1998). Production of neurotrophic factors by activated astrocytes and microglia contribute to the neuroprotective effects of group II mGlu receptor activation, a mechanism that could feasibly be extended to group III receptor activation (Matarredona *et al.* 2001). Indeed, Liang and colleagues recently demonstrated that activation of microglial group III mGlu receptors by glutamate itself results in the release of neurotrophic factors via increased iCa²⁺ release and subsequent activation of the PKC pathway. They proposed that this is a self-regulating process to limit microglial neurotoxicity under conditions of excess glutamate seen in disease pathology (Liang et al. 2010). McMullan and colleagues have suggested that activation of group II and III mGlu receptors encourages microglial phenotype to swap from a pro-inflammatory activated state towards an activated yet beneficial phenotype, and that this may be modulated by a reduction in cAMP signalling (McMullan et al. 2012). As microglial priming by chronic CNS disease or inflammation is thought to induce a pro-inflammatory, detrimental microglial phenotype (Cunningham 2013), the potential to alter activated microglial behaviour with mGlu receptor ligands has therapeutic implications for PD.

Some mGlu receptor ligands displaying neuroprotective properties have reduced the innate inflammatory response in PD models (Betts et al. 2012, Chan 2010). Supra-nigral treatment in rats with VU0155041, an mGlu4 receptor selective allosteric agonist/PAM, was not only protective against 6-OHDA-induced neuronal cell loss but it also caused a significant reduction in microglial activation, suggesting a strong anti-inflammatory mechanism of mGlu4 activation (Betts et al. 2012). However, as VU0155041 was applied prior to 6-OHDA lesioning, it is hard to determine whether the reduced microglial response was due to direct effects on microglia to alter their phenotype, or through direct

protective actions of mGlu4 activation on neurons, reducing the cell death and debris that activates microglia. Studies with delayed administration of ligands following lesioning are needed to resolve this question. Targeting the mGlu8 receptor subtype on microglia might also offer therapeutic benefits in PD, although this has yet to be investigated.

Microglial activation precedes dopaminergic cell loss in PD animal models, and research suggests that degenerating neurons are phagocytosed prematurely by activated microglia, when they might otherwise have remained alive or been rescuable (Marinova-Mutafchieva *et al.* 2009). Neher and colleagues suggest that blocking phagocytosis may prevent some forms of inflammatory-mediated neurodegeneration as inflammation causes viable neurons to externalise phosphatidylserine residues, an 'eat me' signal, resulting in phagocytosis by activated microglia (Neher *et al.* 2011, Neher *et al.* 2012). By reducing microglial activation with mGlu receptor ligands, it may be possible to stop destruction of potentially viable neurons. Importantly, group III mGlu receptor activation with L-AP4 has been shown to both prevent and reverse NO-induced phosphatidylserine externalisation at the neuronal membrane surface, as illustrated in Figure 1.6 (Vincent & Maiese 2000).

As pro-inflammatory stimuli and OS can cause excess glutamate release from microglia (Barger *et al.* 2007, McMullan *et al.* 2012), targeting this additional neurotoxic process could be beneficial. Indeed, excess glutamate itself can activate microglial group III mGlu receptors, a feedback mechanism that might serve to allow activated microglia to control their own export of glutamate, attenuating glutamate excitotoxicity (McMullan *et al.* 2012). This neuroprotective process could therefore potentially be enhanced by group III mGlu receptor activation. Supporting this, McMullan and colleagues recently demonstrated that L-AP4 can block LPS-induced glutamate export from microglia (McMullan *et al.* 2012). It was suggested that stimulation of group III mGlu receptors by L-AP4 lead to suppression of cellular cAMP levels, likely via activation of G α i and inhibition of AC, and consequently inhibited further LPS-induced glutamate export from microglia (McMullan *et al.*, 2012).

Both mGlu4 and mGlu8 receptor subtypes have been detected on activated astrocytes (Geurts *et al.* 2005, Tang & Lee 2001). Due to the involvement of activated astrocytes in the degenerative process, it is possible that group III mGlu receptor activation on astrocytes could contribute to the neuroprotective actions of these compounds. Activated astrocytes can produce cytotoxic and pro-inflammatory factors (Mennicken *et al.* 1999), so inhibiting the release of these factors may provide neuroprotection. Supporting this, prolonged (28 days) treatment with L-AP4 significantly increased recovery rate in rats with experimental autoimmune encephalomyelitis (EAE), a finding associated with inhibition of the astrocytic production of RANTES, a chemokine critically involved in inflammation (Besong *et al.* 2002), suggesting group III mGlu receptor stimulation can

reduce the vicious cycle of inflammatory signalling. This effect was suggested to be mediated mainly via the mGlu4 receptor as astrocyte cultures from mGlu4 knockout mice showed a limited reduction in production of pro-inflammatory chemokines when treated with L-AP4 (Besong et al. 2002).

1.7 Summary and Aims

Recent advances in the development of subtype-selective ligands that cross the blood-brain barrier provides the ability to explore the variety of functions associated with targeting individual group III mGlu receptor subtypes in a clinically relevant manner. The overarching hypothesis of this thesis is that targeting specific group III mGlu receptors may hold therapeutic promise in PD, both by modulating aberrant neurotransmission in the basal ganglia to provide symptom relief and by providing neuroprotective effects in the nigrostriatal system through a variety of mechanisms including attenuation of inflammatory-induced damage.

Many studies have investigated the effects of targeting group III mGlu receptors in animal models of PD, however these models are all lacking one of the key pathological features of PD: the presence and accumulation of altered proteins. Hence, it is critical to test the efficacy of targeting these receptors in an animal model reproducing the central pathological characteristics of PD including altered protein accumulation. Additionally, the majority of studies discussed above used either pre-treatment or co-treatment of ligands at the onset of toxin administration. However, as PD patients receive drugs long after the initiation of disease pathology, these studies have limited clinical relevance. This thesis aims to address these gaps in knowledge by testing selective group III mGlu receptor ligands in the lactacystin model, which features altered protein formation and accumulation associated with a slower rate of neuronal death and development of motor deficits, allowing a delayed start to therapeutic administration and therefore providing a more clinically relevant study design. Novel subtype-selective compounds will be used in order to further elucidate which receptor subtypes are effective when targeted.

The objectives of this thesis are:

1. To investigate the effects of chronic delayed-start treatment with subtype-selective mGlu receptor ligands on behavioural deficits in the lactacystin-lesioned rat model of PD.
2. To examine the effects of chronic delayed-start treatment with subtype-selective mGlu receptor ligands on neuroprotection of the dopaminergic neurons of the SNc in the lactacystin-lesioned rat model of PD.
3. To establish whether any neuroprotection detected in aim 2 was due to anti-inflammatory actions of subtype-selective mGlu receptor ligands using the LPS rat model of PD.
4. To investigate the possible anti-inflammatory mechanisms of neuroprotection by subtype-selective mGlu receptor ligands using primary microglial cultures *in vitro*.
5. To use acute doses of subtype-selective mGlu receptor ligands to determine whether they have symptomatic effects in the lactacystin-lesioned rat model of PD.

To achieve these objectives, neuroprotection and antiparkinsonian effects of chronic delayed-start systemic dosing with group III mGlu receptor ligands were investigated in the lactacystin model using a battery of behavioural tests to measure motor deficits such as forelimb usage and rotational behaviour, combined with neuropathological assessments using immunohistochemical staining and stereological cell counting. Compounds which showed any efficacy/neuroprotection in these studies were then tested in the LPS model to elucidate whether neuroprotection was mediated via anti-inflammatory effects. The anti-inflammatory effects of mGlu receptor ligands were then further investigated *in vitro* using primary microglial cell cultures and analysis of culture medium with ELISAs. The ability of selective mGlu receptor ligands to provide rapid symptomatic relief was tested using acute single doses of compounds in the lactacystin model followed by a battery of behavioural tests measuring motor deficits such as forelimb usage.

Chapter 2: Materials and Methods

2.1 In vivo methods

2.1.1 The lactacystin model of Parkinson's disease

Lactacystin is an irreversible, rapid and specific inhibitor of the UPS that is synthesised naturally by the *Streptomyces* strain of bacteria (see Figure 2.1) (Fenteany *et al.* 1995, Omura *et al.* 1991). Lactacystin binds to the catalytic subunits of the 20S catalytic core and 26S proteasome complex and inhibits specific peptidase activities responsible for proteolysis (Fenteany & Schreiber 1998). Inhibition of the UPS results in an accumulation of misfolded ubiquitinated proteins within the cytosol, eventually leading to cell dysfunction and death (Betarbet *et al.* 2005a). Impairment of the UPS is thought to be key in the pathogenesis of PD due to its vital role in protein degradation within cells (McNaught *et al.* 2001).

Lactacystin has been used in a number of studies to induce a Parkinson's-like pathology in rodents, and is able to reproduce several pathological features of the disease (see section 1.3.2). Lactacystin causes dopaminergic cell death *in vitro*, as well as inducing the formation of α -synuclein and ubiquitin-positive cytoplasmic inclusions (McNaught *et al.* 2002c, Rideout *et al.* 2001). *In vivo*, lactacystin is injected locally within the brain in animals as it is unable to cross the BBB, and many studies have demonstrated its ability to cause degeneration of dopaminergic neurons when injected into the SNc, MFB or striatum, as well as inducing measurable motor dysfunction (Fornai *et al.* 2003, Lorenc-Koci *et al.* 2011, Mackey *et al.* 2013, McNaught *et al.* 2002b, Miwa *et al.* 2005, Niu *et al.* 2009, Vernon *et al.* 2011, Vernon *et al.* 2010). Furthermore, several studies have detected α -synuclein and ubiquitin immunopositive inclusions within degenerating dopaminergic neurons in the SNc (McNaught *et al.* 2002b, Niu *et al.* 2009, Vernon *et al.* 2010, Xie *et al.* 2010, Zhu *et al.* 2007). Additional to the induction of protein aggregation, lactacystin injection induces a significant inflammatory response which likely serves to further contribute to cell loss and is associated with PD pathogenesis. Zhu and colleagues (2007) reported a dramatic activation of microglia following lactacystin lesioning in the rat, with these cells remaining in a highly activated state when examined 28 days later.

Although lactacystin is a non-specific neuronal toxin, nigrostriatal dopaminergic neurons seem to be preferentially sensitive to damage. Following intranigral injection of lactacystin in rodents, striatal levels of dopamine and its metabolites are significantly reduced, whereas other neurotransmitter levels and their metabolites do not change (Fornai *et al.* 2003, Zhang *et al.* 2005). Nigral dopaminergic neurons may be more susceptible to UPS impairment than other neuronal populations (McNaught *et al.* 2002b), although some studies have shown a loss of GABAergic neurons (Reaney *et al.* 2006) or a spreading of pathology to extranigral areas following high doses of lactacystin (Mackey *et al.* 2013, Vernon *et al.* 2010). Importantly, protein inclusions are found to localise to the cytoplasm and processes of neurons but have not been detected within glial cells (Fornai *et al.* 2003,

McNaught et al. 2002b). Indeed, dopaminergic cells seem to be significantly more vulnerable to degeneration than glia in this model (McNaught et al. 2002b).

Following unilateral lesioning with lactacystin, rodents develop spontaneous behavioural dysfunction. Abnormalities include bradykinetic, akinetic or cataleptic behaviour, displaying a stooped posture with ipsiversive tilting of the head or postural asymmetry (Miwa et al. 2005, Konieczny *et al.* 2014a). An asymmetry in forelimb and hindlimb use and a loss of sensorimotor integration on the contralateral side have also been observed (Konieczny et al. 2014a, Schallert *et al.* 2000). Additionally, a reduction in locomotor activity and motor coordination is common (Zhu et al. 2007). Drug-induced rotational behaviour is commonly used to determine the amount of dopaminergic neuronal loss with either apomorphine or amphetamine (McNaught et al. 2002b) as the magnitude of the lesion correlates with the measured circling motor behaviour (Ungerstedt & Arbuthnott 1970). Studies using toxin-based lesion models have measured a variety of behaviours covering general motor activity, coordination, gait and lack of movement such as akinesia or catalepsy (Tieu 2011). It has been recently shown that treatment with the commonly used antiparkinsonian drug L-DOPA can alleviate lactacystin-induced motor and biochemical deficits in rats (Konieczny et al. 2014a), demonstrating its usefulness as a model for testing antiparkinsonian therapies.

Evidence suggests that the concentration of lactacystin used, the site of injection and the duration of the experiment all contribute to the extent and speed of lesion development and motor impairment. Administration of proteasome inhibitors to the nigrostriatal system can produce rodent models displaying robust Parkinsonian-like symptoms and pathology. Direct intranigral lesions are most common with lactacystin (Lorenc-Koci et al. 2011, Mackey et al. 2013, McNaught & Jenner 2001, Niu et al. 2009, Vernon et al. 2010), although intrastriatal lesions have been used to induce retrograde dopaminergic neuronal degeneration (Fornai et al. 2003, Miwa et al. 2005). However, there is some conflicting evidence regarding the effectiveness of striatal lesioning, as dopaminergic nerve terminals may not be as vulnerable to proteasomal inhibition as their cell bodies in the SNc (Lorenc-Koci et al. 2011). Lesions to the MFB demonstrate a similar pathology to that of intranigral lesions although are often more complete, leaving few surviving dopaminergic neurons and reflecting a more advanced stage of PD (Du *et al.* 2008, Vernon et al. 2011, Wu *et al.* 2010, Zhu et al. 2007). Additionally, due to the non-selective cytotoxicity of lactacystin, intrastriatal and MFB lesions can result in toxicity to many other cell types and spread to extranigral areas (Vernon et al. 2011). It is therefore more effective to inject lactacystin directly into the SNc in order to limit its non-specific toxicity on other cell types. Furthermore, the pattern of cell loss and dopamine depletion by targeting this site more closely reflects the pathology seen in human PD (Deumens et al. 2002).

Classical acute toxin-based models of PD such as MPTP and 6-OHDA cause a rapid and selective destruction of nigrostriatal neurons. Time course experiments reveal that although dopaminergic degeneration does not begin fully until a few days after 6-OHDA injection into the SN or MFB, cell death after this point is swift (Walsh *et al.* 2011, Zuch *et al.* 2000). Similarly, in acute MPTP-treated animals, extensive neuronal cell death is seen by 24 hours post injection (Jackson-Lewis *et al.* 1995, Jeon *et al.* 1995). These models therefore fail to replicate the progressive nature of PD. Neuroprotective treatments in studies utilising these models are often applied prior to or during lesioning in order to provide any efficacy, but these findings cannot be easily translated for clinical use. Instead, it is more clinically relevant to start therapeutic intervention after lesioning has taken place but prior to extensive degeneration. For studies investigating potential neuroprotective treatments at a point mirroring early to mid-stage PD, a progressive model demonstrating a gradual degeneration of nigrostriatal neurons with a delayed start to treatment may reveal potential neuroprotective agents that would otherwise not be detected in models where degeneration is rapid and the majority of cell death has already taken place. Lactacystin causes a more protracted and progressive neurodegeneration than that seen in traditional acute toxin-based models, providing a larger window for delayed-start therapy. Indeed, successful neuroprotective and neurorestorative studies have been carried out using a delayed start to treatment design following lactacystin lesioning in mice (Pan *et al.* 2008, Zhu *et al.* 2007).

The lactacystin model recapitulates important pathological aspects of PD such as intracellular protein inclusions and inflammation, alongside a progressive loss of nigral dopaminergic neurons and the development of measurable motor deficits. This makes the lactacystin model an ideal platform to test delayed-start neuroprotective and antiparkinsonian therapies, especially as cell loss is more protracted when injected locally into the SN than in other classical models. To determine the ideal dose of lactacystin necessary to produce robust nigrostriatal degeneration and measurable motor deficits, Mackey and colleagues injected a range of lactacystin doses into the SN of rats (Mackey *et al.* 2013). A dose-dependent loss of dopaminergic neurons from SNc and VTA was shown, with a 10µg dose producing a consistent severe lesion and motor deficits after 2-3 weeks. This dose has however been shown to eventually spread to many extra-nigral regions due to its nonspecific toxicity (Vernon *et al.* 2011, Vernon *et al.* 2010). To limit this non-specific toxicity and extra-nigral spreading of pathology, a lower dose of 7.5µg of lactacystin was chosen for intranigral lesioning in several studies for this PhD, which produced a robust lesion of the SN alongside measurable motor deficits. Additionally, a low dose of 2.5µg of lactacystin was also selected for lesioning of the SNc in order to provide a more partial lesion model of PD to determine if neuroprotective therapies were more efficacious in this less severe low-dose model. In order to test the efficacy of chosen compounds in

this project in a more clinically relevant manner, it was important to identify a time point for drug therapy to begin after lactacystin lesioning, when lactacystin-induced cell death has begun but no overt behavioural deficits are observable.

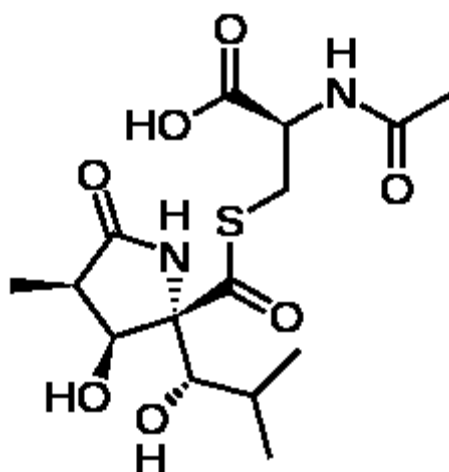


Figure 2.1: Chemical structure of lactacystin

(2R,3S,4R)-3-Hydroxy-2-[(1S)-1-hydroxy-2-methylpropyl]-4-methyl-5-oxo-2-pyrrolidinecarboxy-N-acetyl-L-cysteine thioester (lactacystin) irreversibly binds to the N-terminal threonine residue of the catalytic β -subunit of the 20S proteasome, inhibiting its function. Lactacystin stock was stored at -80°C in dimethyl sulfoxide (DMSO) and reconstituted in 0.9% sterile saline and stored on ice prior to intranigral administration.

2.1.2 The LPS model of Parkinson's disease

LPS is a component of gram-negative bacteria cell walls and is a potent inducer of inflammation in the brain. LPS is not directly toxic to neurons but instead induces an inflammatory reaction which causes cell death; activation of glial cells via the toll-like-receptor 4 (TLR4) receptor contributes to neurotoxicity through the secretion of toxic molecules including proinflammatory cytokines and chemokines (Herrera et al. 2000). The SN has been shown to be extremely sensitive to LPS-induced inflammation compared to the MFB, striatum and dorsal raphe nucleus (Herrera et al. 2000). Intranigral injection of LPS causes strong activation of resident parenchymal microglia/macrophages and recruitment of peripheral blood monocytes into the brain (Montero-Menei *et al.* 1996), inducing degeneration of the nigrostriatal dopaminergic system (Castaño et al. 1998, Herrera et al. 2000, Machado et al. 2011). It seems that dopaminergic neurons are particularly sensitive to inflammation-induced damage, as GABAergic and serotonergic neurons are spared (Herrera et al. 2000). Intranigral administration of LPS induces degeneration of nigrostriatal dopaminergic neurons in a dose and time-dependent way, with 5µg LPS causing cell loss from day 3 and nearly complete neuronal loss by day 30 (Gang *et al.* 2004). Systemic administration of LPS has also been shown to induce progressive loss of nigral dopaminergic neurons, with persistent neuroinflammation and microglial activation ongoing after peripheral inflammation has subsided and significant (47%) dopaminergic cell loss by 10 months (Qin et al. 2007).

Intranigral administration of LPS induces measurable motor deficits, although symptoms are dependent on the extent of the nigrostriatal lesion. Indeed, dysfunctional motor coordination and decreased motor speed have both been demonstrated in LPS-lesioned rodents (Tanaka et al. 2013, Zhou et al. 2012). LPS is particularly useful for studying the effects of the innate inflammatory reaction on dopaminergic cells as a model of PD, particularly due to the sensitivity of nigral neurons to LPS-induced damage. It provides a model to test the effects of novel anti-inflammatory and potentially neuroprotective therapies on the neuroinflammatory causes of cell death in PD. LPS has been used extensively as a model of inflammation and microglial activation in PD, being used *in vitro* to induce microglial activation, and *in vivo* to cause inflammatory-induced neuronal cell death. A dose of 5µg LPS was chosen for intranigral lesioning in this PhD based on findings by Gang et al. (2004), which produced a robust lesion of the SN alongside measurable motor deficits.

2.1.3 Stereotaxic surgery

2.1.3.1 Animals

Male Sprague-Dawley rats (Charles River, UK and Harlan, UK) were housed in groups of 2-3 with access to food and water *ad libitum*, and exposed to 12h light/dark cycle at 21°C. All animal

procedures were carried out in accordance with the Home Office Animals (Scientific Procedures) Act, UK, 1986. Recovery surgery was carried out after a period of 7 days acclimatisation following arrival to the animal house. Animals weighed between 240-290g prior to stereotaxic surgery. Prior to and following surgery, animals were routinely handled to ensure reliable behavioural testing. Only male rats were used for experiments due to the altering effects of the oestrus cycle on female rat brains and the possible neuroprotective effects of oestrogen (Gillies & McArthur 2010). Power calculations based on dopaminergic cell counts in the lactacystin model from previous studies in our lab indicated that a sample size of 7 animals per treatment group would detect an effect of 20% and provide >90% power and a significance level of 5%, so this sample size was aimed for when lesioning groups of animals in these studies (see Table 2.1).

2.1.3.2 Stereotaxic surgical procedure

Initially, in order to validate the coordinates selected for lesioning of the SNc and to confirm lesioning placement and accuracy, rats (n=6) were lesioned with 6-OHDA in order to induce rapid and severe dopaminergic cell death within at least one week that could be assessed both behaviourally and histologically (see section 2.2.3.3). Once this technique was validated, lesioning for neuroprotection studies was carried out.

Unilateral lesioning of the left SNc was carried out, allowing the right, unlesioned hemisphere to act as an internal control. Anaesthesia was induced in a chamber with 5% isoflurane (IsoFlo®, Abbot Laboratories, Maidenhead, UK) and oxygen (2L/min) in an induction chamber and maintained at 1-3% isoflurane in oxygen (2L/min) using a nose cone (coaxial bain circuit) when transferred to the stereotaxic frame (Kopf instruments, Tujunga, CA, USA). The incisor bar was set at 3.3mm below the interaural line to match the coordinates of the rat brain atlas (Paxinos 2009). Depth of anaesthesia was monitored throughout the experiment by carefully observing breathing and loss of responsiveness to toe pinches. To help maintain body temperature, rats were kept under a heat lamp. The scalp was shaved and the surgical site was swabbed with povidone-iodine wash (Betadine) for sterilisation prior to surgery. For perioperative care, analgesia was provided by a single intramuscular injection of buprenorphine (Vetergesic, Alstoe Animal Health, York, UK) administered into the gastrocnemius muscle (0.3mg/ml solution at 0.1ml/kg volume). Local anaesthesia was given subcutaneously around the incision area at 3 sites with 0.02ml/site of bupivacaine (bupivacaine hydrochloride 0.25% w/v solution, Taro Pharmaceuticals, Tipperary, Ireland). Eye ointment (Lacrilube, Allergan, Buckinghamshire, UK) was applied to the eyes to reduce post-surgical dryness. 6-OHDA (Sigma-Aldrich, Dorset, UK; 3µg/µl dissolved in 0.1% ascorbic acid and 0.9% sterile saline) was made fresh prior to use, stored on ice and protected from exposure to light to limit oxidation. Lactacystin

(Enzo Life Sciences Ltd., Exeter, UK; 25µg/µl dissolved in dimethyl sulfoxide (DMSO)) was diluted 10-fold in 0.9% saline to a 2.5µg/µl solution prior to use and stored on ice to limit degradation. LPS from E.Coli (Sigma-Aldrich, Dorset, UK; 1mg/ml concentration in sterile 0.9% saline) was stored at -20°C until needed, and stored on ice prior to use. After exposing the skull and drilling a small hole with a dental drill above the intended injection site either, 6-OHDA (12µg/4µl), lactacystin (2.5µg/3µl or 7.5µg/3µl sterile saline (0.9%)) or LPS (5µg/5µl saline) was injected intranigrally using a Hamilton syringe (Esslab, Essex, UK, blunt ended 10µl syringe) into the left SNc at a rate of 1µl/minute at the following coordinates relative to bregma; anteroposterior: -5.2mm, mediolateral: +2.4mm and dorsoventral: -7.6mm relative to dura (see Figure 2.2) (Paxinos 2009). After intranigral injection, the Hamilton syringe was left *in situ* for a further 5 minutes to prevent backflow of the toxin along the needle tract and allow diffusion into the target area. Following this, the syringe was slowly retracted and the skull surface was kept clean and moist with saline (0.9% sterile saline). Approximately 4 sutures were used in a simple interrupted style using 4-0 polyamide suture material (Ethilon, Ethicon, New Jersey, USA). During post-surgical recovery, lost fluids were replaced with 5ml glucosaline (0.18% NaCl + 4% glucose, Baxter, Thetford, UK) intraperitoneally (i.p.) and rats were placed in a heated chamber to recover. Animals were provided with a wet mashed diet when returned to their cage and weight and physical appearance were monitored closely throughout the experiment to assess recovery and general health. Any adverse effects that developed following surgery such as animals losing >20% of their pre-surgical weight or developing incapacitating neurological effects such as a failure to maintain a normal upright posture or engaging in persistent circling were judged as humane endpoints for the termination of animals. Of the 150 animals that underwent surgery, 3 rats died during surgery due to complications, and 3 rats were culled following surgery due to developing adverse effects. Overall, 73.6% of animals that did not develop adverse effects were judged to be accurately lesioned in the SNc based on assessment of needle placement during histological processing.

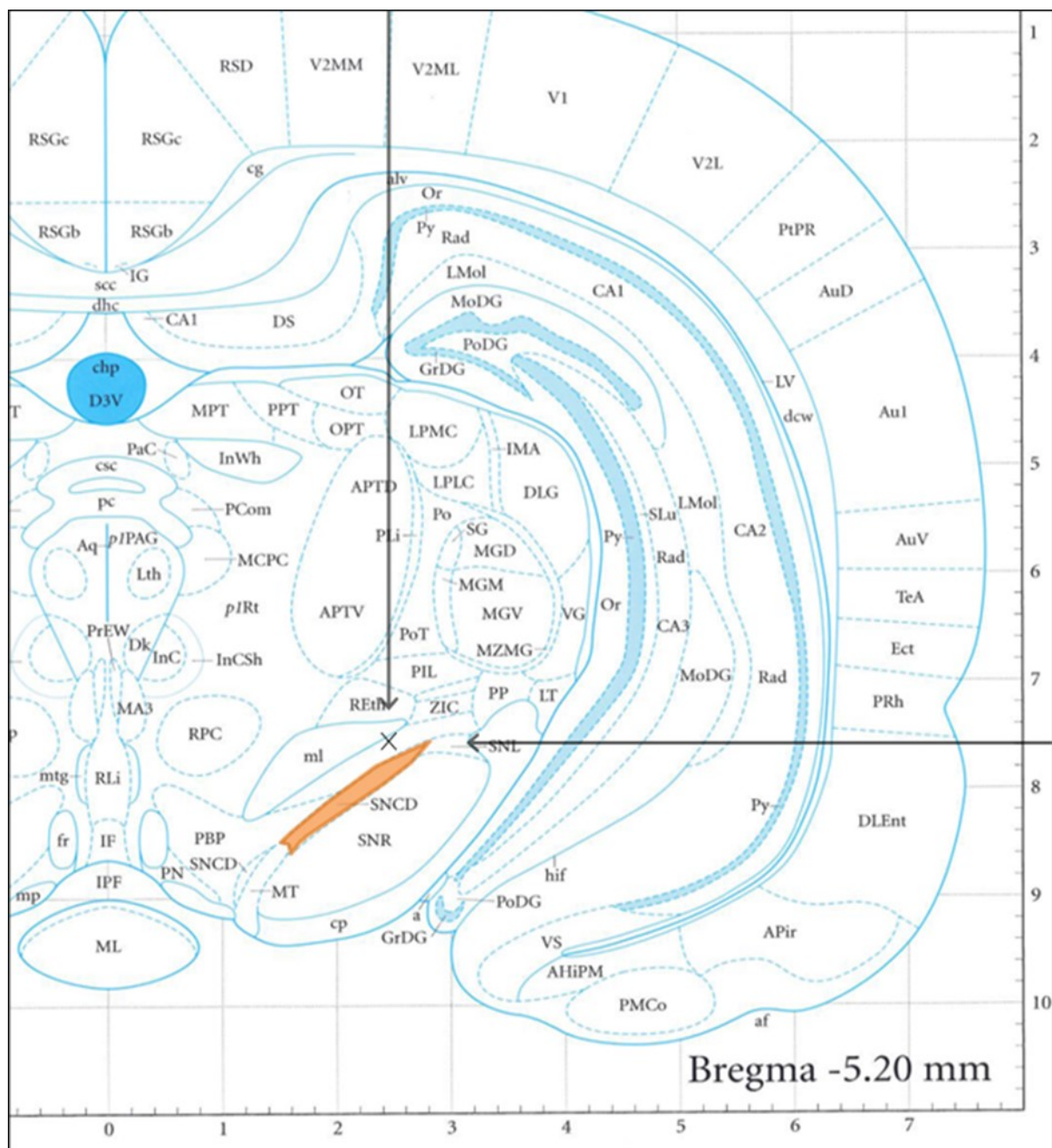


Figure 2.2: Coordinates for stereotaxic lesioning of the substantia nigra pars compacta.

Coronal diagram illustrates the lesion site for injection of 6-OHDA, lactacystin or LPS positioned directly above the lateral part of the SNc (highlighted in orange) which is found between -4.8mm and -6.3mm relative to bregma. The stereotaxic coordinates used were AP: -5.2mm (corresponds with above coronal diagram of rat atlas) and ML: +2.4mm (vertical arrow) relative to bregma, and DV: -7.6mm (horizontal arrow) relative to dura, marked with a cross. The incisor bar was set at 3.3mm below the interaural line to correspond with the coordinates of the atlas measurements. (Image adapted from Paxinos and Watson, 6th edition, 2009)

2.1.4 Drugs

Drug solutions were prepared and used on the same day to ensure full activity. The mGlu8 receptor agonist (S)-3,4-Dicarboxyphenylglycine(DCPG) was obtained from Abcam (Cambridge, UK) and was dissolved in 0.9% sterile saline. Animals were administered 3mg/kg or 15mg/kg DCPG i.p. at a volume of 1 or 3ml/kg.

The mGlu4 receptor PAM VU0364770 was not soluble in DMSO alone at the concentrations used. Stock VU0364770 was dissolved in DMSO, and then diluted to a final concentration of 5% DMSO in a solution of 0.9% sterile saline and 10% Tween 80 (Sigma-Aldrich, Dorset, UK). The solution was sonicated (Soniprep 180, MSE Ltd., London, UK) to distribute the compound through the solution and injected i.p. as a suspension. Animals were administered 10mg/kg, 30mg/kg or 100mg/kg VU0364770 at a volume of 1 or 3ml/kg. Table 2.1 gives an overview of experimental groups and their treatment.

Table 2.1 Overview of experimental groups and treatment

Number of animals lesioned	Lesion toxin and dose	Treatment	Duration of treatment	Behavioural tests	Analysis performed	Number used in final analysis
n=6	12ug 6-OHDA	None	-	VCT and AIR	Behaviour and histology	6
n=12	7.5µg lactacystin	None	Until Day 4	None	Histology and cell quantification	7
n=15	7.5ug lactacystin	Drug vehicle	Day 4-18	VCT and AIR	Behaviour, histology and cell quantification	8
n=11	7.5ug lactacystin	15mg/kg DCPG	Day 4-18	VCT and AIR	Behaviour, histology and cell quantification	7
n=11	7.5ug lactacystin	3mg/kg DCPG	Day 4-18	VCT and AIR	Behaviour, histology and cell quantification	7
n=8	5ug LPS	Saline	Day 1-7	VCT and AIR	Behaviour, histology and cell quantification	5
n=8	5ug LPS	15mg/kg DCPG	Day 1-7	VCT and AIR	Behaviour, histology and cell quantification	5
n=11	7.5ug lactacystin	Drug vehicle	Day 4-18	VCT, AIR, AST, VeFP and SC	Behaviour, histology and cell quantification	8
n=9	7.5ug lactacystin	30mg/kg VU0364770	Day 4-18	VCT, AIR, AST, VeFP and SC	Behaviour, histology and cell quantification	7
n=9	7.5ug lactacystin	10mg/kg VU0364770	Day 4-18	VCT, AIR, AST, VeFP and SC	Behaviour, histology and cell quantification	7
n=8	2.5ug lactacystin	Drug vehicle	Day 4-18	VCT, AIR, AST, VeFP and SC	Behaviour, histology and cell quantification	6
n=8	2.5ug lactacystin	30mg/kg VU0364770	Day 4-18	VCT, AIR, AST, VeFP and SC	Behaviour, histology and cell quantification	7
n=8	2.5ug lactacystin	15mg/kg DCPG	Day 4-18	VCT, AIR, AST, VeFP and SC	Behaviour, histology and cell quantification	5
n=26	7.5µg lactacystin	Acute doses of DCPG and VU03647870 + drug vehicle	From Day 21 onwards, acute doses	VCT, AST and VeFP	Behaviour	21

Vertical cylinder test: VCT, amphetamine-induced rotations: AIR, adjusted stepping test: AST, vibrissae-evoked forelimb placement: VeFP, spontaneous circling: SC. Day of lesioning surgery was Day 0. Drug vehicle= diluent (5% DMSO and 10% Tween 80 in saline) or saline.

2.1.5 Motor impairment assessment

2.1.5.1 Vertical cylinder

When placed into a small enclosure, rats will naturally explore their environment by rearing. Forelimb use asymmetry develops following unilateral lesioning of the SNc, and the vertical cylinder test, illustrated in Figure 2.3 A, allows sensitive measurement of the degree of forelimb usage during rearing behaviour (Schallert et al. 2000). Performance in the vertical cylinder test to measure forelimb use asymmetry during vertical exploration was assessed prior to lesioning surgery to ascertain a baseline score. Animals were then re-tested post-surgery to monitor deficit development. Animals were placed in a clear Perspex cylinder (height: 300mm, diameter: 360mm) and forelimb use was evaluated during rearing behaviour. Animals were digitally recorded for up to 6 minutes, or until 10 complete rearings had occurred, and subsequently scored using slow playback. A mirror was placed at an angle behind the cylinder to allow scoring of limb use when the animal faced away from the camera. The first weight-bearing contact of the ipsilateral and/or contralateral forelimb with a surface was scored for 3 stages of rearing; pushing off the ground, exploring the cylinder and landing back on the ground. As the animals were lesioned in the left hemisphere, a functional deficit in dopaminergic transmission would result, and this would be displayed behaviourally as a reduction in the use of the right, contralateral forelimb. The use of the contralateral forepaw was calculated as a percentage of the total number of contacts for all 3 stages of rearing combined. The percentage of forelimb asymmetry was calculated using the formula below:

$$\frac{(\text{total number of contralateral forelimb contacts} + \frac{1}{2} \text{ total number of both forelimb contacts}) \times 100}{(\text{total number of forelimb use contacts})}$$

Additionally, the percentage of forelimb asymmetry for wall exploration of the vertical cylinder during rearing was calculated alone as this was sometimes more sensitive to early motor deficit changes due to animals showing some compensatory behaviour during push-off and landing. When both analyses of the same vertical cylinder test were directly compared (see Figure 2.3 B), significantly lower contralateral forelimb use was seen when measuring wall exploration in the vertical cylinder alone compared to measuring all three aspects of rearing: push-off, cylinder wall exploration and landing combined. This difference confirms previous findings by Schallert et al. (2000) in 6-OHDA lesioned rats that suggest wall exploration is a more sensitive marker of forelimb use asymmetry. A similarly stronger asymmetry was seen in unilaterally lesioned rats when assessing vertical wall exploration in the cylinder test by Konieczny et al. (2014a), an effect seen for up to 6 weeks after lesioning.

Animals were discounted from the test if they failed to make more than 5 full rears in 6 minutes. In some cases, animals were encouraged to rear using food pellets, as some animals displayed a lesion-induced catalepsy or akinesia over time. Care was taken to avoid over-exposure of rats to this test as habituation could occur, reducing exploratory behaviour. During initial use and analysis of this test, the method for assessment of recordings was validated for reliability by repeatedly analysing the same test recording several times and checking its reproducibility. Statistical analysis in SPSS (IBM SPSS statistics, version 22, New York, USA) confirmed the intra-class correlation coefficient value was >0.99 , indicating a very high degree of intra-rater reliability and indicating strong reproducibility.

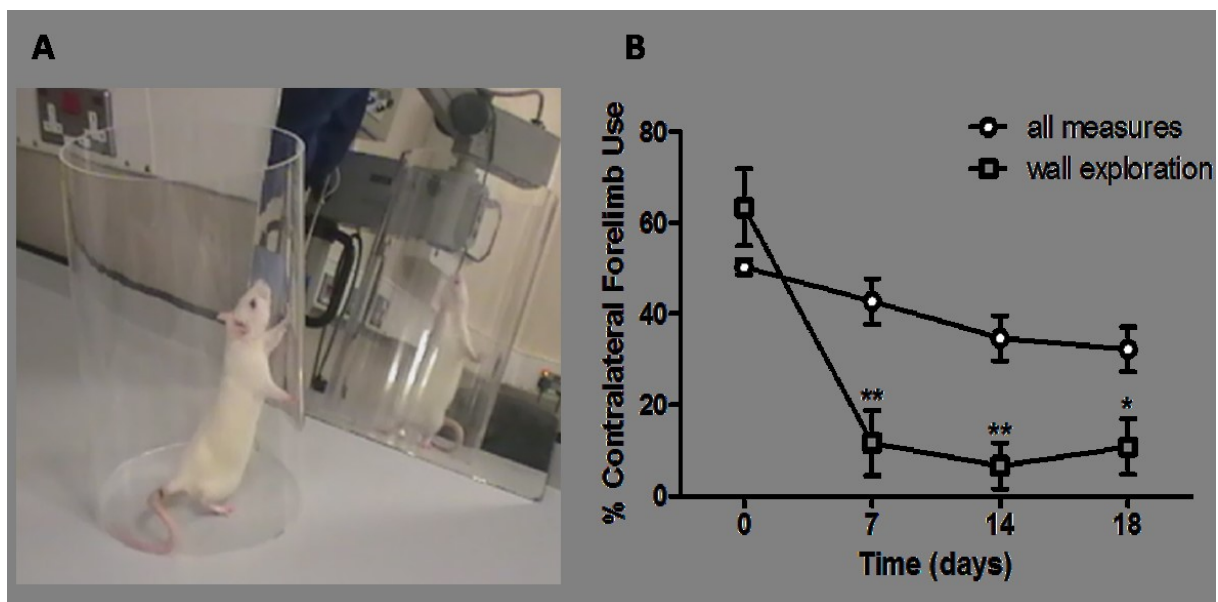


Figure 2.3: Assessment of forelimb use asymmetry in the vertical cylinder test. A: animals were placed in a Perspex vertical cylinder (height: 300mm, diameter: 360mm) with a mirror positioned at an angle behind to show forepaw placements facing away from the video camera. Rearing was measured as push-off from the ground, exploration of the cylinder wall and landing on the ground after rearing. B: Assessment of forelimb use asymmetry in the vertical cylinder test was carried out prior to 7.5 μ g lactacystin lesioning, and repeated on days 7, 14 and 18 post-lesioning using two different analysis methods; either all 3 parameters of rearing were assessed (push-off, exploration of the cylinder wall and landing) or exploration of the vertical cylinder wall was assessed alone. A significant difference in contralateral forelimb use in 7.5 μ g lactacystin-lesioned rats ($n=8$) can be detected between the two types of analysis of the same test; when analysing all 3 parameters (all measures) of the vertical cylinder test compared to analysing wall exploration alone. ** $p=0.01$ at days 7 and 14 post-lactacystin lesioning, * $p=0.05$ at day 18 post-lactacystin lesioning using a two-way ANOVA test and Bonferroni post-hoc tests.

2.1.5.2 Amphetamine-induced rotational asymmetry

Amphetamine increases synaptic levels of dopamine by three major mechanisms. First, it is a substrate for the DAT that competitively inhibits dopamine uptake; second, it facilitates the movement of dopamine out of vesicles and into the cytoplasm; and third, it promotes DAT-mediated reverse-transport of dopamine into the synaptic cleft independently of action-potential-induced vesicular release (Jones *et al.* 1998). Following treatment with amphetamine, an increase in dopaminergic activity in the nigrostriatal system occurs, and unilaterally lesioned rats preferentially rotate towards their lesioned side (ipsiversively) due to the imbalance in levels of striatal dopamine between the lesioned and unlesioned hemisphere. Amphetamine was used to assess rotational asymmetry in lesioned animals as an indicator of nigrostriatal function, and therefore the extent of the nigral lesion (Ungerstedt & Arbuthnott 1970). Amphetamine (5mg/kg D-amphetamine sulphate, Sigma, dissolved in 0.9% saline given at 1ml/kg i.p.) was administered 30 minutes prior to testing to induce rotations. Animals were digitally recorded in a Perspex circling bowl (height: 360mm, diameter: 400mm) for 30 minutes as illustrated in Figure 2.4, and the net number of full 360° ipsilateral turns was later calculated. As repeated exposure to amphetamine can be neurotoxic to the dopaminergic neurons (Bowyer *et al.* 1998), only a single dose was given to rats during each experiment. Reliability of rotational assessment was initially checked by analysing the same behavioural recording repeatedly 3 times to validate consistency, and was found to be extremely reproducible.

In order to test the acute impact of drug treatment on motor deficits in animals, acute dosing studies were carried out from 21 days post-lesioning. In order to determine criteria for inclusion or exclusion in the acute dosing study, rotational asymmetry testing with amphetamine was carried out on day 14 (14 days post-lesioning with 7.5µg lactacystin) as an indication of accurate lesioning and measurable motor deficits. Animals that failed to rotate ipsiversively on average <5 times in 5 minutes in the amphetamine-induced rotation test were excluded from the study. Final inclusion for analysis was based on accurate lesioning as assessed by histology (as described in 2.2.2.6).

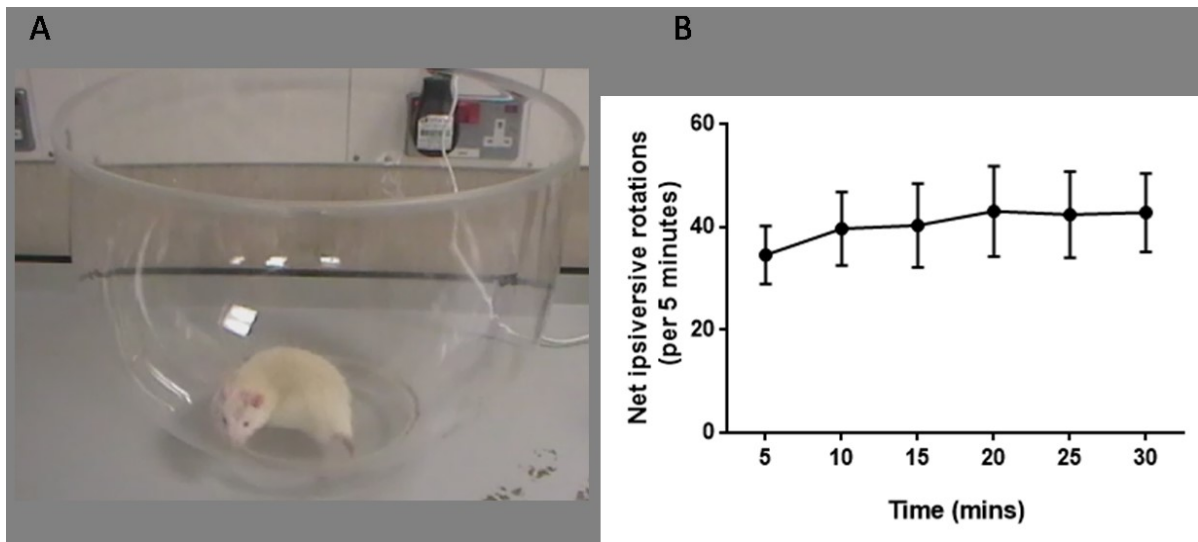


Figure 2.4: The circling bowl for testing rotational asymmetry. Animals were administered amphetamine (5mg/kg i/p.) and placed in a Perspex circling bowl (A) (height: 360mm, diameter: 400mm) 30 minutes later to measure amphetamine-induced rotational asymmetry for 30 minutes. Effectively lesioned animals rotated in a net ipsiversive direction, which was digitally recorded and analysed afterwards. (B) Data is shown as net ipsiversive rotations per 5 minutes $\pm$ SEM over 30 minutes for 7.5 μ g lactacystin-lesioned vehicle-treated animals (n=8) following amphetamine administration 18 days post-lesioning.

2.1.5.3 Adjusted stepping

Due to the lack of rearing activity seen in some rats following lesioning, a forced behavioural test was introduced in later studies; the adjusted stepping test (Schallert *et al.* 1979, Schallert *et al.* 1992) was adapted and used to test contralateral forelimb akinesia and weight-bearing strength. Both rat hindlimbs and the contralateral forepaw were gently held off the ground while the remaining unimpaired forepaw was slowly pushed forwards along a surface in a straight line of 100cm distance over 30 seconds (approx. 3.3cm/s), as illustrated in Figure 2.5 below. This was then repeated for the contralateral forepaw over the same distance. This test was repeated 3 times on both sides, and an average score was taken. The number of full weight-bearing adjusting steps taken by the contralateral forelimb was calculated as the percentage of steps relative to the unaffected ipsilateral forelimb, and gave an indication of forelimb akinesia.

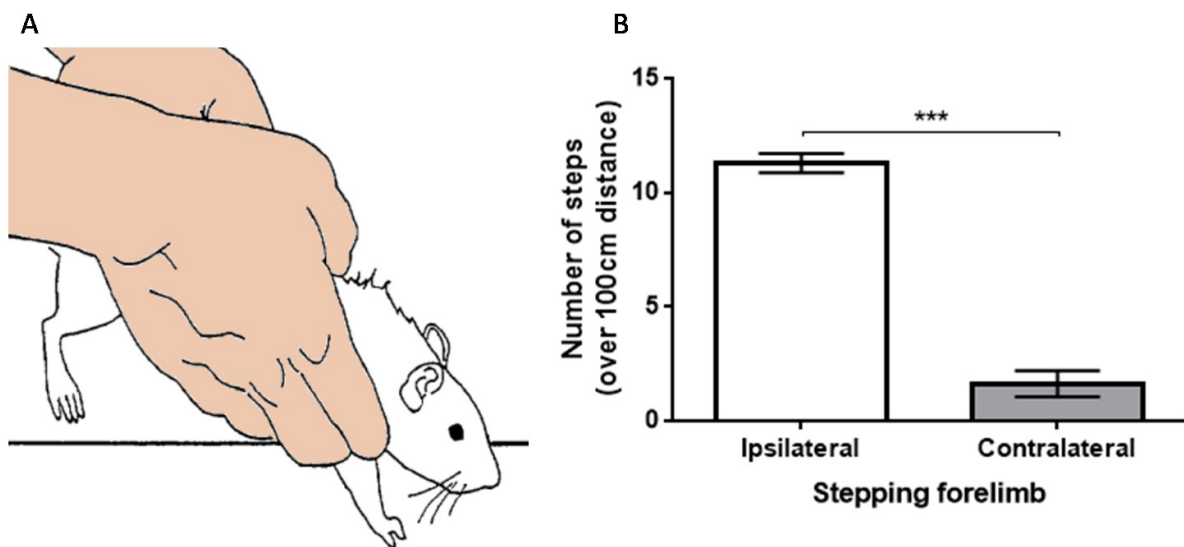


Figure 2.5: Diagram of the adjusted stepping test to measure forelimb akinesia. (A) Rats were gently restrained and all limbs were held off the surface except for the forelimb being tested, so that the animal's weight was supported by the single forelimb. Animals were gently pushed forwards in a straight line for ~30 seconds over a 100cm distance and the number of adjusted steps taken was recorded for each forelimb 3 times to give an average score. (B) A significant reduction in stepping with the contralateral forepaw is demonstrated using a paired t-test 18 days after lesioning with 7.5µg lactacystin, n=8, *** $p < 0.001$. Data in B is shown as average steps taken $\pm$ SEM. Image A adapted from Tillerson *et al.* (2001).

2.1.5.4 Vibrissae-evoked forelimb placement

The vibrissae-elicited forelimb placement test was also used to assess akinesia in response to a sensory stimulus in unilaterally lesioned rats. The test can be used to detect deficits in sensorimotor integration, for example in models of stroke, where animals will successfully place forelimbs on a surface during same-side vibrissae stimulation, but not during cross-midline testing. In comparison, in rats unilaterally lesioned in the SNc, placement of the contralateral forelimb is expected to be impaired in both same-side or cross-midline vibrissae stimulation, whereas the ipsilateral forelimb is unimpaired and can be successfully placed in response to both same-side and cross-midline stimulation, indicating contralateral motor initiation deficits rather than sensorimotor deficits (Schallert et al. 2000). In the present studies, animals were tested for same-side vibrissae-evoked placements to measure deficits in motor initiation that indicate a lesion-induced akinesia, and to detect any treatment-induced improvements in forelimb akinesia. As illustrated in Figure 2.6, rat hindlimbs were restrained with one hand and the upper body was supported with the other, allowing forelimbs free movement. With the head angled slightly downwards, the animal was moved slowly in an upward direction towards the corner of a flat surface to allow the vibrissae on one side of the body to brush the edge and trigger same-side limb placement on the surface. Where animals responded quickly to place their same-side forelimb on the surface, it was counted as one correct forelimb placement and scored 1 point. Independent testing of each forelimb was repeated five times, and the number of contralateral forelimb placements was calculated as a percentage of correct forelimb placements relative to the unaffected ipsilateral forepaw. Prior to lesioning, rats would quickly and instinctively place forelimbs upon the nearby surface in response to vibrissae stimulation on both sides of the body (Woodlee *et al.* 2005), usually placing forelimbs successfully 100% of the time. After lesioning, an inability to place the impaired forelimb contralateral to the lesioned hemisphere was seen following vibrissae stimulation. Animals were handled regularly prior to testing to reduce struggling behaviour and stress during the test, however if animals did struggle during the test, they were released and gently handled until struggling had subsided.

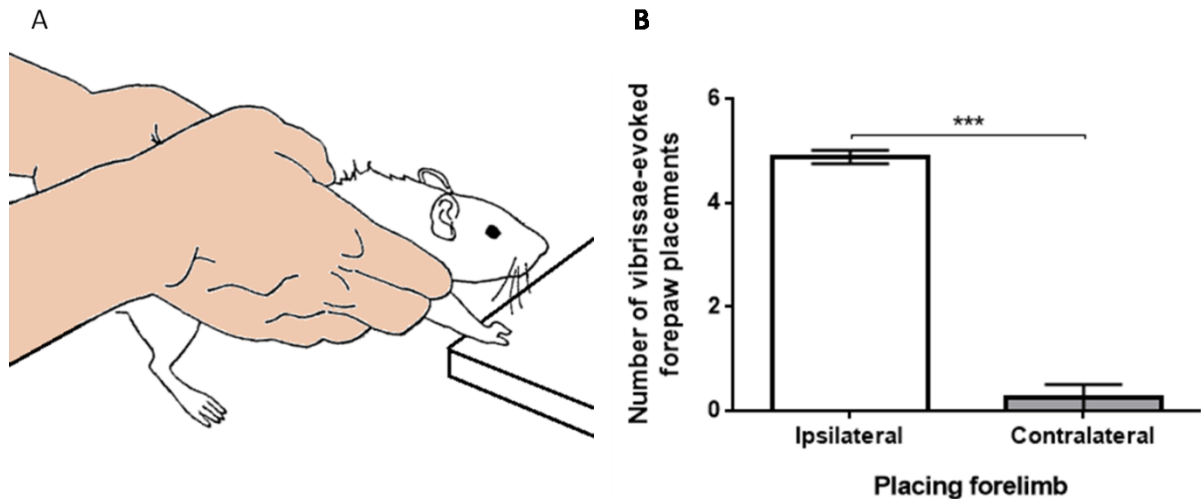


Figure 2.6: Diagram of the vibrissae-evoked forelimb placement test for forelimb akinesia. (A) Animals were positioned with their head tilted slightly downwards, their hindlimbs gently restrained and their upper body supported, allowing free movement of the forelimbs. Animals were slowly moved in an upward direction so that the vibrissae on one side of the body brushed a surface, evoking same-side limb placement on that surface. This was repeated 5 times on both sides of the body. (B) A significant reduction in forelimb placing with the contralateral forepaw is demonstrated using a paired t-test 18 days after lesioning with 7.5 μ g lactacystin, $n=8$, *** $p<0.001$. Data in B is shown as total number of successful forelimb placements $\pm$ SEM. Image A adapted from Tillerson et al. (2001).

2.1.5.5 Spontaneous circling

To further assess motor deficits and response to drug treatment, later studies also included a test to monitor spontaneous rotations throughout the course of the experiment. This test was adapted from the Bederson neurological scoring test (Bederson *et al.* 1986), where rats with striatal damage are assessed for the development of spontaneous asymmetric rotations to help determine the extent of the unilateral deficit. Rats were placed in the Perspex circling bowl (see section 2.1.5.2) as this encouraged rotational behaviour and were observed for 2 minutes freely moving around the bowl. The number of full 360° rotations in both ipsilateral and contralateral directions was measured to detect an asymmetry. A duration of 2 minutes was chosen to limit habituation to the test, and because many animals stopped exploring the rotation bowl after this time, remaining inactive for longer periods, a finding similar to that of Ungerstedt and Arbuthnott (1970). Each full rotation towards the lesioned side (ipsiversive) was scored 1 point, and each full rotation away from the lesioned side (contraversive) was scored -1 point. The final score after 2 minutes of rotations was calculated.

2.2 Ex-vivo methods

2.2.1 Post mortem procedures and tissue collection

At the conclusion of the study, animals were sacrificed by CO₂ overdose and brains were removed immediately and fixed in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) for 72 hours. Subsequently, brains were cryoprotected in 30% sucrose solution in PBS for up to 3 days at 4°C until brains had sunk, snap frozen in isopentane on dry ice and stored at -80°C until needed.

2.2.2 Histology and immunohistochemistry

2.2.2.1 Overview

Histological processing allows visualisation of microscopic events occurring at the time of tissue collection. Immunohistochemistry is a technique allowing the visualisation and localisation of cellular antigens using selective antibodies and enzyme labels or fluorescent dyes. Immunostaining techniques allow localisation of single and multiple antigens in the same tissue. Antibodies can be raised against any antigen and the specific signal can be augmented with a second antibody raised against the primary. The first fluorescence immunohistochemical study was reported in 1942 (Coons *et al.* 1942), with the peroxidase-labelled antibody technique being introduced in 1968 (Nakane 1968) which provides a more stable marker. Immunostaining of tissue allows the quantitative measurement of

specific cell types and the localisation of these cells or proteins to specific regions, allowing pathological investigations to be carried out.

The avidin-biotin (ABC) method of immunohistochemistry is a standard way to specifically label antigens of interest with a peroxidase enzyme reaction (see Figure 2.7). The glycoprotein avidin contains four identical subunits, with each subunit capable of binding a molecule of biotin. The high affinity of avidin for biotin means the bond is effectively irreversible. The horseradish peroxidase (HRP) enzyme can be conjugated to biomolecules as a label, which can then be visualised with a chromogen such as 3,3'-diaminobenzidine (DAB), which provides the substrate hydrogen peroxide to react with the biotinylated HRP enzyme. This forms an insoluble brown precipitate which labels the antigen of interest. The macromolecular complex formed between avidin and biotinylated enzymes mean that there is an increased concentration of enzyme at the antigenic site, amplifying the original signal and improving the sensitivity of the technique.

In fluorescent immunohistochemical staining, instead of an enzymatic reaction, a fluorescently-conjugated secondary antibody can be used to visualise antigens. Fluorescent dyes are visible when excited by specific wavelengths of light, emitting light of a different wavelength through selective filters. Double immunofluorescence labelling techniques can be employed to stain multiple antigens in the same tissue with fluorescent dyes excited by different wavelengths to detect co-localisation of proteins of interest.

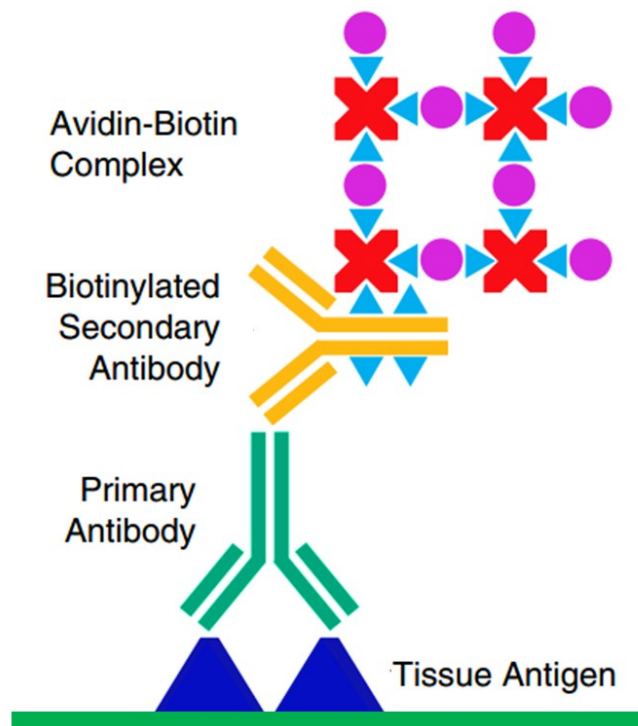


Figure 2.7 Schematic representation of Avidin-Biotin Complex immunohistochemical staining method. Three layers of macromolecules are built upon the antigen of interest. The first layer is the primary antibody raised against the antigen in a different species. The second layer constitutes the biotinylated secondary antibody raised against the primary antibody. The third layer is the avidin-biotin peroxidase complex. The signal is amplified through these steps as several biotin molecules can be conjugated to the secondary antibody, and there are four binding sites available on the avidin molecule for biotinylated enzymes to bind to. This complex can then be visualised with a chromogen such as DAB, which provides the substrate hydrogen peroxide to react with the biotinylated horseradish peroxidase enzyme, forming an insoluble brown precipitate.

2.2.2.2 Cryostat sectioning of brain tissue

Brains were removed from -80°C and mounted for coronal sectioning in Optimal Cutting Temperature compound (Tissue-Tek, Sakura, Alphen aan den Rijn, Netherlands), and left to equilibrate to -20°C for >1 hour. 20µm thick serial coronal sections through the entire SNc were cut on a cryostat (-18°C, Bright Instruments, Cambridge, UK) and mounted onto charged glass slides (Superfrost plus, VWR) and stored at -80°C for later immunohistochemical processing. The rat atlas was referred to whilst sections were cut as the SNc is found between -4.8mm and -6.3mm relative to bregma.

2.2.2.3 ABC immunohistochemical staining protocol

Optimal primary and secondary antibody concentrations were selected for all immunohistochemical stains (see Table 2.2) through trialling a variety of conditions on test tissue and selecting the concentrations giving the clearest staining and lowest background. Selecting the first section at random, a systematic subset of sections- in this case every 7th section through the SNc- was immunostained to detect a variety of cell types and proteins throughout the entire SNc. Occasionally, where a section was damaged or not well enough stained for accurate quantification, the section 20µm prior to or following that section was stained to be used instead. Reagents used were from Sigma, unless otherwise stated.

Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol or PBS for 45 minutes; methanol perforates cell membranes so was not used for staining of membrane bound antigens such as OX-6 and CD68. Sections exposed to methanol were subsequently rehydrated in 90% then 70% ethanol for 5 mins, followed by distilled water. Nonspecific binding was blocked by incubating slides in a blocking solution of 20% serum from the species the secondary antibody was raised in diluted in 0.1% Triton X-100 in PBS (PBST, to permeabilise the tissue) for 1 hour. Blocking solution was removed and sections were incubated overnight at room temperature (RT) with primary antibody (see Table 2.2 for antibodies used) in blocking solution. The following day, slides were washed in PBST before being incubated with the secondary antibody in PBST for 1 hour. Following this, slides were washed with PBST and incubated with the Avidin Biotin Complex (ABC) solution (Vectastain Elite ABC kit, Vector Labs) for 1 hour at RT. Sections were washed in Tris buffer (0.1M Tris) for 5 mins before being stained with DAB solution (DAB Peroxidase Substrate Kit, Vector Labs). Following DAB incubation, sections were washed thoroughly in distilled water and Nissl bodies of all neurons were counterstained with cresyl violet solution for 5 mins. Slides were then washed in distilled water and dehydrated in ascending concentrations of ethanol (70%, 90%, 2x 100%), before being cleared in Xylene and coverslipped with DPX mounting medium. Confirmation of correct lesion placement in the SNc was carried out by locating the needle tract in each rat brain. Where placement

of the needle was outside of the desired region and a full lesion had not occurred, animals were discounted from the study. Negative control tissue was treated with the primary antibody omitted and showed no immunoreactivity, as shown in Figure 2.8 below.

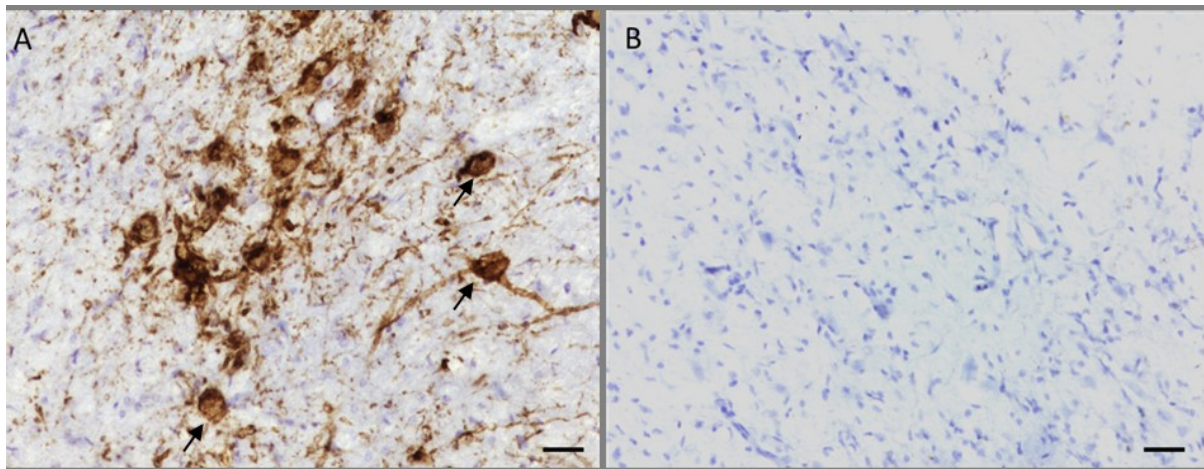


Figure 2.8: Immunostaining of TH+ dopaminergic neurons in the SNc. Example images of (A) TH+ Immunostaining of dopaminergic neurons in the SNc (brown DAB staining) and (B) negative control tissue that was exposed to the same immunostaining protocol but with the primary antibody omitted. Negative control SNc tissue showed no specific brown DAB staining, only blue Nissl+ staining of cells. Arrows show example TH+ neuronal cell bodies. Scale bar: 30 μ m.

Table 2.2 Antibodies employed for immunohistochemistry and immunocytochemistry.

Target	Antigen	1° Antibody and working dilution	Source	2° Antibody and working dilution	Source
Dopaminergic neurons	Tyrosine hydroxylase (TH)	Rabbit polyclonal 1:1000	Millipore (AB152)	Biotinylated Gt X Rb 1:200	Vector Labs (BA-1000)
Activated microglia	MHC class II (OX-6)	Mouse monoclonal 1:500	ABD Serotec (MCA46G)	Biotinylated Hs X Ms 1:200	Vector Labs (BA-2000)
Macrophages and phagocytic microglia	CD-68 (ED1)	Mouse monoclonal 1:500	ABD Serotec (MCA341)	Biotinylated Hs X Ms 1:200	Vector Labs (BA-2000)
mGlu8 receptor	mGlu8 receptor	Rabbit polyclonal 1:500	St John's Laboratory (STJ30108)	Fluorescent: AlexaFluor 488 (green) Gt x Rb 1:200	Invitrogen (A11008)
Microglia	CD11b (OX-42)	Mouse monoclonal 1:500	ABD Serotec (MCA275)	Fluorescent: CY3 Gt x Ms 1:200	Invitrogen (A10521)
DAPI: nuclear stain	DNA, fluorescent (blue)	1:1000 (4mg/ml stock)	Invitrogen (D1306)	-	-
Neurons	NeuN	Mouse monoclonal 1:500	Millipore (MAB377)	Fluorescent: CY3 Gt x Ms 1:200	Invitrogen (A10521)
Astrocytes	Glial fibrillary acidic protein (GFAP)	Mouse monoclonal 1:500	Sigma (G3893)	Fluorescent: AlexaFluor 488 (green) Gt x Ms 1:200	Invitrogen (A11001)

Abbreviations: DAPI: 4',6-Diamidino-2-Phenylindole, Dihydrochloride, Gt: Goat, Rb: Rabbit, Hs: Horse, Ms: Mouse. Sources: Millipore, Watford, UK; ABD Serotec, Kidlington, UK; Vector Labs, Peterborough, UK; Invitrogen, Paisley, UK; St John's Laboratory, London, UK; Sigma Aldrich, Dorset, UK.

2.2.2.4 Immunostaining of dopaminergic neurons

To assess the efficacy of neuroprotective treatments, specific immunostaining and quantification of dopaminergic neurons within the SNc was carried out. Dopaminergic neurons within the SNc were visualised by staining for the tyrosine hydroxylase (TH) enzyme. TH is necessary for catalysing the conversion of L-tyrosine to the precursor of dopamine, L-3,4-dihydroxyphenylalanine (L-DOPA), and is therefore present in cells requiring dopamine synthesis as a rate-limiting enzyme. This means immunostaining of TH will also pick up noradrenergic neurons, however, as the SNc predominantly contains dopaminergic neurons this would not affect dopaminergic cell counts of the SNc. Additionally, positive staining of the VTA directly adjacent to the SNc was seen in tissue, and this area was delineated from the SNc during cell counting according to Carman et al. (1991), as illustrated in Figure 2.12.

2.2.2.5 Immunostaining of microglial cells

To investigate the effects of mGlu receptor ligands on the innate immune response, immunostaining of microglia in different activation states was carried out. OX-6, an antibody against the rat major histocompatibility complex (MHC) class II antigen expressed by activated but not resting microglia, was used to identify classically activated microglia, as illustrated in Figure 2.9 (left image). In separate sections, an anti-CD68 antibody targeting the lysosomal glycoprotein of monocytes and macrophages was used to label phagocytic microglia and macrophages as illustrated in Figure 2.9 (right image).

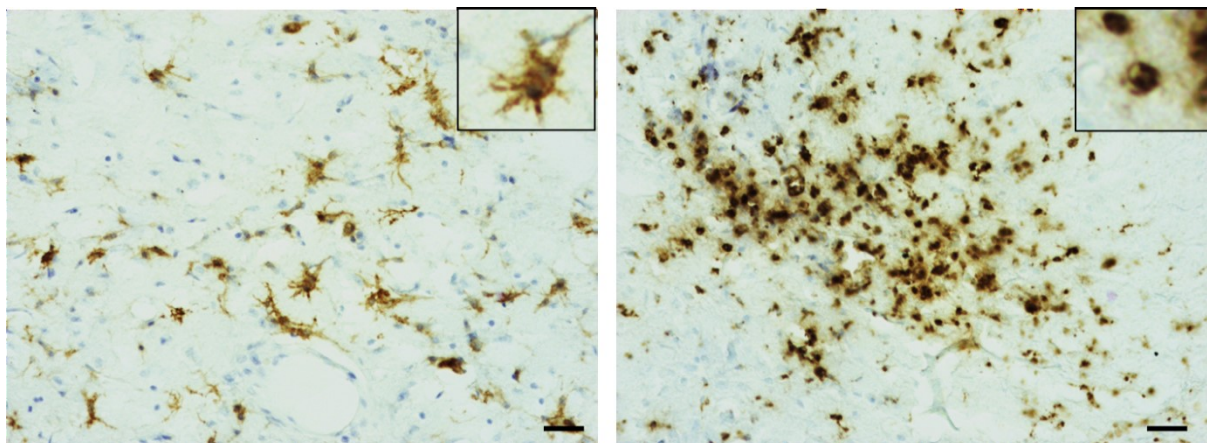


Figure 2.9 Representative photomicrographs of immunopositive microglia and macrophages. OX-6 immunopositive microglia (left, brown staining) displayed a clearly activated morphology following lactacystin lesioning, with swollen cell bodies and thick processes monitoring the environment. Staining for CD68 immunopositive phagocytic microglia/macrophages (right, brown staining) was localised to amoeboid cell bodies, and had a more intense punctate staining. Differences in cell morphology are highlighted by enlarged corner images. Images taken at 20x magnification, scale bars: 30µm.

2.2.2.6 Assessment of accurate lesioning and neuronal loss

Following staining for TH-immunoreactive (TH+) neurons, sections were counterstained with cresyl violet (section 2.2.2.3) to stain Nissl bodies and for visualisation of anatomical landmarks. The needle tract could be clearly seen in several sections of each brain, and its location within the target area of the SNc was assessed using the rat atlas for guidance. Where the needle tract was not found to be in the vicinity of the left SNc, animals were considered poorly lesioned and discounted from analysis of the study (see below Figure 2.10 for examples). The number of animals included in each final analysis is shown in Table 2.1. Nissl staining was also carried out to control for the potential loss of TH expression in dopaminergic neurons following lesioning, thus verifying true neuronal loss.

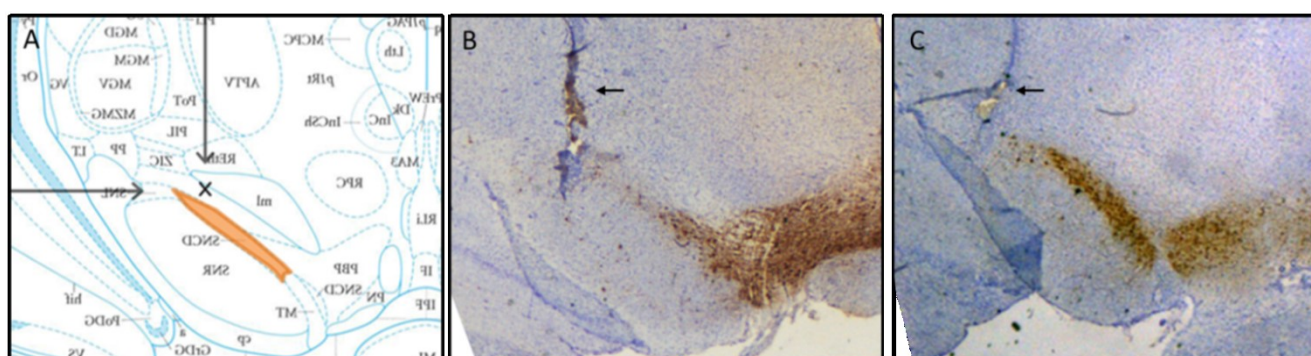


Figure 2.10: Representative lesioning tract sites in left hemisphere of lactacystin lesioned rats. To determine the accuracy of needle placement in the SNc, sections were Nissl stained to highlight the needle tract through the brain, and TH+ dopaminergic neurons were immunostained to highlight the remaining SNc. The accuracy of lesioning was based on the rat atlas coordinates (A, SNc highlighted in orange), with lesion tracts in animals compared directly to this. B demonstrates an accurately lesioned animal, whereas in C, the needle tract is at an angle and is placed too laterally to accurately lesion the SNc, thus discounting the animal from analysis. Arrows denote lesion site. Images taken at 4x magnification.

2.2.3 Quantification of immunohistochemical staining

2.2.3.1 Stereological cell counting

Stereological cell counting in the SNc was performed with stereology computer software (ImagePro Version 7, Media Cybernetics, PA, USA) attached to a Nikon Eclipse E8 microscope with a motorised stage (Nikon Instruments, Surrey, UK) and JVC 3CCD camera (JVC, London, UK) for visualisation of tissue. The fractionator method (Gundersen 2002, West 1999, West *et al.* 1991, Gundersen 1986) with systematic random sampling was used to make efficient, unbiased and precise estimates of the total number of cells within a given region. Selecting the first section at random, a systematic subset of sections- in this case every 7th section- was used to estimate the cell number within each sub-volume (7 sections thick). The neuroanatomical limits of the SNc were defined as the region of interest (ROI) with reference to the rat atlas (Paxinos 2009) and research carried out by Carman *et al.* (1991) which defines the boundaries between the SNc and its neighbouring dopaminergic area, the VTA. Initially, practice counting was carried out on a number of brains to optimise parameters and evaluate accuracy and consistency (see Figure 2.11). Magnification for counting was carried out under a 10x objective for neurons and a 20x objective for microglia/macrophages due to their smaller size and need for closer morphological assessment. The shrinkage that occurred during dehydration and processing of the tissue meant that all cells could be seen in one focal plane of the section, thus removing the need for 3-dimensional cell counting for each section. Fractionator sampling allows a known fraction of the ROI area to be sampled with all parts of the region having an equal probability of being sampled, thus making the selection unbiased (West, 1993). Counting frames (160 x 140µm for neurons and 125 x 100µm for microglia) were superimposed over the ROI in a uniform systematic random manner to cover between 40-60% of the area. The total area of the ROI was measured in each hemisphere for each section counted, and used to calculate the area sampling fraction (asf) to determine the proportion of the sectional area investigated. The asf for each ROI is calculated as below:

$$\text{asf} = \frac{\text{number of counting frames} \times \text{area of counting frame}}{\text{total area of ROI}}$$

Approximately 12-16 sections per rat spanning the entire SNc were counted. The number of cells within each sub-volume (n) was calculated by multiplying the number of cells counted in the ROI by the reciprocal of the serial sampling fraction (ssf) and by the reciprocal of the asf as below:

$$n = \text{number of cells in ROI} \times (1/\text{ssf}) \times (1/\text{asf})$$

This n value was calculated for each section counted and summed to determine the total cell number for the entire volume of the SNc in both hemispheres. The degree of cell loss in the lesioned hemisphere was calculated as a percentage of the total cell number of the unlesioned hemisphere. A minimum of 100 systematic samples (i.e. counting frames) were taken from the SNc in each hemisphere in each individual rat brain in order to obtain precise estimations of the total SNc cell number and produce a Coefficient of Error of <0.1 (Gundersen *et al.* 1999). To ensure consistently accurate and reproducible cell counts, 10 sections were counted three times using the same parameters as described above. To assess intra-rater reliability, the Coefficient of Variance was calculated using the percentage deviation from the mean to demonstrate a Coefficient of Variance of $<5\%$, indicating estimates are precise, reliable and reproducible when conducted by a single rater, as illustrated in Figure 2.11 below. Statistical analysis in SPSS confirmed the intra-class correlation coefficient value was >0.99 , indicating a very high degree of intra-rater reliability.

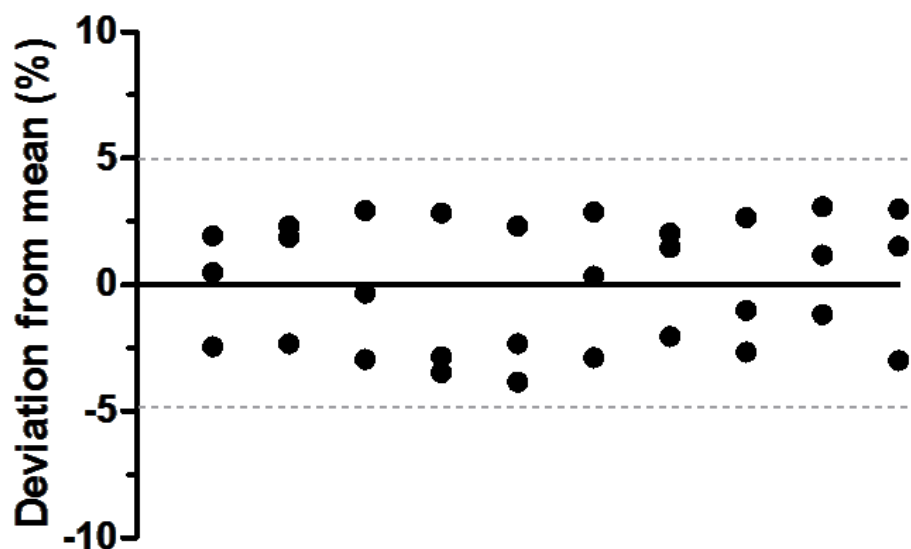
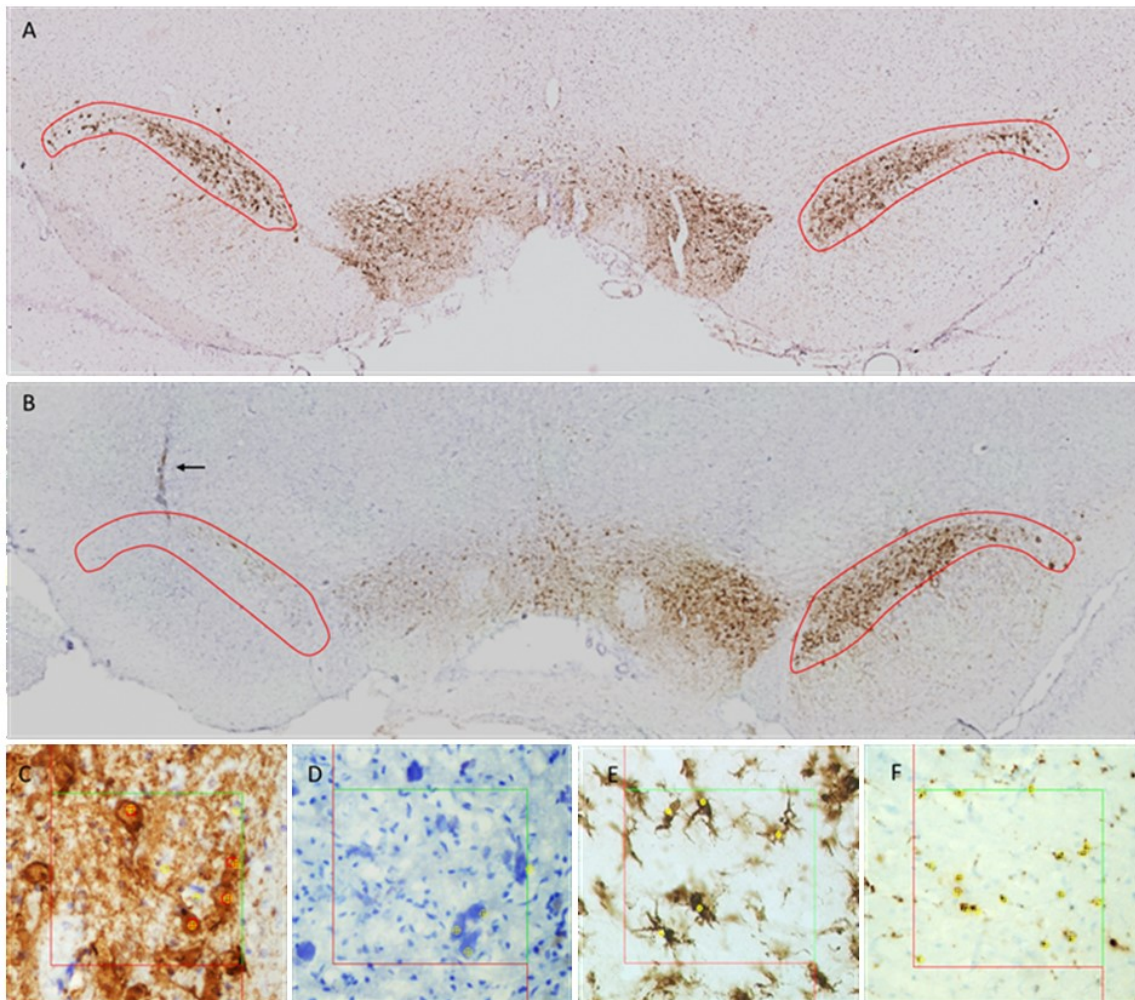
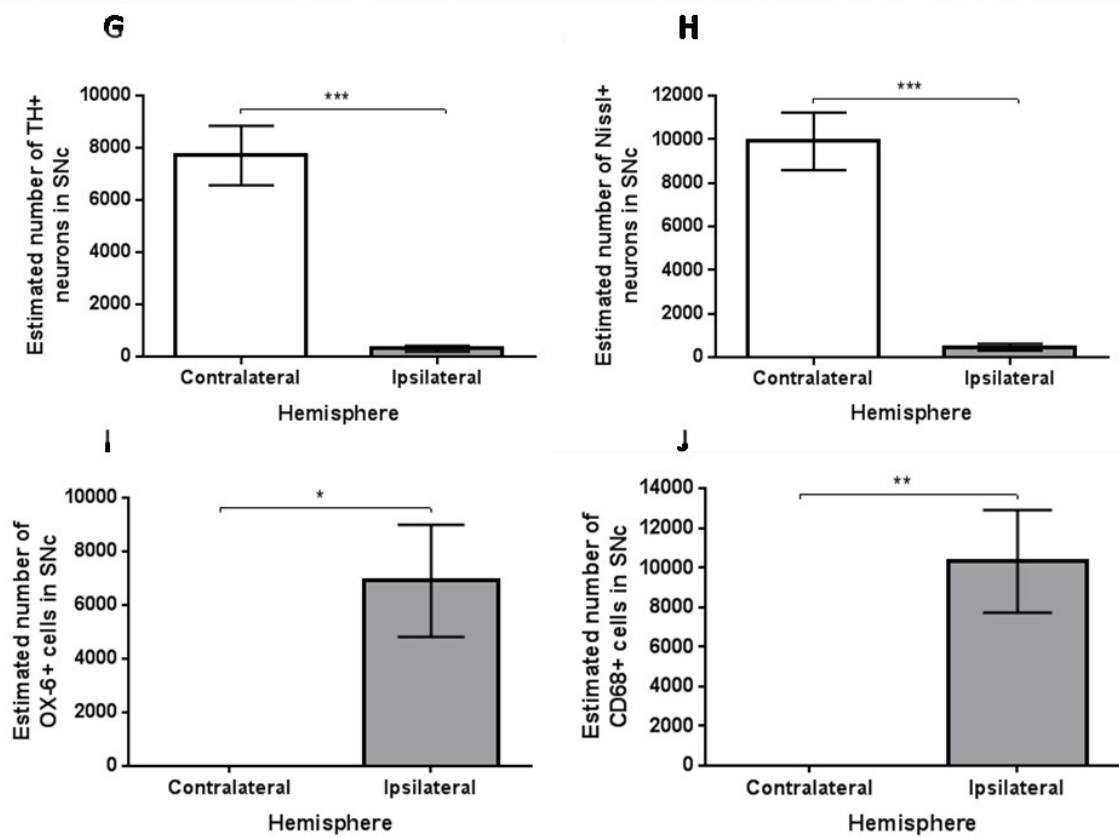


Figure 2.11: Validation of stereological cell counting reliability. Intra-rater reliability was validated for repeated cell counts of TH+ neurons within the SNc of 10 sections. The Coefficient of Variance was calculated from the percentage deviation from the mean and all counts fell within the 5% threshold, indicating reproducible and reliable cell counts were achieved by a single rater.

2.2.3.2 Criteria for stereological cell counting: morphological assessment of neurons and microglia

The counting frame used for stereology has clear boundaries with regions of inclusion and exclusion. The areas within the counting frame and to the right are inclusion zones, providing cell bodies do not cross the red line of exclusion (see Figure 2.12, C-F). Cell bodies crossing the red line were always excluded, whereas cell bodies crossing the green line were included. Neurons were counted only if they had TH+ or Nissl+ staining within the cell body. To avoid overestimation of cell numbers, TH+ neurons were assessed as whole or untransected if the nucleus or darkly stained nucleolus was visible, surrounded by an immunopositive cell body. Nissl positive (Nissl+) neurons were assessed on their size and morphology; cells with a defined nucleus and larger, clearly defined cell body (>15µm diameter) were counted as neurons, whereas cells with a less clearly defined shape and smaller diameter (<15µm) were considered to be glia and not counted (Davanlou & Smith 2004). Activated microglial cells displaying OX-6 positive (OX-6+) immunostaining were characterised by thickened processes and a larger cell body compared to ramified microglial cells, or as fully activated 'reactive' microglia with a globular morphology and few/no processes (Castaño et al. 1998, Kreutzberg 1996). CD-68 positive (CD68+) phagocytic cells were characterised by their dark brown, punctate staining of cell bodies depicted in Figure 2.12, F.

Figure 2.12 (next page) Representative photomicrographs demonstrating delineation of the SNc and the fractionator method of stereological cell counting. The ROI was delineated (red lines) using TH+ neurons as the neuroanatomical border for stereological cell counts. **A** demonstrates the ROI for an unlesioned animal with an intact SNc in both hemispheres, whereas **B** is a well lesioned vehicle-treated animal with much cell loss in the lesioned hemisphere (left) although a few cells remain allowing the SNc to be delineated. Counting frames were superimposed over the SNc of sections and cells were counted with markers: **C** shows TH+ dopaminergic neurons, **D** shows Nissl+ neurons, **E** shows OX6+ activated microglia and **F** shows CD68+ macrophages in the SNc. Cells with a complete cell body containing a nuclei/nucleolus within the frame or crossing the green inclusion line were counted (red and yellow markers), whereas incomplete cells or cells found outside the counting frame or crossing the red exclusion line were ignored. The entire depth of the section could be seen in one plane of focus. Low magnification images were taken at 4x magnification (A and B). High magnification cell counting images were taken at 10x (C and D) and 20x (E and F) magnification. **G** demonstrates total estimated TH+ cell numbers in the contralateral and ipsilateral hemispheres of 7.5µg lactacystin-lesioned rats 19 days post-lesioning surgery (n=8). Similarly, **H** demonstrates total estimated Nissl+ cell numbers, **I** demonstrates total estimated OX-6+ cell numbers and **J** demonstrates total estimated CD-68+ cell numbers in the same group of animals. A significant loss of cells is shown in the ipsilateral lesioned SNc. Data is shown as total cell number ± SEM and analysed with paired t-tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, contralateral vs ipsilateral SNc.



2.2.3.3 Validation of coordinates and lesioning accuracy in 6-OHDA lesioned animals

To determine the accuracy of SNc lesioning, rats were unilaterally lesioned with 6-OHDA (2.1.3.2), and development of behavioural deficits was monitored over 10 days in the vertical cylinder test for forelimb-use asymmetry (2.1.5.1) and for asymmetric rotational behaviour in response to amphetamine (2.1.5.2). A repeated measures ANOVA and Bonferroni post-hoc test was used to compare baseline measurements of contralateral forepaw use to days 7 and 10 post-lesion (Figure 2.13 A). A significant decrease ($p<0.0001$) in contralateral forelimb use between pre- and post-lesioned animals was seen by day 7. The amphetamine-induced rotational asymmetry test (Figure 2.13 B) revealed the average number of full ipsiversive rotations per 5 minutes was significantly higher than contraversive rotations (paired t-test: $p<0.0001$). This suggests that the animals had much lower levels of striatal dopamine in the ipsilateral, lesioned hemisphere compared to the contralateral hemisphere, causing rats to rotate towards the lesioned side. As animals showed clear behavioural alterations in 7-9 days in both behavioural tests, a loss of nigrostriatal function was confirmed. Additionally, dopaminergic neurons in the ipsilateral SNc were selectively killed and histological assessment of the needle tract confirmed anatomically correct needle placement in the SNc, as illustrated previously in Figure 2.10.

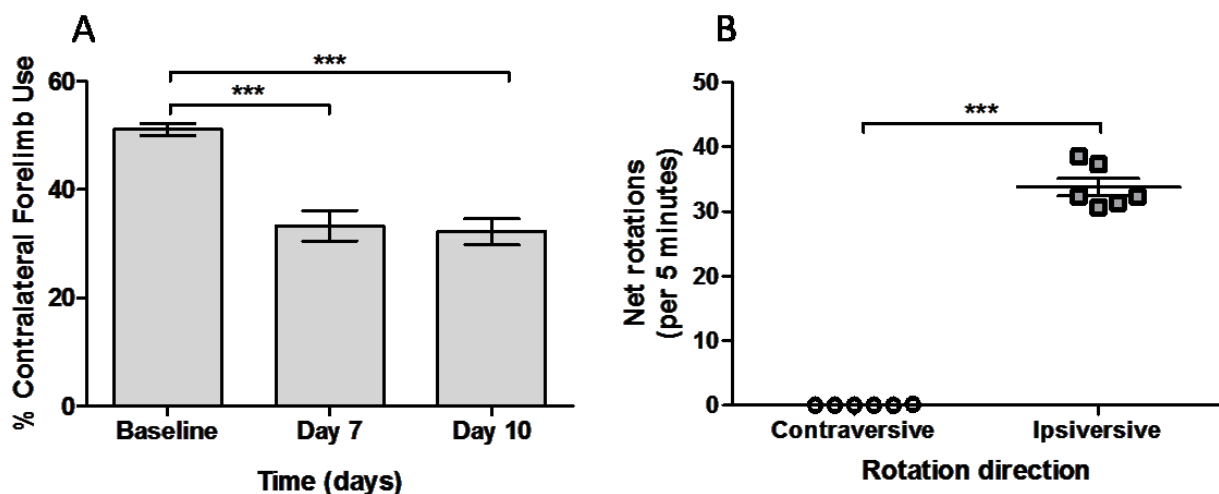


Figure 2.13: Unilateral 6-OHDA lesioning of the SNc resulted in time-dependent development of behavioural deficits. (A) Baseline measurements in the vertical cylinder test were taken prior to lesioning surgery, and testing was subsequently carried out at days 7 and 10 post-lesioning. 6-OHDA lesioned rats ($n=6$) demonstrated a significant reduction in contralateral forepaw use by day 7. Data are shown as mean $\pm$ SEM, one-way ANOVA: *** $p<0.001$. (B) On day 10, amphetamine-induced rotations were measured over 30 minutes with all animals clearly rotating towards the lesioned side (ipsiversive). Data is shown as net mean rotations $\pm$ SEM, paired t-test: *** $p<0.001$.

2.2.4 Progression of TH+ dopaminergic cell loss in the SNc following 7.5µg and 2.5µg lactacystin lesioning

A delayed start to therapeutic intervention was used in neuroprotection studies in this thesis to ensure the clinical relevance of findings. Based on the progression of motor deficits in the lactacystin model seen in previous studies in our lab, day 4 was selected as the optimal time to begin therapeutic treatment, when lactacystin-induced cell death has begun but no overt motor deficits are observable (Chan. H, unpublished observation 2010 and Harrison. I, unpublished observation, 2011, Vernon et al. 2010). As the animals used for later neuroprotection studies were intranigally lesioned with 2.5µg or 7.5µg lactacystin, the degree of nigral cell loss at day 4 was examined to further understand the degree of neurodegeneration at the time of therapeutic intervention. This data was compared to the dopaminergic cell loss in lesioned animals at day 19 in order to profile the progression of dopaminergic neuronal loss throughout a drug study (Figure 2.14).

In order to assess the pathological changes occurring in the brain at the time drug treatment would begin, male Sprague-Dawley rats (240-290g) were unilaterally lesioned in the SNc with either 2.5µg (n=6) or 7.5µg (n=6) lactacystin. Animals were then sacrificed at day 4 and brains were removed and fixed for histological processing and stereological analysis. Histological assessment of rats lesioned intranigally with 2.5µg or 7.5µg lactacystin at day 4 post-lesioning revealed visible neuronal cell loss in the ipsilateral SNc compared to the contralateral SNc in both models, as shown in Figure 2.15. To examine the progression of TH+ dopaminergic cell loss from the SNc by 19 days after lesioning (the length of neuroprotection studies) with both 2.5µg and 7.5µg lactacystin, brains from animals treated with saline for 14 days from day 4 post- lactacystin lesioning (19 days after lesioning) were compared to brains of lesioned animals at day 4 post-lesioning, as illustrated in Figure 2.14. Correct needle placement was verified after Nissl counterstaining, as described in section 2.2.2.6. Staining of TH+ neurons and Nissl cell bodies was carried out to quantify dopaminergic cell loss in the SNc with stereology. These animals were treated with saline (i.p.) from day 4-18 due to their later use as control animals in Chapter 3 when testing neuroprotective therapies, but were used here to examine the progression of the model.

Stereological estimates of the total number of TH+ neurons and Nissl+ neurons lost in the ipsilateral SNc following lactacystin lesioning at both day 4 and day 19 were analysed using a two-way ANOVA (Figure 2.14 A and B). Results showed a significant effect of time after lesioning on TH+ cell loss in the ipsilateral SNc for both lactacystin concentrations ($F_{(2,29)}=47.25$, $p<0.0001$), and showed a small but non-significant ($F_{(1,29)}=0.87$, $p=0.35$) effect of lactacystin concentration overall, although a visible difference between lactacystin concentrations at day 4 post-lesioning was apparent, with 2.5µg lactacystin demonstrating a slightly lesser degree of neurodegeneration at this time point. Nissl+ cell

loss mirrored TH+ cell loss, demonstrating a significant effect of time after lesioning for both lactacystin concentrations ($F_{(2,29)}=59.52$, $p<0.0001$), and a small but non-significant effect ($F_{(1,29)}=1.37$, $p=0.25$) of lactacystin concentration overall.

Comparing percentage loss of neurons in the ipsilateral SNc vs contralateral SNc at both day 4 and day 19 demonstrated a small difference in the percentage of TH+ dopaminergic neurons and Nissl+ neurons remaining in the ipsilateral SNc between 7.5 μ g and 2.5 μ g lactacystin-lesioned rats (Figure 2.14 C and D). As illustrated in Figure 2.14 C, the percentage of TH+ neurons remaining in the ipsilateral SNc (compared to the contralateral SNc) at day 4 after 7.5 μ g lactacystin lesioning was $46.3 \pm 2.6\%$ ($n=3$) compared to $60 \pm 1.8\%$ following 2.5 μ g lactacystin ($n=4$), indicating a small concentration-dependent effect of lactacystin on dopaminergic neuronal cell death in the SNc, with a difference of ~14% between lesion concentrations at this stage. Similarly, the percentage of Nissl+ neurons remaining in the ipsilateral SNc at day 4 after 7.5 μ g lactacystin was $46.1 \pm 1.5\%$ compared to $61 \pm 2.3\%$ following 2.5 μ g lactacystin (Figure 2.14 D), indicating dopaminergic and some non-dopaminergic neuronal cell death occurred in response to lactacystin lesioning by day 4. This finding is supported by that of Reaney et al. (2006) who showed a loss of GABAergic neurons following lactacystin treatment, and suggests that some non-dopaminergic neurons are also susceptible to lactacystin-induced UPS inhibition. By day 19 post-lesioning, just $3.7 \pm 0.95\%$ of TH+ dopaminergic neurons remained in the lesioned SNc following lesioning with 7.5 μ g lactacystin ($n=8$). Similarly, $4.3 \pm 1\%$ of Nissl+ neurons remained in the 7.5 μ g lactacystin lesioned SNc at day 19 post-lesioning. In comparison, following lesioning with 2.5 μ g lactacystin ($n=6$), $14 \pm 1.9\%$ of TH+ dopaminergic neurons remained in the lesioned SNc at day 19 post-lesioning. Similarly, $14.4 \pm 2.02\%$ of Nissl+ neurons remained in the 2.5 μ g lactacystin lesioned SNc at day 19 post-lesioning. Equivalent levels of Nissl+ cell loss indicated that lactacystin induced cell death of dopaminergic neurons, rather than inhibiting the TH enzyme.

Interestingly, the loss of 54% of SNc dopaminergic neurons by day 4 seen here following 7.5 μ g lactacystin lesioning corresponds to the early motor phase of PD, with around 50-60% of SNc dopaminergic neurons lost by the onset of motor symptoms in patients (Dauer & Przedborski 2003, Fearnley & Lees 1991). The loss of 40% of nigral dopaminergic neurons by day 4 in 2.5 μ g lactacystin corresponds with the presymptomatic phase of PD, so may be a useful model to determine the efficacy of earlier therapeutic intervention, as well as allowing neuroprotective treatments to take effect without the nigrostriatal system being overwhelmed by toxin. Lesioning with 2.5 μ g lactacystin induced a progressive dopaminergic neuronal loss similar to that of 7.5 μ g lactacystin lesioned animals, although the loss was less extensive; a difference of 14% was apparent between models at day 4, and a difference of 10% was apparent between models by day 19 post-lesioning. Collectively, these findings suggest that intranigral administration of 2.5 μ g and 7.5 μ g lactacystin is sufficient to produce

a model of progressive and extensive nigral neurodegeneration, and that day 4 is a clinically relevant time point for therapeutic intervention with neuroprotective compounds in the lactacystin model of PD and corresponds best with the pre-motor/early motor stages of PD, when protective therapies may be most effective.

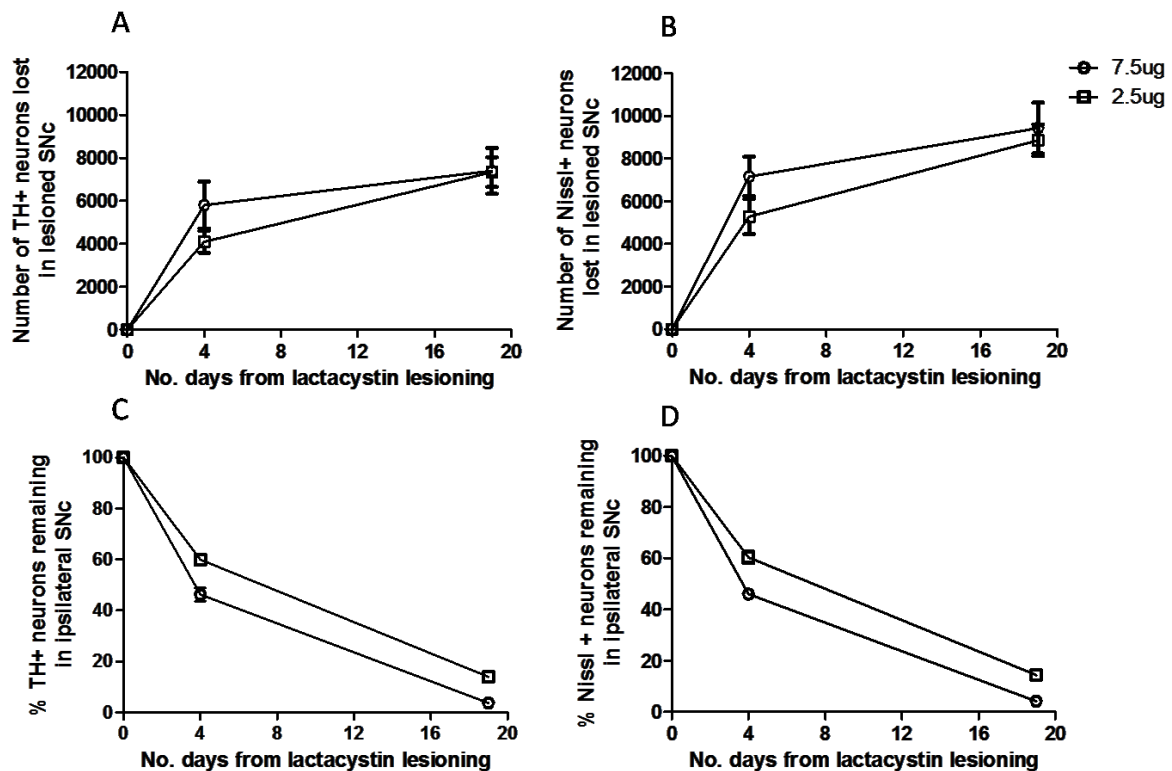


Figure 2.14: Progression of TH+ dopaminergic and Nissl+ cell loss in the ipsilateral SNc following unilateral lesioning with 7.5µg and 2.5µg lactacystin. Animals were unilaterally lesioned in the SNc with either 7.5µg or 2.5µg lactacystin, and the loss of TH+ dopaminergic neurons and Nissl+ neurons in the SNc was assessed at day 4 or day 19 post-lesioning. Brains were removed and processed for immunohistochemical staining and stereological cell counting. Stereological estimates of the total number of TH+ neurons (A) and Nissl+ neurons (B) lost in the SNc following lactacystin lesioning were analysed using two-way ANOVA and show a small but non-significant ($p=0.35$) difference between lactacystin concentrations at day 4 post-lesioning. Comparing percentage loss of TH+ (C) and Nissl+ (D) neurons in the ipsilateral SNc vs contralateral SNc at both day 4 and day 19, a small difference in the percentage of TH+ dopaminergic neurons remaining in the ipsilateral SNc was observed between 7.5µg and 2.5µg lactacystin-lesioned rats (7.5µg lactacystin: day 4 $n=3$, day 19 $n=8$; 2.5µg lactacystin: day 4 $n=4$, day 19 $n=6$).

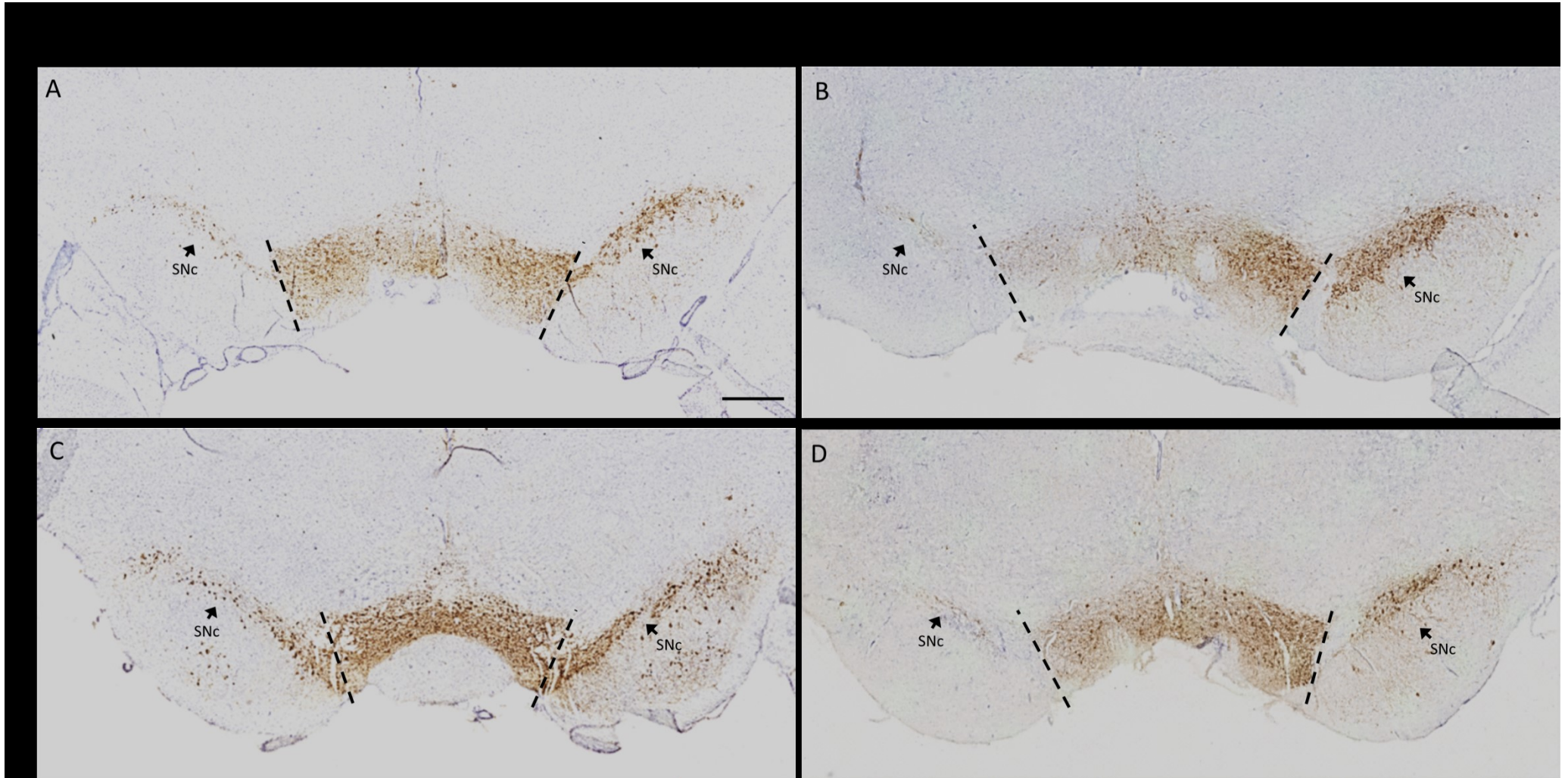


Figure 2.15: Effect of SNc lesioning with 7.5µg and 2.5µg lactacystin lesioning of the SNc on TH+ dopaminergic neuronal loss at day 4 and day 19 post-lesioning. Representative photomicrographs of nigral tissue from lesioned animals, Rats unilaterally lesioned in the SNc with 7.5µg or 2.5µg lactacystin were either left untreated (day 4) or treated with saline (day 19) i.p. daily from day 4 post-surgery for 14 days. At day 4 or day 19 post-lesioning, brains were removed and processed for immunohistochemical staining. By day 4 post-lesioning, a lactacystin concentration-dependent loss of TH+ dopaminergic neurons (brown neuronal staining) from the ipsilateral SNc was visible (A and C). By day 19 post-lesioning, an extensive loss of TH+ dopaminergic neurons was seen in the ipsilateral SNc compared to the contralateral SNc of animals lesioned with both 2.5µg and 7.5µg lactacystin by day 19 (B and D). Arrows denote the SNc in the ipsilateral lesioned and contralateral unlesioned hemisphere. Images taken at 4x magnification, scale bar: 100µm for all images.

2.3 In vitro methods

2.3.1 Primary microglial cell culture

2.3.1.1 Introduction

To further investigate the anti-inflammatory effects of mGlu receptor activation on microglia, *in vitro* experiments were conducted. In this study, primary rat microglial cells were isolated and activated by treatment with LPS in order to reproduce the *in vivo* inflammatory situation associated with the pathogenesis of PD. Primary rat microglia have previously been shown to express the mGlu8 receptor (Taylor et al. 2003), and more accurately represent the natural phenotype of microglial cells *in vivo* than microglial cell lines. By using primary cells *in vitro*, activating conditions could be tightly controlled and assays could be performed on cell medium to quantify the microglial activation state. When treated with LPS, primary microglia become activated, adopting an amoeboid morphology and activating iNOS which results in the production of NO, as well as producing pro-inflammatory cytokines such as TNF α , IL-6 and IL-1 β (see Introduction section 1.2.4). Following 18h incubation with LPS, NO levels were quantified in culture medium using the Griess assay to detect nitrite levels (as an index of NO production) as described in section 2.3.1.6 below, and TNF α levels in medium were detected with enzyme linked immunosorbent assays (ELISAs) in order to measure the level of activation induced by incubation with LPS, as described in section 2.3.1.7 below. This time point (18h) was chosen based on previous findings within our lab that indicated 18h of LPS treatment causes robust microglial activation, and was also based on literature that demonstrates 18 hours is a midway point between the peak expression of the proinflammatory cytokine TNF α and NO synthesis (Byrnes *et al.* 2009, Zujovic *et al.* 2000). Cell viability assays were performed directly on remaining cells to detect possible cytotoxic or proliferative effects of LPS treatment. Cells were also stained using immunofluorescence to confirm the purity of microglial cultures, as described in section 2.3.1.4 below.

2.3.1.2 Reagents and consumables

In vitro experiments were carried out with autoclaved or ethanol-sterilized equipment and reagents in ventilated sterile hoods that had been U.V. light irradiated and cleaned with 70% ethanol. Sterile conditions were carefully maintained throughout the experiments by ensuring suitable ventilation level and hood heights were used. Incubators for primary cell culture only were regularly cleaned with 70% ethanol and water reservoirs replaced weekly with autoclaved water containing SigmaClean® Water Bath Treatment (Sigma). Sterile cell culture plates (24 and 96 well plates), sterile pipette tips, sterile serological pipettes, sterile eppendorf tubes (2.5ml) and sterile Falcon tubes (50ml) were all purchased from VWR (Lutterworth, UK). LPS derived from Salmonella Minnesota was obtained from Enzo Life Sciences Ltd. (Exeter, UK). L-glutamine, Dulbecco's modified Eagle medium (DMEM),

penicillin/streptomycin solution, foetal calf serum, trypan blue, bovine serum albumin (BSA), paraformaldehyde powder, Griess reagent (sulfanilamide plus N-1-naphthylethylenediamine) and Neutral Red dye were purchased from Sigma-Aldrich (Dorset, UK). Primary microglia were maintained in a medium (complete microglial medium) comprised of DMEM (with phenol red) supplemented with 10% filter-sterilized heat-inactivated foetal calf serum, 4mM L-glutamine, 50u/ml Penicillin and 50µg/ml Streptomycin and 8mM L-glutamine in a humidified incubator at 37°C and with 5% CO₂ ventilation.

2.3.1.3 Isolation of rat primary microglia

Primary microglia were isolated from Wistar rat pups at post-natal day 7 (p7) (pregnant dams purchased from Charles River Laboratories, UK) with a non-enzymatic method utilising Percoll (Sigma, P1644), a low viscosity solution that can be used to isolate different cells, organelles and viruses using varying density gradients to separate them (Kingham & Pocock 2000). Pups were quickly sacrificed by decapitation with sterile scissors and brains were immediately removed, the cerebellum was detached and discarded and the remaining brains removed into cold 1x PBS (137 mM NaCl, 5 mM KCl, 25 mM Na₂HPO₄, 11 mM glucose and 0.2% bovine serum albumin, pH = 7.4) on ice. Collected brains were subsequently moved into fresh cold PBS to eliminate any blood contamination, and were homogenized in a sterile manual glass pestle tissue homogeniser (Thomas Scientific, New Jersey, USA) to break up and liquidise the tissue. Tissue homogenate from all pups was then transferred into a Falcon tube and made up to 50ml total volume with PBS, before being centrifuged at 450g at 20°C for 10 minutes. Pelleted homogenate was gently re-suspended in 12ml of 66% Percoll solution in PBS, then carefully overlaid with 12ml of the less dense 34% Percoll solution in PBS, before overlaying this with 12ml PBS (1x); overlaying was done extremely slowly to avoid mixing of density layers. To separate cells into different density layers, the gradient was centrifuged at 4°C at 1800g for 45 minutes with the centrifuge brake off, to avoid gradient disturbance. Once cells had separated into layers at the interface of each gradient, as illustrated in Figure 2.16, the myelin layer at the PBS: 34% Percoll interface was removed and discarded. The microglial layer was then collected at the 34%:66% Percoll interface with a pipette, and transferred into a fresh 50ml Falcon tube already containing 30ml PBS before being centrifuged at 800g at RT for 10 minutes. The microglial pellet was re-suspended in warm complete microglial medium and live cell numbers were counted using the Trypan blue exclusion assay. Trypan blue is a diazo dye which is not absorbed by viable cells with intact membranes, but is absorbed by dead or dying cells with compromised membranes. Therefore, cells excluding the blue dye were counted with a haemocytometer to quantify the number of viable cells in suspension. Cells were seeded onto 24 well plates at a density of 1x10⁵ live cells per well with 750µl complete microglial medium and placed in the incubator (37°C, 5% CO₂) overnight to attach and re-establish. Medium was

changed the following morning to remove any dead or unattached cells. Primary microglia were left to attach for 24 hours before experiments began. On average, each rat pup brain yielded approximately 200,000 cells.

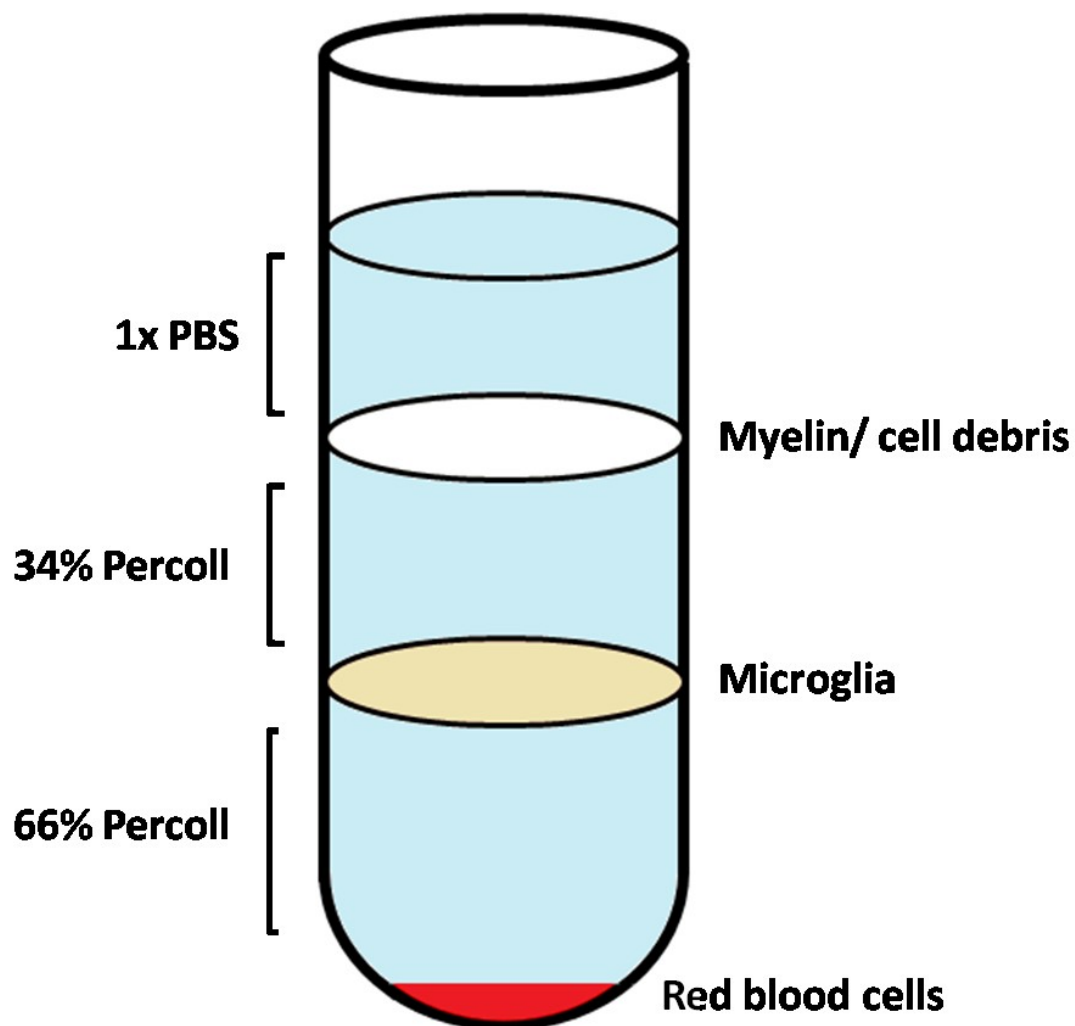


Figure 2.16: Isolation of primary microglia using the Percoll gradient separation technique. Wistar rat pups' brains are removed, dissected on ice and homogenised. Homogenate is slowly overlaid with layers of 66% and 34% Percoll, followed by 1x PBS. Following centrifugation at 4°C, red blood cells form a sediment at the bottom of the falcon tube, myelin and cell debris form a layer at the top PBS:34% Percoll interface and microglial cells form a layer at the middle 34%:66% Percoll interface.

2.3.1.4 Fluorescent immunocytochemical staining of primary microglia

The concentrations of antibodies used for fluorescence immunostaining were slightly higher than those used in the ABC method (see Table 2.2), as the signal can be less amplified. The staining protocol was the same as that used for the ABC method in section 2.2.2.3 until the secondary antibody incubation steps where the fluorophore-conjugated secondary antibody was incubated for 1 hour in the dark. After washing in PBS, cells were incubated in the fluorescent nucleic acid stain DAPI (1:1000, Invitrogen) for 5 minutes to counterstain all cell nuclei. Cells on coverslips were mounted in Vectasheild mounting medium for fluorescence (Vector Laboratories) after washing and stored in the dark at 4°C before being studied under the fluorescence microscope (Nikon Eclipse 80i, Surrey, UK) equipped with a QICAM camera (QImaging, British Columbia, Canada). For double immunofluorescence, staining with primary and secondary antibodies could be carried out in parallel where they were from different species and would not cross-react.

To confirm that isolated cells were microglia, some cells were plated onto glass coverslips in wells to aid removal for immunostaining. Cells were fixed in ice cold 4% PFA for 5 minutes, before being washed in PBST and immunostained with an anti-OX42 antibody to specifically label cells expressing the pan microglial marker CD11b, as described in section 2.2.2.3. Immunofluorescent staining of untreated primary microglial cells 24 hours after isolation revealed no contamination by OX42 negative non-microglial cells, as illustrated in Figure 2.17 A, confirming the purity of isolated cells. Cells activated with 125ng/ml LPS showed similar levels of OX42 positive staining with no OX42 negative cells visible (Figure 2.17 B). Specificity of OX42+ staining was confirmed by omitting the primary antibody to ensure no non-specific binding was occurring (negative control, Figure 2.17 D). Additional staining to detect neurons (NeuN) and astrocytes (GFAP) revealed no staining of these cell types as shown in Figure 2.17 D, confirming the purity of primary microglial cell cultures.

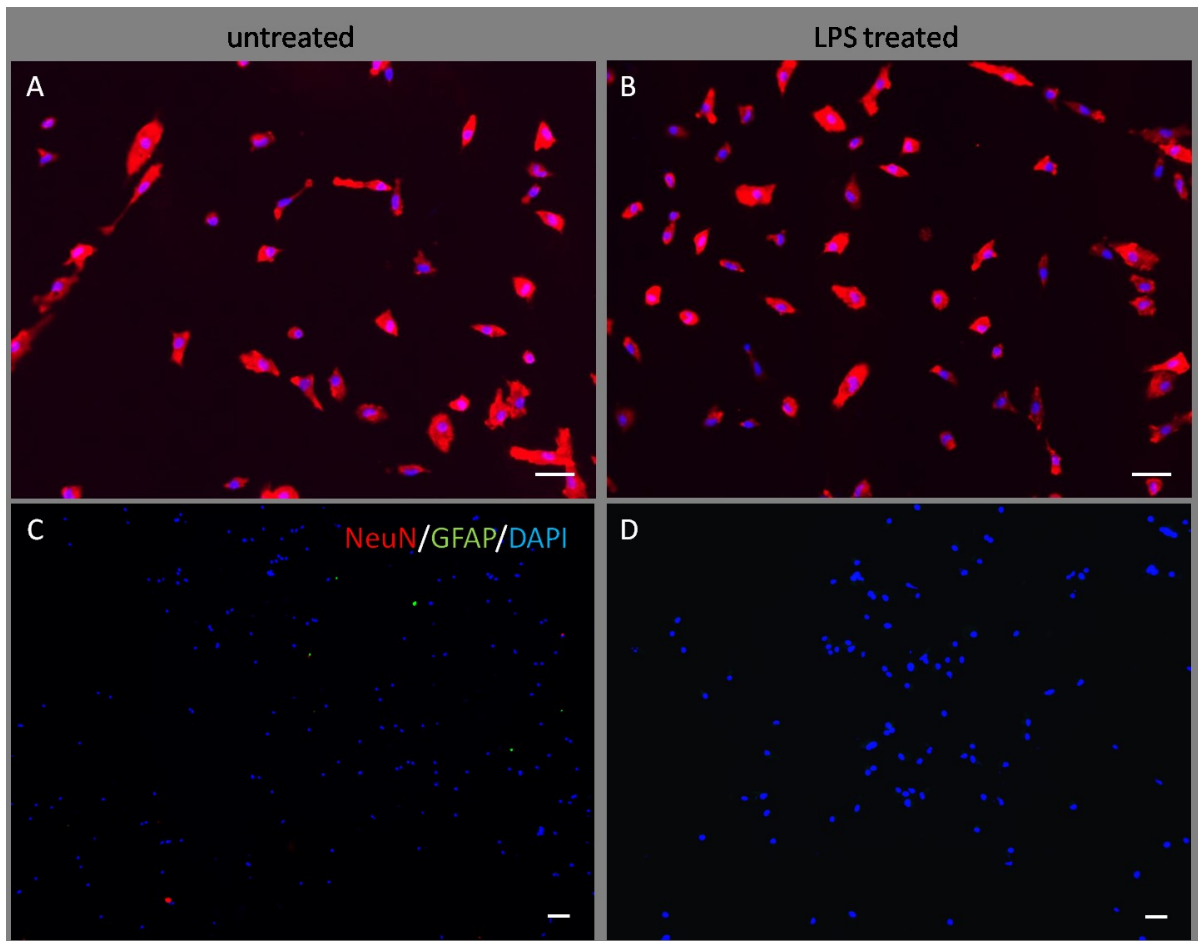


Figure 2.17: Confirming purity of isolation of primary microglial cells. Rat primary microglia were isolated from pup brains at P7 and plated in 24 well plates (on glass coverslips) at a density of 1×10^5 before being left to re-establish for 24 hours. Cells were then left untreated or treated with LPS (125ng/ml) for 18 hours in order to induce activation. Cells were subsequently fixed and stained with immunofluorescence. All visually checked isolated primary cells in culture stained positive for OX42, a pan microglial marker (red), confirming their purity. OX42+ staining was present in untreated cells (A) and cells activated with 125ng/ml LPS (B). No positive staining for neurons (NeuN, red) or astrocytes (GFAP, green) was apparent in cultures (C) and specificity of staining was confirmed by excluding primary antibody (D, OX42 negative (red), DAPI+ staining all nuclei (blue)). Images taken at 2x and 4x magnification. Scale bar: 30 μ m.

2.3.1.5 Cell viability assessment with the Neutral Red assay

The neutral red assay (Parish & Müllbacher 1983) was used at the conclusion of cell culture experiments to quantify cell viability, and determine if treatment had cytotoxic or proliferative effects in microglia. At physiological pH, neutral red dye has a weak cationic charge allowing it to passively enter viable cells. As the lysosome has a lower pH than the cytoplasm due to proton gradients, the neutral red dye becomes charged and accumulates there (Repetto *et al.* 2008). Dying or dead cells are unable to maintain the pH gradient between the cytoplasm and lysosome, and therefore the dye does not accumulate in these cells. An acidified ethanol solution then releases the accumulated dye from viable cells, and can be quantified by spectrophotometry at 540nm. The absorbance reading of the solubilised dye is therefore directly proportional to the number of viable cells remaining in wells. Neutral red stock solution (4mg/ml neutral red dye in PBS) was dissolved in complete microglial medium to give a final concentration of 40µg/ml neutral red solution. At the conclusion of the cell culture experiment, cell medium was collected for further analysis, and cells were incubated in 100µl neutral red solution for 3 hours in a humidified incubator at 37°C and with 5% CO² ventilation. Neutral red solution was subsequently aspirated from wells and cells were washed with sterile PBS, before adding 150µl of destain solution (50% ethanol, 49% water, 1% glacial acetic acid) for 10 minutes with rapid shaking of the plate to release the dye. The dye solution was then transferred into a 96 well plate for spectrophotometric reading in a microplate reader (VersaMax Microplate Reader, Molecular devices) at 540nm. Absorbance at 540nm was converted to a percentage of the control group and viability data was expressed as mean ± SEM, as illustrated in Figure 2.18 below.

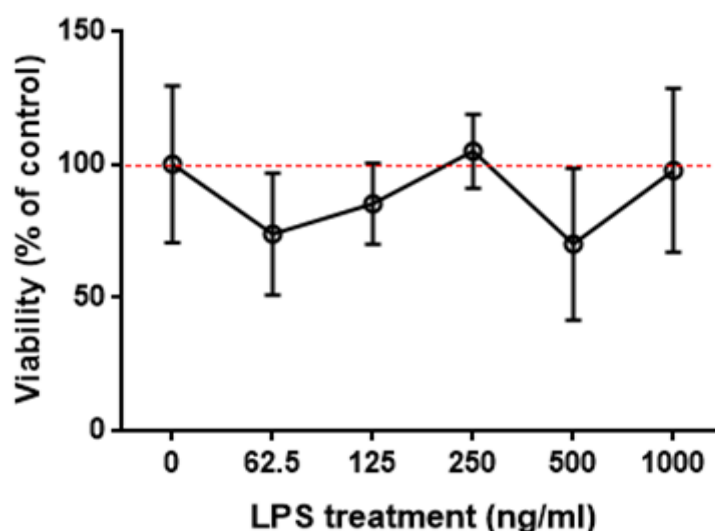


Figure 2.18: Cell viability as measured by the Neutral Red assay. Viability of rat primary microglia was examined following treatment with increasing concentrations of LPS (62-1000ng/ml) for 18 hours. LPS did not affect the viability of cells when compared to control in a one-way ANOVA. Results are displayed as mean $\pm$ SEM of an independent experiment measured in triplicate.

2.3.1.6 Quantification of NO with the Griess Assay

NO has a variety of functions in the body, acting as a vasodilator, a neurotransmitter and as a defence against pathogens (for review see Schmidt and Walter, 1994). Upon activation, microglia up-regulate iNOS, which results in NO production and can be cytotoxic when it reacts with ROS such as superoxide, to produce peroxynitrite (Beckman & Koppenol 1996). Due to its short half-life and unstable nature, NO is often quantified indirectly; in medium, NO decomposes to form the stable end products of nitrate (NO_3^-) and nitrite (NO_2^-) which can then be quantified in a spectrophotometric assay. The Griess assay, developed by J.P Griess (Griess 1879), allows detection of the stable metabolite nitrite, as an indirect measure of NO levels in culture medium. At the experimental endpoint of microglial cell culture experiments in this thesis, the Griess assay was used as an indicator of the degree of microglial activation due to their increased NO production upon activation.

In the Griess reaction, NO_2^- reacts with sulfanilamide to produce a diazonium salt intermediate, which reacts with the azo dye N-1-naphthylethylenediamine, and results in the formation of a coloured azo chromophore, as illustrated in Figure 2.19 below. The chromophore levels can then be measured in a spectrophotometer at 540nm to quantify the levels of nitrite. At the experimental endpoint when microglial cells had been activated by LPS and treated with the test compound, culture medium was collected and centrifuged at 1200g for 5 minutes to remove cell debris. 50 μ l of medium from each condition was plated in a clear 96 well plate in triplicate. A set of standards of known nitrite concentrations (0-50 μ M sodium nitrite in complete microglial medium, see Figure 2.18) were plated

alongside samples in triplicate in order to later create a standard curve. Finally, 50µl Griess reagent was added to each well, and plates were incubated for 10 minutes on a plate shaker. Optical density was measured at 540nm using a 96-well plate reader (VersaMax Microplate Reader, Molecular Devices). A standard curve of optical density readings was made using the known sodium nitrite standards, and the slope of the line of best fit was used to calculate nitrite concentrations from optical density readings from medium samples (Figure 2.20). A separate standard curve was created for each separate plate to control for variance in optical density readings by the spectrophotometer. LPS treatment showed a trend of increased nitrite levels by primary microglial cultures at concentrations >62.5ng/ml LPS, as illustrated in Figure 2.21, with the highest level of nitrite (3.8µM) detected following exposure to 1000ng/ml LPS, although statistical analysis could not be performed due to low n-numbers.

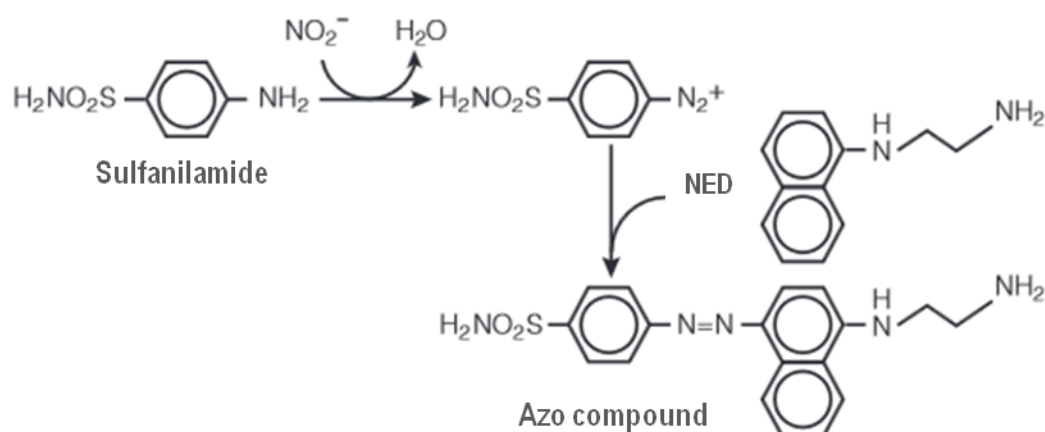


Figure 2.19: Chemistry of nitrite quantification in the Griess Assay. NO is measured indirectly by quantifying levels of one of its metabolites, nitrite (NO_2^-), using the Griess assay. NO_2^- in collected supernatant reacts with sulfanilamide to produce a diazonium salt intermediate, which then reacts with the azo dye N-1-naphthylethylenediamine (NED), and results in the formation of a coloured azo compound which can be measured in a spectrophotometer at 540nm.

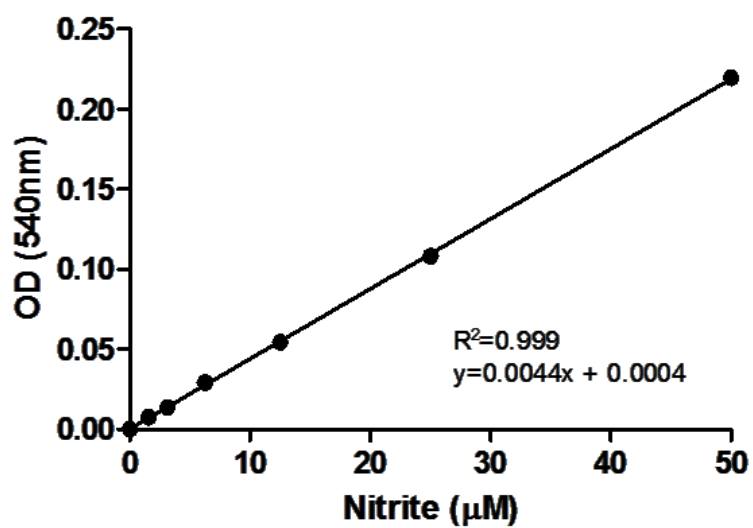


Figure 2.20: Nitrite standard curve for Griess assay. A standard curve of known nitrite concentrations (0-50μM sodium nitrite in complete microglial medium) was created for each Griess assay plate in order to translate optical density (OD) readings at 540nm into nitrite concentrations.

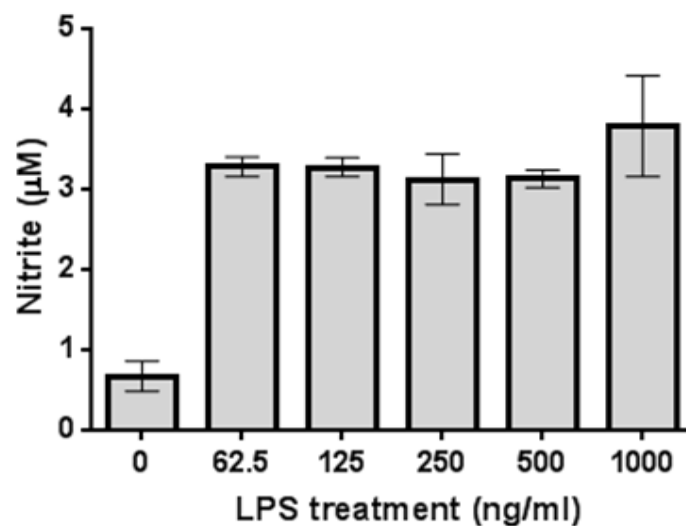


Figure 2.21: Nitrite quantification using the Griess assay. Levels of nitrite in culture medium produced by rat primary microglia were examined using the Griess assay as an indirect measure of NO production following treatment with increasing concentrations of LPS (62.5-1000 ng/ml) for 18 hours. Treatment with >62.5 ng/ml LPS tended to increase nitrite concentration although statistical analysis could not be performed due to low n-numbers.

2.3.1.7 TNF α ELISA

At the experimental endpoint of microglial cell culture experiments in this thesis, levels of the pro-inflammatory cytokine tumour necrosis factor alpha (TNF α) released by microglia into the medium were quantified using an enzyme linked immunosorbent assay (ELISA) as an indicator of microglial activation. ELISAs use antibodies against a specific protein of interest to capture and detect that protein. A series of amplification steps are then applied in order to detect small amounts (pg/ml) of the target protein reliably and effectively, as illustrated in Figure 2.22. A capture antibody captures TNF α from the cell culture medium sample, and a secondary biotinylated detection antibody then binds to this capture antibody. The addition of an avidin peroxidase-conjugate then amplifies the biotin signal by binding 4 avidin molecules to each biotin molecule, creating a complex of peroxidase enzymes. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate solution is then added and is catalysed by peroxidase enzymes to a coloured soluble substrate which can be quantified by a spectrophotometer. TNF α ELISAs were performed using a murine TNF α ELISA development kit (Peprotech Ltd., London, UK) which also cross reacts with rat TNF α and was carried out following the manufacturer's protocol (eBioscience, #88-7324).

Culture medium was collected from wells at the experimental endpoint and stored at -20°C until needed. High binding EIR/RIA 96 well ELISA plates (Corning) were coated overnight at RT with 100 μ l/well of purified anti-TNF α capture antibody (1 μ g/ml in PBS). Wells were then washed with 250 μ l of wash buffer (0.05% Tween-20 in PBS) five times (5x), and non-specific binding was blocked with block buffer (1% BSA in PBS) for 1 hour at RT. Wells were again washed with 250 μ l of wash buffer 5x and were ready for the addition of samples/standards. Medium samples were thawed on ice and standards were made up (0–2ng/ml TNF α in diluent (0.05% Tween-20 and 0.1% BSA in PBS), before 100 μ l of each was added to wells in triplicate and incubated for 2 hours at RT. Following this, wells were again washed in wash buffer x5 and incubated with biotinylated anti-TNF α detection antibody (0.25 μ g/ml in diluent) for another 2 hours at RT. After washing in wash buffer 5x, the signal was amplified by incubating wells for 30 mins at RT with 100 μ l avidin HRP-conjugate (1:2000 in diluent) which binds to biotin. Finally, wells were washed in wash buffer 5x and 100 μ l of ABTS substrate solution (at RT) was added to each well to form a coloured substrate. Absorbance was read in a 96-well plate reader (VersaMax Microplate Reader, Molecular Devices) at both 405nm and 650nm. Colour development was monitored via spectrophotometric readings at 5 minute intervals for 45 minutes, until readings at 405nm were ≤ 0.2 for the 0ng/ml TNF α standard and ≤ 1.4 for the 2ng/ml TNF α standard, as instructed by the manufacturer's protocol. The spectrophotometric reading at 650nm was subtracted from the reading at 405nm for wavelength. Readings of standards were plotted against their known TNF α concentrations to create a standard curve (see Figure 2.23) and the equation from

the line of best fit was used for calculation of TNF α concentrations in medium samples. Data was expressed as mean $\pm$ SEM of triplicate samples. LPS treatment showed a trend towards increased TNF α levels in primary microglial cultures at all concentrations of LPS, as illustrated in Figure 2.24 below, with the highest levels of TNF α detected following exposure to 500ng/ml and 1000ng/ml LPS, although statistical analysis could not be performed due to low n-numbers.

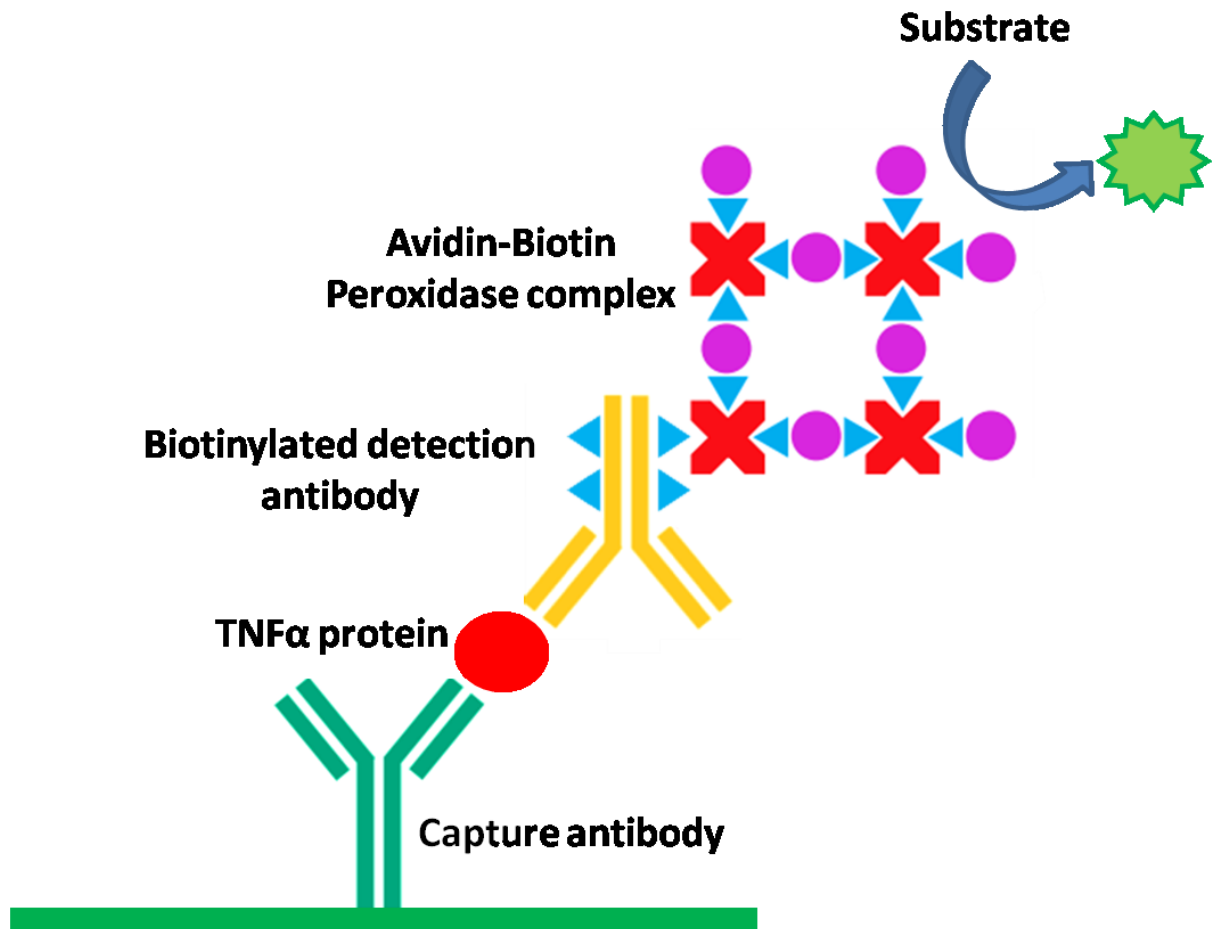


Figure 2.22: Schematic of enzyme linked immunosorbent assay (ELISA) for TNFα. TNFα in the supernatant is bound by the capture antibody which coats the well. A biotinylated detection antibody is then added, which binds to the captured TNFα protein. Addition of the avidin peroxidase-conjugate amplifies the biotin signal, creating a complex of peroxidase enzymes which convert the substrate ABTS into a coloured soluble substrate. The coloured substrate can be quantified in a spectrophotometer at 405nm.

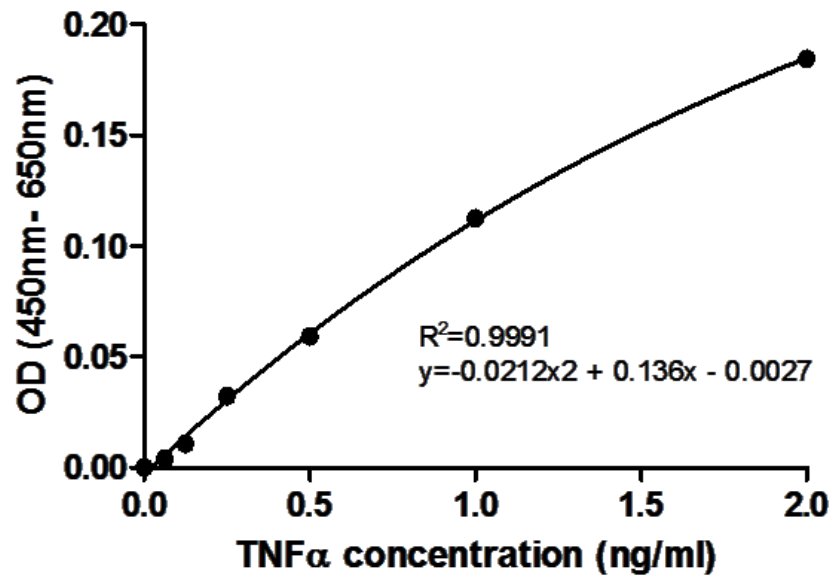


Figure 2.23: TNFα standard curve. A standard curve was created for each TNFα ELISA plate using samples of known TNFα concentrations (0-2ng/ml diluted in 0.05% Tween-20 and 0.1% BSA in PBS). The line of best fit equation was used to convert the optical density (OD) readings for each sample into TNFα concentrations. Data shown as mean ± SEM, n=3.

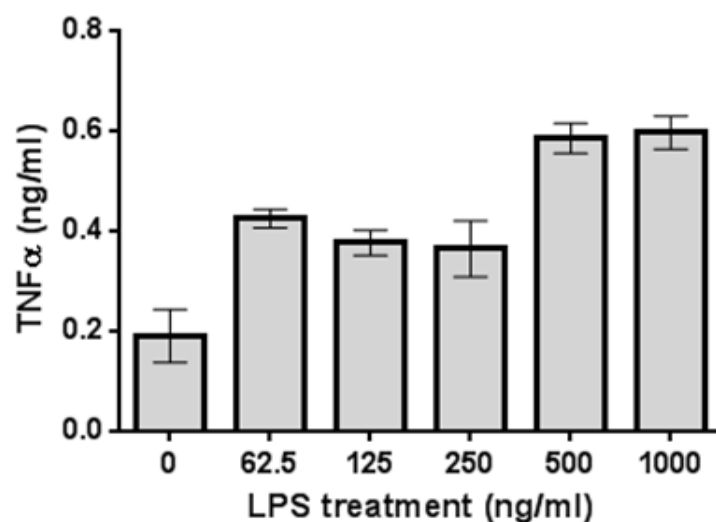


Figure 2.24: TNFα quantification using ELISA method. Levels of TNFα in culture medium produced by rat primary microglia were examined using the ELISA method to quantify TNFα production following treatment with increasing concentrations of LPS (62-1000ng/ml) for 18 hours. Treatment with >62.5ng/ml LPS tended to increase TNFα concentrations although statistical tests could not be performed due to low n-numbers.

Chapter 3: The effects of acute and chronic administration of the mGlu8 receptor agonist DCPG *in vivo* on motor deficits and neuroprotection in the lactacystin model of Parkinson's disease

3.1 Introduction

The effects of mGlu8 receptor activation have been less well studied than mGlu4, due to the limited numbers of sub-type selective compounds available so far (see Table 1.5) and the fact that the antiparkinsonian and neuroprotective effects of broad group III agonists have mainly been attributed to their actions at the mGlu4 receptor (MacInnes et al. 2004, Marino et al. 2003, Valenti et al. 2003). Within the BG motor circuit, mGlu8 receptor immunoreactivity has been detected in the striatum and SNr (Austin et al. 2010) in addition to mGlu8 receptor mRNA being found in high levels in the cortex, moderate levels in the STN, striatum, and SNc, and low levels in the GP and SNr (Messenger et al. 2002), indicating its ideal placement for targeting the overactive BG pathways seen in PD. Additionally, expression of the receptor is up-regulated on activated microglia in multiple sclerosis (MS) lesions in patient tissue (Geurts et al. 2005) as well as being strongly detected on primary microglia from rat pups (Taylor et al. 2003), although this expression may be age-dependent. The localisation of mGlu8 on microglia, particularly activated microglia, may provide a useful target to attenuate the detrimental effects or enhance the beneficial effects of the activated microglia seen in PD and many other neurodegenerative diseases.

Recent findings show that mGlu8 receptor activation with the selective agonist (S)-3,4-dicarboxyphenylglycine (DCPG) provides antiparkinsonian effects in rodent models with prolonged dopamine depletion (Johnson et al. 2013). In this study DCPG, when injected into cerebral ventricula (i.c.v), can robustly reverse reserpine-induced akinesia and haloperidol-induced catalepsy in rats, but only if prolonged pre-treatment of reserpine or haloperidol is given (several doses of either reserpine or haloperidol over 18-20 hours). In addition, DCPG was able to improve forelimb-use asymmetry in unilateral 6-OHDA-lesioned rats, suggesting that DCPG only provides antiparkinsonian effects in models with prolonged dopamine depletion (Johnson et al. 2013). It may be that following prolonged dopamine depletion, changes in mGlu8 receptor function or expression occur, allowing it to be effectively targeted. These findings may explain the contradictory lack of antiparkinsonian efficacy by DCPG found in other previously published studies using short-term drug-induced akinesia or catalepsy models (Broadstock et al. 2012, Lopez et al. 2007).

DCPG (see Figure 3.1) is a potent and selective mGlu8a receptor agonist with an estimated EC₅₀ of 31nM, with >100 fold selectivity for mGlu8 over the other group III receptor subtypes (Thomas et al. 2001). DCPG is more potent and selective for the mGlu8 receptor than other previously reported agonists, and is centrally active when administered systemically (Marabese *et al.* 2007, Robbins et al. 2007). DCPG has a presynaptic mode of action in neonatal rat spinal cord, and inhibits forskolin-stimulated cAMP production in mGlu8 receptor expressing cells (Thomas et al. 2001). Additionally, DCPG has been shown to have anticonvulsant and anxiolytic properties when injected i.c.v and into

the amygdala respectively (Moldrich et al. 2001, Schmid & Fendt 2006). These anxiolytic properties may be of particular use in PD as non-motor symptoms often include anxiety (see Chapter 1, section 1.1.4.2). AZ12216052 is an mGlu8 PAM which also crosses the BBB and shows promise as an anxiolytic agent *in vivo* (Duvoisin et al. 2011). It has recently been found to protect human neuroblastoma cells *in vitro* from MPP+ toxicity (Jantas et al. 2014), although there is no published research on its efficacy in animal models of PD as yet. Additionally, AZ12216052 has been suggested to have actions at other receptors as well as modulating mGlu8 (Duvoisin et al. 2011), so it was not considered for use in this study.

To date, no published studies have examined the putative neuroprotective effects of DCPG *in vivo* in models of PD. According to the anatomical distribution of mGlu8 within the BG and the functional effects of potentiating glutamate's activity at this receptor, DCPG could theoretically provide protection against excitotoxic damage in the SNc. Additionally, the localisation of mGlu8 receptors on microglia may provide additional neuroprotection against lesioning via beneficial anti-inflammatory mechanisms. Previous work investigating DCPG's anticonvulsant effects demonstrated substantial neuroprotection following induced seizures when injected i.c.v (Folbergrová et al. 2008). DCPG has also recently been shown to be neuroprotective against MPP+ toxicity in neuroblastoma SH-SY5Y cells *in vitro* at a range of concentrations (Jantas et al. 2014). However, it remains to be seen if selective activation of mGlu8 receptors *in vivo* results in levels of neuroprotection similar to that described of mGlu4. Preliminary studies by our group with DCPG suggests it has mildly neuroprotective effects (around 20% protection) on SNc dopaminergic neurons *in vivo* when given peripherally in lateral MFB 6-OHDA lesioned rats (Chan, H., unpublished observation, 2010).

The hypothesis of this Study is that activation of the mGlu8 receptor with the mGlu8 selective agonist DCPG can provide neuroprotection and symptomatic relief in the lactacystin rat model of PD. In order to test this hypothesis, in this study, the aim was to demonstrate the potential antiparkinsonian effects of targeting the mGlu8 receptor with systemically administered DCPG in the lactacystin model of PD, and to reveal any neuroprotection elicited by mGlu8 receptor activation with this compound in order to assess its effectiveness as a potential novel treatment for PD. Doses of 3mg/kg and 15mg/kg DCPG were selected based on previous findings within our lab indicating that a higher dose of 30mg/kg did not enhance neuroprotection and had negative effects on the animals' weight (Chan, H., unpublished observation, 2010). Using two doses allowed investigation into a possible dose-dependent relationship and aided identification of the minimum dose needed for significant neuroprotection. Additionally, the expense of the compound when given systemically in prolonged studies limited the use of higher dosages. Most published neuroprotection studies begin drug administration prior to toxic insult; however, this is not particularly informative or clinically

relevant since at present neuroprotective therapies can only be administered at a stage where disease progression and neuronal damage is already established. Additionally, due the differences in early lesion progression following lactacystin injection into the SNc (see Chapter 2, section 2.2.4), two different concentrations of lactacystin were used for lesioning in animal models. Therefore, the antiparkinsonian and neuroprotective effects of chronic systemic administration of DCPG were investigated using a delayed-start study design in rats unilaterally lesioned with both high (7.5µg) and low (2.5µg) concentrations of lactacystin in order to test DCPG's effects in models with different rates and degrees of neurodegeneration. Additionally, more acute symptomatic improvements were investigated in response to single doses of DCPG in order to determine the effect of targeting mGlu8 receptors on symptomatic relief alone.

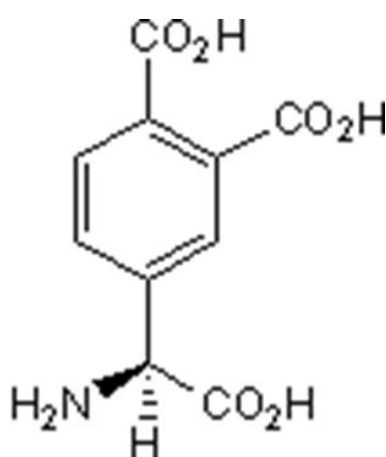


Figure 3.1: Chemical structure of (S)-3,4-Dicarboxyphenylglycine (DCPG). DCPG was dissolved in 5% DMSO and further diluted in a solution of 0.9% sterile saline before being administered i.p.

3.2 Experimental design

3.2.1 Chronic systemic DCPG treatment in high dose (7.5µg) lactacystin-lesioned rats: behavioural and neuroprotective effects

To investigate the neuroprotective and behavioural effects of the mGlu8 receptor agonist DCPG *in vivo*, male Sprague-Dawley rats (240-290g) were unilaterally lesioned in the SNc with 7.5µg lactacystin at day 0 (see experimental design, Figure 3.2). Animals were randomly assigned to a treatment group. Dosing began at day 4 post-lesioning, when lactacystin-induced cell death had begun but no overt behavioural deficits were observable, allowing the drug to be tested in a more clinically relevant manner. Treatment groups were as follows; 3mg/kg DCPG (n=7), 15mg/kg DCPG (n=7) and drug vehicle (n=8). Drug or drug vehicle treatment was carried out daily before midday from day 4 post-lesioning for 14 days, as illustrated in Figure 3.2: D4-D17. Forelimb-use asymmetry was regularly assessed using the vertical cylinder test (see section 2.1.5.1) to detect changes in motor symptoms in response to lesioning and drug treatment. Performance in the vertical cylinder test was assessed prior to lesioning surgery to ascertain a baseline score. Animals were then re-tested behaviourally at intervals on days 7, 14 and 18 post-surgery to monitor for motor deficit development and detect possible neuroprotective effects of DCPG. DCPG was administered i.p. immediately after behavioural testing so that the drug did not directly impact on behaviour, and any functional neuroprotection-driven behavioural changes could be detected. On day 18, at the conclusion of the experiment and following vertical cylinder testing, amphetamine-induced rotations (see section 2.1.5.2) were carried out to assess rotational asymmetry and determine the extent of the lesion and potential neuroprotection. On day 19, brains were removed and fixed for histological processing and immunohistochemical analysis. Correct needle placement was verified after Nissl counterstaining using the rat atlas to guide assessment (see section 2.2.2.6). Where lesioning was not in the correct place, animals were discounted from analysis (see Table 2.1 for lesion success rate). Staining of dopaminergic neurons was carried out on serial sections throughout the SNc utilising TH immunohistochemistry with Nissl counterstaining (see section 2.2.2.3), followed by stereological quantification (see section 2.2.3.1). Additionally, immunolabelling was performed on serial sections throughout the SNc to detect OX-6+ activated microglia and CD68+ phagocytic microglia, followed by stereological quantification.

3.2.2 Chronic systemic DCPG treatment in low dose (2.5µg) lactacystin-lesioned rats: behavioural and neuroprotective effects

Due to the high degree of dopaminergic neuronal loss detected following lesioning with 7.5µg lactacystin after 19 days post-lesioning, a lower concentration of 2.5µg lactacystin was used for unilateral lesioning to create more of a partial lesion model which may unmask any neuroprotective effects of DCPG. Only the higher 15mg/kg dose of DCPG was used in this study due to the limited amount of drug available and the lack of neuroprotection by DCPG administration at 3mg/kg in rats lesioned with 7.5µg of lactacystin. The same experimental design as that of the chronic study in 3.2.1 was employed (Figure 3.2). Male Sprague-Dawley rats (240-270g) were unilaterally lesioned in the SNc with 2.5µg lactacystin at day 0 and treated with either 15mg/kg DCPG (n=5) or drug vehicle (n=6) daily from day 4 post lesioning for a further 14 days. A larger battery of behavioural tests was introduced to regularly assess changes in motor symptoms in response to lesioning and drug treatment, in order to detect subtler changes in motor deficits. Performance in the vertical cylinder test, vibrissae-evoked forelimb placement test, adjusted stepping test and spontaneous circling (see methods in section 2.1.5) were all assessed prior to lesioning surgery to ascertain a baseline score. Animals were then re-tested behaviourally on days 7, 14 and 18 post-lesioning to monitor motor deficit development and detect possible neuroprotective effects of DCPG. DCPG was administered i.p. immediately after behavioural testing so that the drug did not directly impact on behaviour, and any neuroprotection-driven behavioural changes could be detected. On day 18 at the conclusion of the experiment, amphetamine-induced rotations were carried out, in addition to the above battery of behavioural tests to assess rotational asymmetry and determine the extent of the lesion and any potential neuroprotection. Brains were removed and fixed for histological processing and immunohistological analysis on day 19, and correct needle placement was verified after Nissl counterstaining using the rat atlas to guide assessment. Where lesioning was not in the correct place, animals were discounted from analysis (see Table 2.1 for lesion success rate). Again, immunostaining of TH+ dopaminergic neurons was carried out alongside staining for OX-6+ and CD-68+ microglia on serial sections throughout the SNc, followed by stereological quantification.

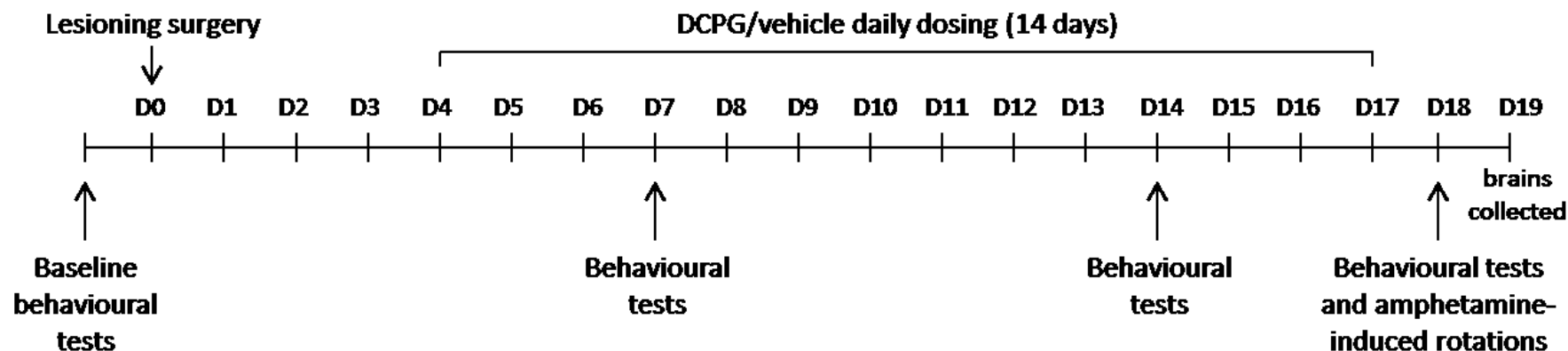


Figure 3.2: Schematic of experimental design for prolonged DCPG treatment in lactacystin-lesioned animals

Rats were unilaterally lesioned with either 7.5µg or 2.5µg lactacystin at day 0. Animals were peripherally administered either drug vehicle or DCPG (3mg/kg or 15mg/kg) i.p. once daily from day 4 post-lesioning, for a duration of 14 days. Baseline behaviour was assessed prior to lesioning and again at days 7, 14 and 18 post-surgery to monitor motor deficit development and detect any neuroprotective effects of DCPG. Amphetamine-induced rotations were also incorporated at day 18 to assess rotational asymmetry and therefore determine the extent of the lesion and detect any potential neuroprotection by DCPG. DCPG was administered following scheduled behavioural tests so that the drug did not directly influence behavioural assessment and potential neuroprotection-driven changes could be detected.

3.2.3 Acute systemic DCPG treatment in lactacystin-lesioned rats: symptomatic changes

The effect of a single systemic dose of DCPG on motor deficits was investigated in lactacystin-lesioned rats to determine the acute anti-parkinsonian effects of activating mGlu8 receptors. In the previous chronic drug treatment experiments, detailed above, motor behaviour was assessed prior to the daily administration of DCPG thus facilitating the assessment of any neuroprotective effects of chronic DCPG administration. However, through its capability to modulate glutamatergic activity, DCPG may induce direct alleviation of motor deficits shortly after acute administration. Hence, any potential symptomatic alleviation of motor deficits by DCPG was subsequently assessed in fully lactacystin lesioned rats.

To investigate the acute effects of DCPG on motor deficits, male Sprague-Dawley rats (250-290g) were unilaterally lesioned in the SNc with 7.5µg lactacystin at day 0 (see experimental design, Figure 3.3). In order to ensure that the lactacystin had induced an extensive lesion of the nigrostriatal tract, amphetamine-induced rotations were carried out at day 14 for 30 minutes (see section 2.1.5.2) and animals that rotated on average <1 net ipsiversive rotation per minute were discounted as poorly lesioned. Animals that would not rear in the vertical cylinder test (n=3) were also discounted from analysis. From day 21 post-lesioning onwards, animals (n=7 per group) were acutely treated with a single i.p. administration of 3mg/kg or 15mg/kg of DCPG or drug vehicle and behaviourally assessed 30 minutes later during peak brain activity based on pharmacokinetic data from Robbins et al. (2007). A minimum of a 4-day washout period between each test treatment was utilised to ensure no drug remained in the animal's system. As behavioural assessments were carried out at different time points after lesioning to allow wash out of test drugs (i.e. day 22, day 28, day 34), behavioural deficits gradually increased over the testing period, necessitating separate baseline measurements prior to each test, and data was normalised to a percentage of mean baseline scores. Baseline behaviour was assessed following drug vehicle administration 1 day prior to drug treatment to ascertain a baseline score and minimise habituation to tests. The following morning, animals were administered the test treatment (3mg/kg, 15mg/kg DCPG or drug vehicle i.p.) and behaviourally assessed 30 minutes later (based on DCPG's pharmacokinetics demonstrated by Robbins et al. (2007) and Johnson et al. (2013)). Behavioural testing with a battery of tests took approximately 15 minutes per animal, so drug administration was staggered between animals on the testing day. Performance in the vertical cylinder test, adjusted stepping test and vibrissae-evoked forelimb placement test were all assessed during behavioural testing in the same order for each animal to control for changes in the concentration of DCPG in the brain over time. Spontaneous circling was not assessed in this study as well-lesioned animals tended not to rotate spontaneously or explore in the circling bowl. Drug vehicle testing was carried out to verify that repeat testing did not alter behaviour.

3.2.4 Preparation of drug solution for peripheral administration

DCPG solution was made daily from aliquots of stock DCPG prepared in sterile saline and used on the day of administration as described in section 2.1.4. Where a small amount of drug solution remained after dosing, it was frozen at 20°C overnight and mixed with fresh compound for the following day's dosing. This was to minimise the wastage of the compound and was not thought to affect its efficacy.

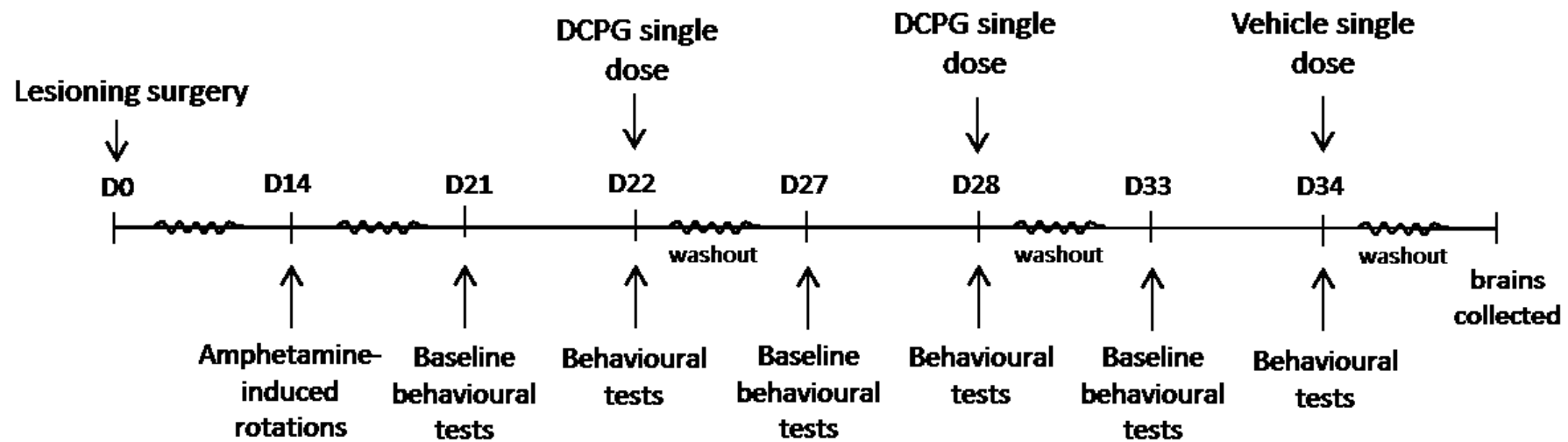


Figure 3.3: Schematic of experimental design for acute DCPG treatment in lactacystin-lesioned animals

Rats were unilaterally lesioned with 7.5ug lactacystin administered into the SNc at day 0 (D0). Amphetamine-induced rotations were used to confirm accurate lesioning 14 days after lesioning surgery (D14). At 21 days (D21) post-lesioning, behavioural deficits were clearly observable and testing was begun. Baseline behaviour was assessed with a battery of behavioural tests, and testing with DCPG or drug vehicle took place the following day on the 'test' day. Animals were peripherally administered either drug vehicle or DCPG (3mg/kg or 15mg/kg) as a single i.p. injection. 30 minutes later, the same battery of behavioural tests was again performed to detect any drug-induced changes to motor deficits.

3.2.5 Data analysis and statistics

All raw data was analysed in Microsoft Excel and statistical analysis was performed in Graphpad Prism software, Version 5 (San Diego, CA, USA). Behavioural tests were analysed according to the methods outlined in 2.1.5. Forelimb-use asymmetry is shown as percentage forelimb use in the vertical cylinder test, spontaneous circling is displayed as net rotations over 2 minutes, forelimb akinesia is shown as percentage contralateral steps relative to ipsilateral steps, and contralateral forelimb placements are shown relative to ipsilateral placements in the vibrissae-evoked forelimb placing test. In some cases, animals were excluded from vertical cylinder test analysis due to a lack of sufficient rearing activity, but were included in all other behavioural and histological analysis. The number of animals included in each test is shown in figure legends.

The effect of time and treatment was analysed by two-way repeated measures ANOVA followed by Bonferroni post hoc tests. Additionally, the area under the curve was calculated for each treatment group over time in each test: for all tests except the spontaneous rotations (for which regular area under the curve was carried out due to positive peaks), negative area under the curve was calculated from baseline to detect the effect of DCPG, and comparisons between groups were carried out with unpaired t-tests (2 groups) or one-way ANOVA (>2 groups). Amphetamine-induced rotational asymmetry was analysed as net mean ipsiversive rotations per 5 minutes (over a 30 minute time period) and comparisons between treatment groups were carried out with unpaired t-tests or one-way ANOVA. Cell counts were analysed using two-way ANOVAs to compare contralateral and ipsilateral total cell estimates between hemispheres and between treatment groups. Data regarding cell loss is represented as percentage cell loss $\pm$ SEM relative to the unlesioned hemisphere.

For analysis of the effect of DCPG on motor behaviour in the acute treatment study, baseline data was normalised to 100% and 'test' data following drug vehicle or drug treatment was calculated as a percentage of the mean baseline score. Two-way ANOVAs were used to compare baseline and 'test' values across treatments. All data in this chapter are presented as mean $\pm$ SEM.

3.3 Results

3.3.1 Chronic systemic DCPG treatment in 7.5µg lactacystin-lesioned rats

3.3.1.1 Effects of chronic peripheral DCPG on behavioural deficits

Prior to lesioning, all animals demonstrated an equal use of both forelimbs at baseline in the vertical cylinder test for forelimb use asymmetry ($48.7 \pm 1\%$ contralateral forelimb use). Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 3.4A, $F_{(3,51)}=6.59$, $p=0.0008$) but no effect of DCPG treatment on forelimb-use asymmetry ($F_{(2,51)}=0.62$, $p=0.55$). Bonferroni post-hoc tests demonstrated a significant effect of lesioning over time from baseline at days 14 and 18 in vehicle-treated animals ($p<0.01$ for both), but no significant effect of lesion was detected in DCPG-treated animals over time. Negative area under the curve (AUC) analysis similarly showed no significant effect of DCPG treatment following lactacystin lesioning (Figure 3.4 B, one-way ANOVA $F_{(2,17)}=1.29$, $p=0.29$).

It was observed that animals demonstrated some compensatory behaviour, such as weight shifting, when pushing off and landing in the vertical cylinder test which could mask their motor deficits during analysis. Therefore, wall exploration of the cylinder was assessed independently in the same tests as a potentially more sensitive marker of forelimb use asymmetry (Figure 3.5). At baseline, all animals again displayed an equal use of both forelimbs for exploration of the cylinder wall ($53.1 \pm 5.2\%$ contralateral forelimb use). Two-way ANOVA analysis of treatment groups again showed an extremely significant effect of lesioning over time (Figure 3.5A, $F_{(3,51)}=22.65$, $p<0.0001$) but no effect of DCPG treatment on forelimb-use asymmetry during wall exploration ($F_{(2,51)}=0.08$, $p=0.91$). Bonferroni post-hoc tests demonstrated a significant effect of lesioning over time from baseline in all three treatment groups (vehicle-treated animals: day 0 vs. days 7, 14 and 18: $p<0.001$. 3mg/kg DCPG-treated group: day 0 vs. day 7 $p<0.05$, day 0 vs. day 14 $p<0.001$, day 0 vs. day 18 $p<0.01$. 15mg/kg DCPG-treated group: day 0 vs. day 7 $p<0.05$). Negative AUC analysis similarly showed no significant effect of DCPG treatment on forelimb-use asymmetry during wall exploration following lactacystin lesioning (Figure 3.5B, one-way ANOVA $F_{(2,17)}=2.270$, $p=0.13$).

Rotational asymmetry was investigated at the conclusion of the experiment (day 18 post lesioning) utilising amphetamine induced rotational behaviour as an indirect measure of lesion magnitude. Drug vehicle treatment daily for 14 days starting 4 days post unilateral SNc lesioning with 7.5µg lactacystin was associated with a marked induction of circling behaviour following amphetamine administration on day 18 of the study. Similar to the effects of DCPG in the cylinder test, administration of 3 or 15mg/kg DCPG i.p. daily for 14 days starting on day 4 post lesioning failed to attenuate the circling behaviour induced after amphetamine administration as shown by one-way ANOVA analysis (net mean ipsiversive rotations/5 minutes for drug vehicle-treated animals: 33.4 ± 8.4 , 3mg/kg-DCPG:

35.1 ± 10.5, 15mg/kg-DCPG: 47.9 ± 8.2, $F_{(2,18)}=0.68$ $p=0.51$) (Figure 3.6). A single animal was excluded from rotational asymmetry analysis in the 15mg/kg DCPG treatment group due to mistakenly administering amphetamine into the bladder.

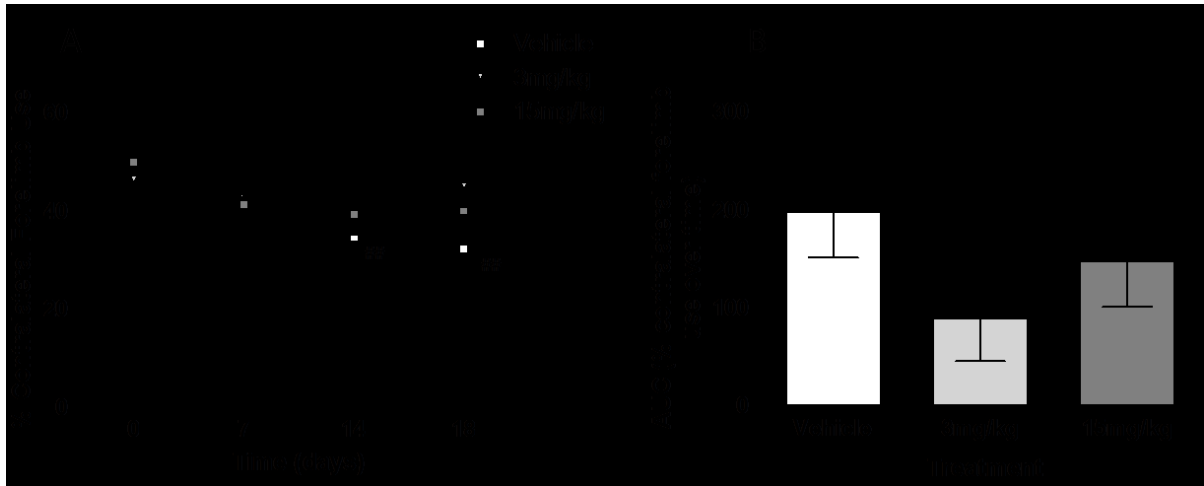


Figure 3.4: Effect of chronic DCPG treatment on forelimb-use asymmetry in the vertical cylinder test.

Rats were unilaterally lesioned in the SNc with 7.5µg lactacystin and treated with drug vehicle, 3mg/kg or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days were tested in a vertical cylinder to determine forelimb-use asymmetry prior to surgery at baseline (day 0), 7, 14 and 18 days after lesioning surgery. Data are shown as % contralateral forelimb use (push-off, cylinder wall exploration and landing combined) ±SEM.

A: There was a significant effect of lesion ($F_{(3,51)}=6.59$ $p=0.0008$) but not DCPG treatment ($F_{(2,51)}=0.62$ $p=0.55$) on contralateral forelimb use when comparing the three treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 14 and 18 compared to baseline in the vehicle-treated group ($^{##}p<0.01$), but not in DCPG-treated groups.

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using one-way ANOVA analysis ($F_{(2,17)}=1.29$, $p=0.29$). AUC units are expressed as time x % contralateral forelimb use.

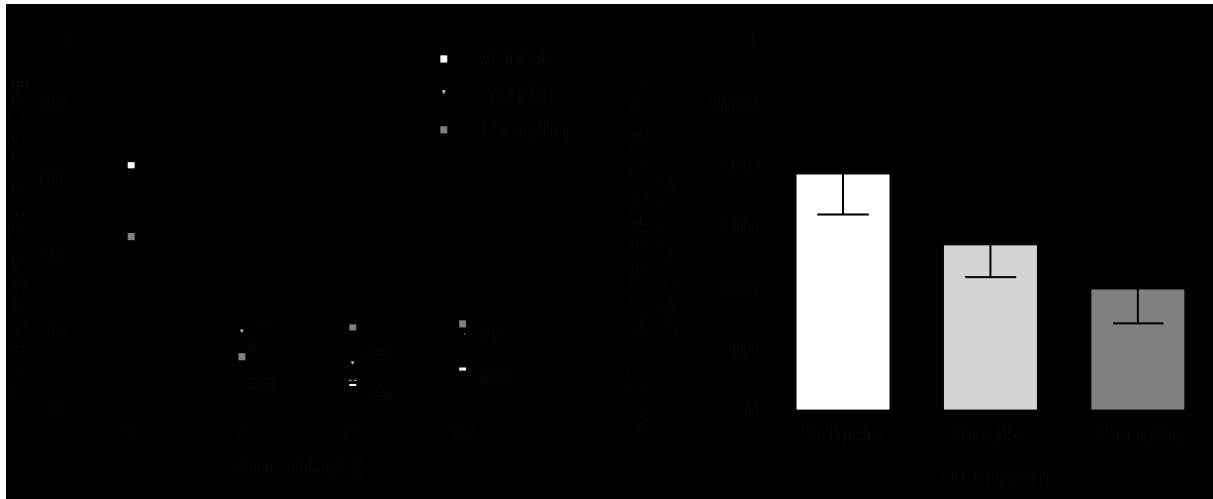


Figure 3.5: Effect of chronic DCPG treatment on forelimb-use asymmetry during wall exploration of the vertical cylinder. Wall exploration of the vertical cylinder was analysed independently of the other measurable parameters in this test (push-off and landing). 7.5µg lactacystin unilateral SNc lesioned rats were treated with drug vehicle, 3mg/kg or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days and tested in a vertical cylinder to determine forelimb-use asymmetry prior to surgery at baseline (day 0), 7, 14 and 18 days after lesioning surgery. Data are shown as % contralateral forelimb use $\pm$ SEM.

A: There was a significant effect of lesion ($F_{(3,51)}=22.65$ $p<0.0001$) but not DCPG treatment ($F_{(2,51)}=0.08$ $p=0.91$) on contralateral forelimb use for exploration of the vertical cylinder when comparing the three treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7, 14 and 18 in the vehicle-treated and 3mg/kg DCPG-treated groups, and at day 7 in the 15mg/kg DCPG-treated group compared to baseline (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using one-way ANOVA analysis. AUC units are expressed as time x % contralateral forelimb use.

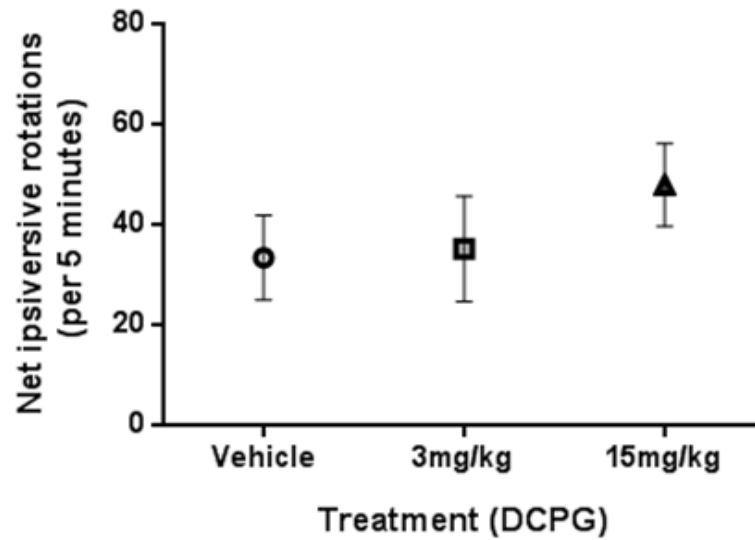


Figure 3.6: Effect of chronic DCPG treatment on rotational asymmetry at day 18 post lactacystin lesioning. Rats unilaterally lesioned in the SNc with 7.5µg lactacystin were treated with 5mg/kg amphetamine (i.p.) at the conclusion of the experiment, 18 days after lactacystin lesioning, to assess rotational asymmetry as an indication of lesion magnitude. Net ipsiversive rotations were measured 30 minutes after amphetamine administration for 30 minutes in 5-minute time-bins. Animals treated with 3mg/kg (n=7) and 15mg/kg (n=6) DCPG from day 4 post-lesioning for 14 days showed no significant reduction in rotational asymmetry from drug vehicle treated animals (n=8) using a one way ANOVA ($F_{(2,18)}=0.68$ $p=0.51$). Data are shown as net mean $\pm$ SEM ipsiversive rotations per 5 minutes $\pm$ SEM.

3.3.1.2 Effects of chronic peripheral DCPG administration on dopaminergic neuronal survival and microglial activation in the lactacystin lesioned SNc

In 7.5µg lactacystin-lesioned animals, the SNc of the unlesioned hemisphere contained approximately 7,700 TH+ dopaminergic neurons, which is comparable to previous studies (Baquet *et al.* 2009, Jackson-Lewis *et al.* 2000, Nair-Roberts *et al.* 2008) (Figure 3.7 & 3.8). Administration of 3 or 15mg/kg DCPG i.p. for 14 days starting from 4 days post lesioning did not affect the numbers of TH+ dopaminergic neurons in the unlesioned SNc (Figure 3.8 A). Numbers of Nissl+ neurons detected in the unlesioned SNc was slightly higher at ~10,000, reflecting the previously described fact that not all neurons in the SNc are dopaminergic. Similarly, DCPG administration at 3 or 15mg/kg daily for 14 days did not affect the numbers of Nissl+ neurons in the unlesioned SNc (Figure 3.8 B).

Two-way ANOVA analysis revealed an extremely significant effect of lesioning with 7.5µg lactacystin on TH+ neurons in the ipsilateral SNc by day 19 for all treatment groups (Figure 3.8 A, $F_{(1,38)}=153.83$ $p<0.0001$), but no effect of DCPG treatment on TH+ neuronal estimates in the ipsilateral SNc ($F_{(2,38)}=0.01$ $p=0.99$). Similarly, two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning with 7.5µg lactacystin on Nissl+ neurons in the ipsilateral SNc by day 19 for all treatment groups (Figure 3.8 B, $F_{(1,38)}=188.07$ $p<0.0001$), but no effect of DCPG treatment on Nissl+ neuronal estimates in the ipsilateral SNc ($F_{(2,38)}=0.12$ $p=0.88$). In the animals receiving drug vehicle treatment for 14 days starting 4 days post-surgery, Bonferroni multiple comparisons tests showed lesioning of the left SNc with 7.5µg lactacystin resulted in a highly significant ($p<0.001$) and severe neuronal loss, as assessed by both TH+ and Nissl+ estimates, in the ipsilateral, lesioned SNc compared to the contralateral, unlesioned SNc at day 19 post-lesioning (Figure 3.7 & 3.8). Bonferroni multiple comparisons tests similarly showed lesioning of the left SNc with 7.5µg lactacystin resulted in a highly significant ($p<0.001$) and severe TH+ and Nissl+ neuronal loss in animals administered DCPG at 3 or 15 mg/kg daily for 14 days starting 4 days post-surgery (Figure 3.7 & 3.8). No significant neuroprotection of TH+ dopaminergic neurons against lactacystin toxicity was detected in the ipsilateral lesioned SNc (Figure 3.8 A, vehicle: 302 ± 111 vs 3mg/kg: 661.6 ± 170 and 15mg/kg: 1070 ± 416 TH+ cells remaining in the lesioned SNc). Similarly, no significant neuroprotection of Nissl+ neurons against lactacystin toxicity was detected in the ipsilateral lesioned SNc (Figure 3.8 B, vehicle: 465 ± 159 vs 3mg/kg: 806 ± 196 and 15mg/kg: 1276 ± 476 Nissl+ cells remaining in the lesioned SNc).

Comparing percentage cell loss in the lesioned relative to the unlesioned SNc revealed no difference between drug vehicle and DCPG-treated groups as illustrated in Figure 3.8 for both TH+ (C) and Nissl+ (D) cell count estimates. Percentage loss of TH+ neurons after drug vehicle treatment was $96.3 \pm 0.95\%$ compared to $91 \pm 2.2\%$ for 3mg/kg DCPG treatment and $86.97 \pm 3.8\%$ for 15mg/kg DCPG-treated animals. Similarly, the percentage loss of Nissl+ neurons following drug vehicle treatment was

95.7 ± 1% compared to 90.9 ± 2.1% following 3mg/kg DCPG treatment, and 86.4 ± 3.96% following 15mg/kg DCPG treatment.

Following unilateral lesioning of the SNc with 7.5µg lactacystin, OX-6+ microglia were found in the lesioned SNc, but no OX-6+ microglia were seen in the contralateral, unlesioned SNc in animals treated with drug vehicle, as illustrated in Figure 3.9. This finding was not affected by treatment for 14 days with 3 or 15mg/kg DCPG from day 4 post lactacystin lesioning. Immunostaining of activated microglia revealed OX-6+ staining within the ipsilateral SNc, but no staining in the contralateral hemisphere, as illustrated in Figure 3.9. Following lactacystin lesioning, a large increase in OX-6+ immunoreactivity was seen in the lesioned SNc in drug vehicle treated animals (6902 ± 2093 OX-6+ microglia, n=8). One-way ANOVA analysis revealed that treatment with 3mg/kg and 15mg/kg DCPG from day 4 post-lesioning for 14 days did not significantly affect the numbers of OX-6+ microglia in the lesioned SNc (Figure 3.11 A, $F_{(2,15)}=1.92$, $p=0.18$), although 14 days administration of 3mg/kg DCPG was associated with a slight but non-significant attenuation in OX-6+ immunoreactivity compared to drug vehicle treated animals (3mg/kg DCPG-treated animals, n=6: 2547 ± 529 OX-6+ microglia, 15mg/kg DCPG-treated animals, n=5: 5216 ± 1542 OX-6+ microglia). Unfortunately, due to over fixation of some tissue, OX-6+ immunoreactivity was not suitably visible for counting in 3 rats, necessitating their exclusion from this data.

Following unilateral lesioning of the SNc with 7.5µg lactacystin, CD68+ macrophages were detected in the lesioned SNc, but no CD68+ macrophages were seen in the contralateral, unlesioned SNc in animals treated with drug vehicle, as illustrated in Figure 3.10. This asymmetric positive immunoreactivity was not affected by treatment for 14 days with 3 or 15mg/kg DCPG from day 4 post lactacystin lesioning (Figure 3.10). In the animals receiving drug vehicle treatment for 14 days starting 4 days post-surgery, lesioning of the left SNc with 7.5µg lactacystin resulted in 10311 ± 2589 CD-68+ macrophages located within the lesioned SNc. One-way ANOVA analysis revealed that administration of DCPG at 3 or 15mg/kg daily for 14 days starting 4 days post-surgery resulted in no significant attenuation in numbers of CD68+ macrophages in the lesioned SNc at day 19 post-lesioning (3mg/kg DCPG-treated animals: 10329 ± 2891 CD-68+ macrophages, 15mg/kg DCPG-treated animals: 10461 ± 2301 CD-68+ macrophages) (Figure 3.11 B, $F_{(2,19)}=0.00$, $p=0.99$).

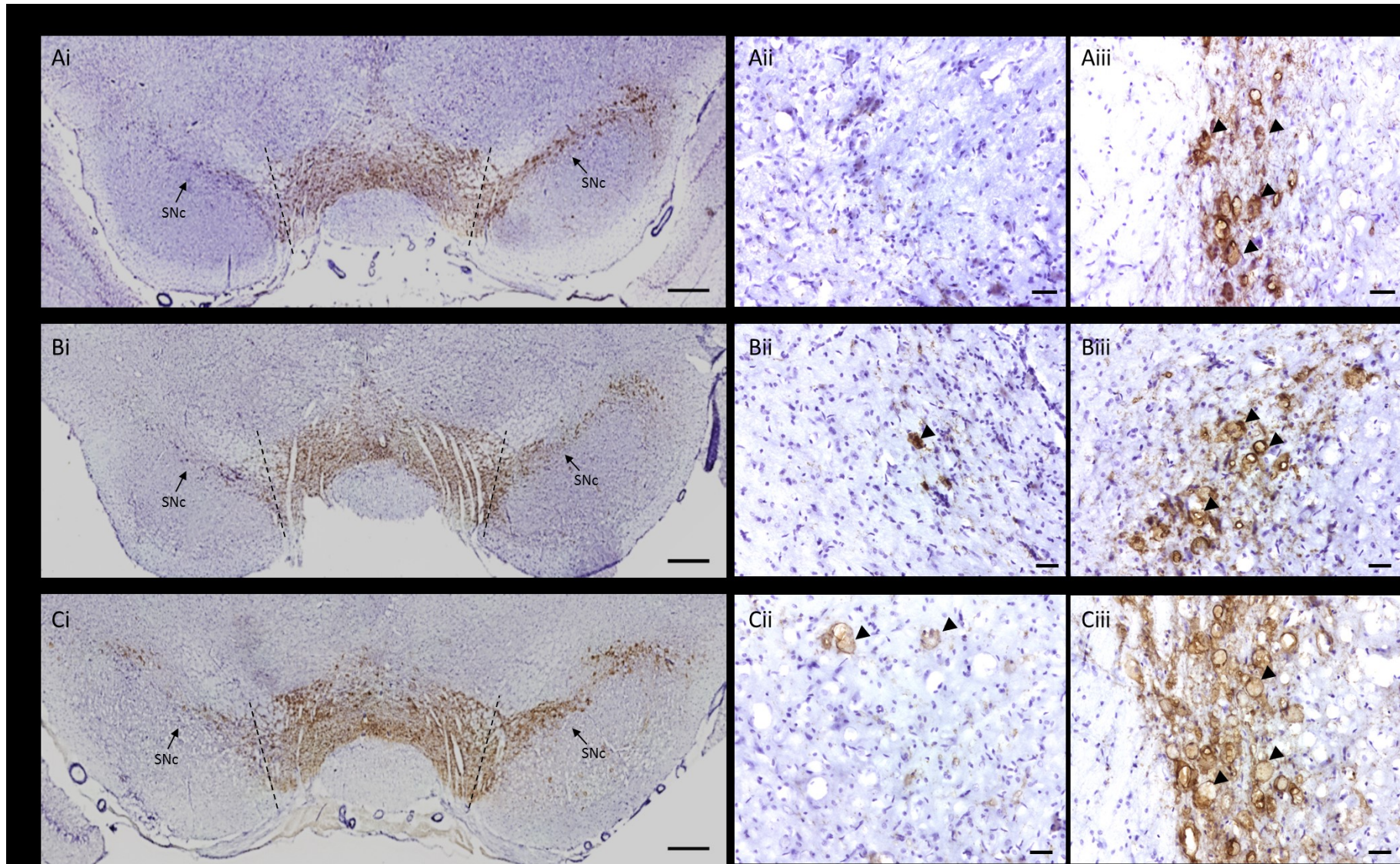


Figure 3.7: Effect of chronic DCPG treatment on TH+ dopaminergic neurons in the SNc of 7.5 μ g lactacystin-lesioned rats at day 19. Rats unilaterally lesioned in the SNc with 7.5 μ g lactacystin were treated with either drug vehicle, 3mg/kg or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining. An extensive loss of TH+ dopaminergic neurons (brown neuronal staining) was seen in the ipsilateral SNc compared to the contralateral SNc of drug vehicle treated animals (Ai-iii). Similarly, an extensive loss of TH+ dopaminergic neurons was seen in the ipsilateral SNc compared to the contralateral SNc of lactacystin lesioned animals treated daily with 3mg/kg and 15mg/kg DCPG (Bi-iii and Ci-iii). Arrows in Ai, Bi and Ci denote the SNc in the ipsilateral lesioned and contralateral unlesioned hemisphere. Arrowheads in magnified images denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100 μ m. High magnification images taken at 20x magnification, scale bar: 30 μ m.

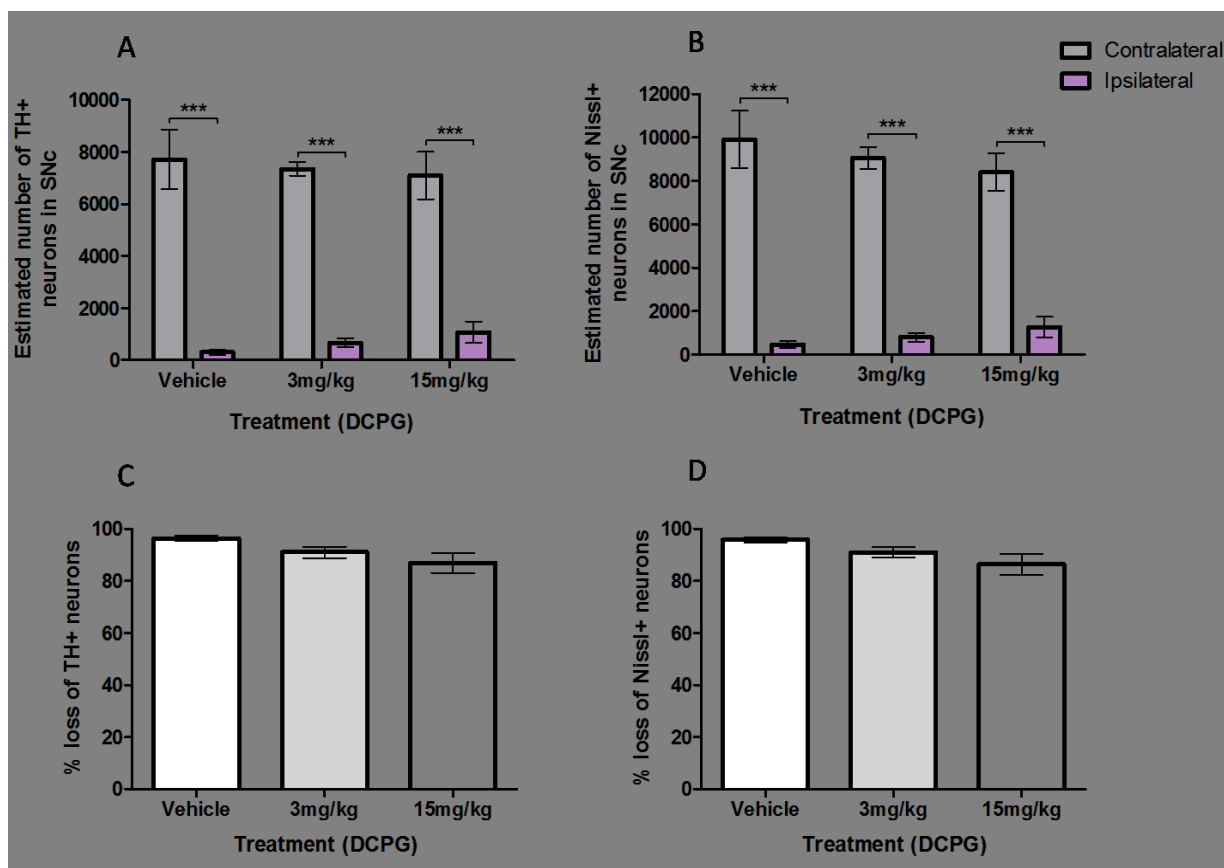


Figure 3.8: Effect of chronic DCPG treatment on the total number of TH+ dopaminergic and Nissl+ neurons in the SNc of 7.5µg lactacystin-lesioned rats at day 19 post-lesioning. Rats unilaterally lesioned in the SNc with 7.5µg lactacystin were treated with either drug vehicle, 3mg/kg or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining and cell quantification. Total neuronal cell numbers in the SNc were estimated in both the ipsilateral lesioned and contralateral unlesioned hemispheres of each 7.5µg lactacystin-lesioned rat and compared. For all comparisons, drug vehicle treated: n=8, 3mg/kg and 15mg/kg DCPG treated: n=7 per group.

A: A significant reduction in the number of TH+ dopaminergic neurons was seen in the ipsilateral SNc compared to the contralateral SNc in all three treatment groups following lesioning ($F_{(1,38)}=153.83$ $p<0.0001$), but no protection of ipsilateral TH+ dopaminergic neurons can be seen in DCPG-treated animals ($F_{(2,38)}=0.01$ $p=0.99$). Data are shown as estimated total cell number $\pm$ SEM and analysed with a two-way ANOVA, *** $p<0.001$ ipsilateral vs. contralateral SNc.

B: A significant reduction in the number of Nissl+ neurons was seen in the ipsilateral SNc compared to the contralateral SNc in all three treatment groups following lesioning ($F_{(1,38)}=188.07$ $p<0.0001$), but no protection of ipsilateral Nissl+ neurons can be seen in DCPG-treated animals ($F_{(2,38)}=0.12$ $p=0.88$). Data are shown as estimated total cell number $\pm$ SEM and analysed with a two-way ANOVA, *** $p<0.001$ ipsilateral vs. contralateral SNc.

C: The percentage loss of TH+ dopaminergic neurons in the ipsilateral SNc relative to the contralateral SNc at day 19 demonstrated no clear protection of ipsilateral TH+ dopaminergic neurons. Data are shown as % cell loss relative to the contralateral SNc $\pm$ SEM.

D: The percentage loss of Nissl+ neurons in the ipsilateral SNc relative to the contralateral SNc at day 19 similarly demonstrated no protection of ipsilateral Nissl+ neurons. Data are shown as % cell loss relative to the contralateral SNc $\pm$ SEM.

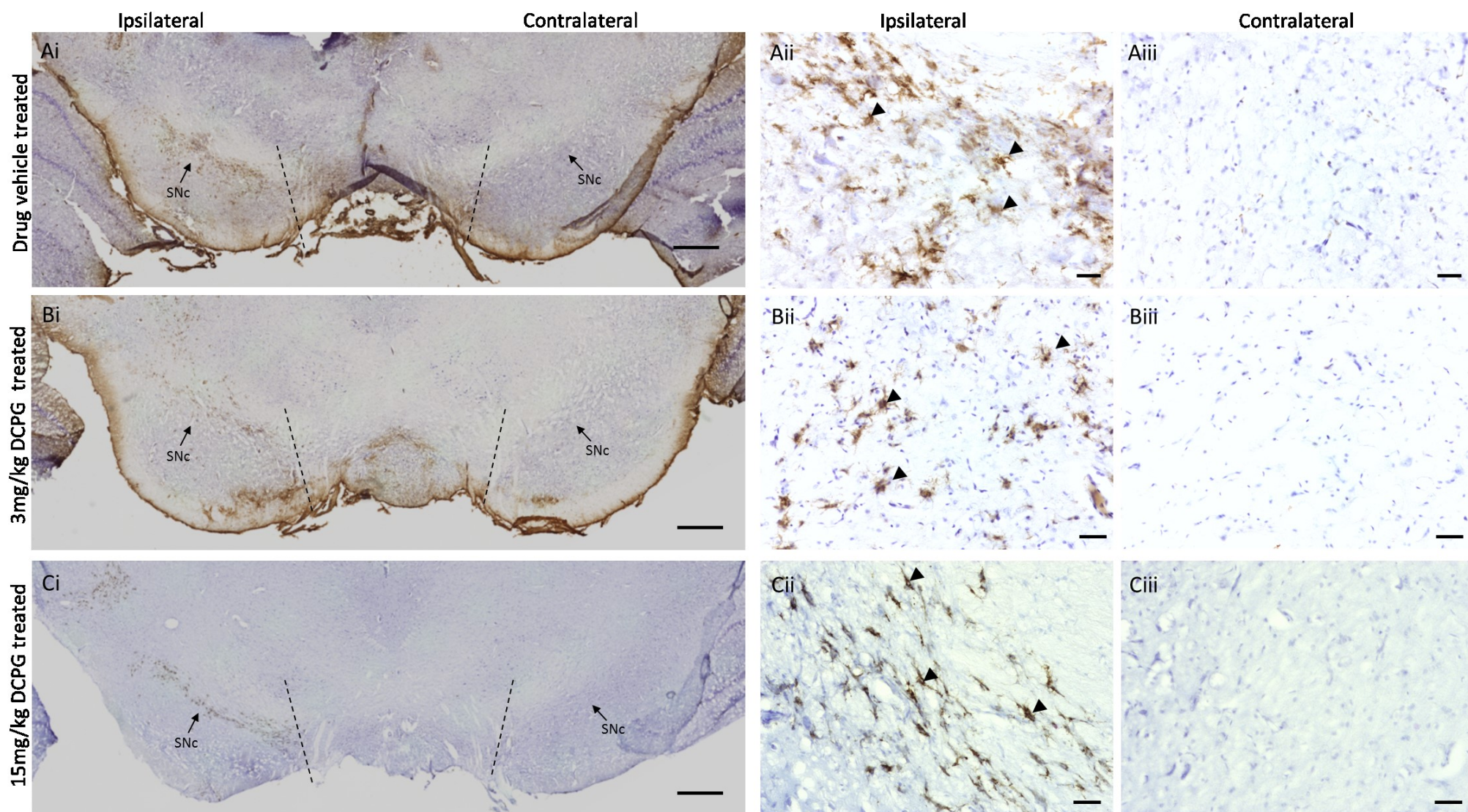


Figure 3.9: Effect of chronic DCPG treatment on OX-6+ microglia in the SNc of 7.5µg lactacystin-lesioned rats at day 19 post-lesioning. Representative photomicrographs of OX-6+ immunostaining. Rats unilaterally lesioned in the SNc with 7.5µg lactacystin were treated with either drug vehicle, 3mg/kg or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining. Lactacystin lesioning resulted in OX-6+ immunostaining (brown cell staining) in the ipsilateral SNc (Aii-Cii) that was not visible in the contralateral SNc (Aiii-Ciii), regardless of treatment. No significant difference in OX-6+ immunostaining in the ipsilateral SNc was detected between drug vehicle treated animals (Ai, n=8) and 3mg/kg (Bi, n=6) or 15mg/kg (Ci, n=5) DCPG treated animals. Arrows in Ai, Bi and Ci denote the SNc in the ipsilateral lesioned and contralateral unlesioned hemisphere. Arrowheads in magnified images (Aii-Cii) denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100µm. High magnification images taken at 20x magnification, scale bar: 30µm.

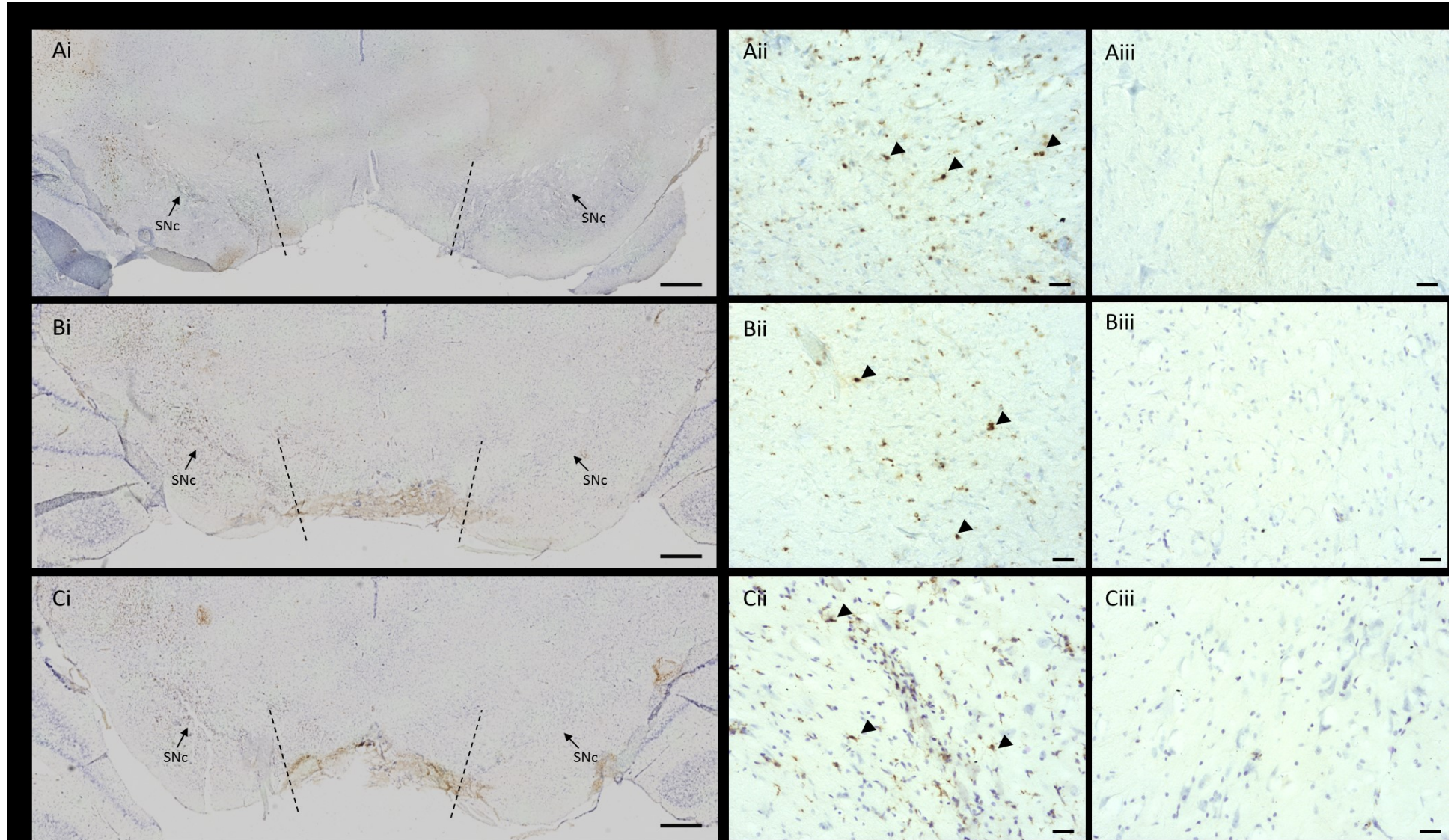


Figure 3.10: Effect of chronic DCPG treatment on CD68+ macrophages in the SNc of 7.5µg lactacystin-lesioned rats at day 19 post-lesioning. Representative photomicrographs of CD68+ immunostaining. Rats unilaterally lesioned in the SNc with 7.5µg lactacystin were treated with either drug vehicle, 3mg/kg or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining. Lactacystin lesioning resulted in CD68+ immunostaining (brown cell staining) in the ipsilateral SNc (Aii-Cii) that was not visible in the contralateral SNc (Aiii-Ciii), regardless of treatment. No significant difference in CD68+ immunostaining in the ipsilateral SNc was detected between drug vehicle treated animals (Ai, n=8) and 3mg/kg (Bi, n=6) or 15mg/kg (Ci, n=5) DCPG treated animals. Arrows in Ai, Bi and Ci denote the SNc in the ipsilateral lesioned and contralateral unlesioned hemisphere. Arrowheads in magnified images (Aii-Cii) denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100µm. High magnification images taken at 20x magnification, scale bar: 30µm.

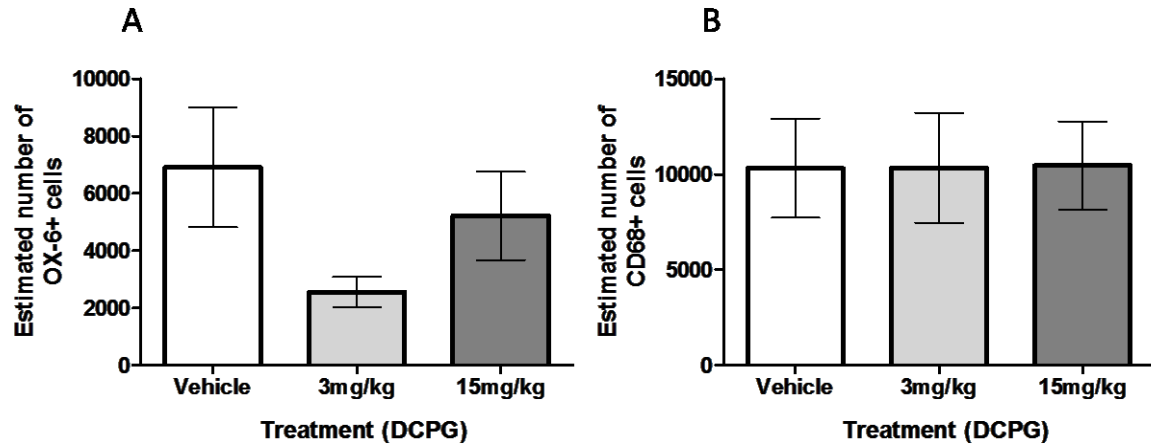


Figure 3.11: Effect of chronic DCPG treatment on microglia in the SNc of 7.5µg lactacystin-lesioned rats at day 19 post-lesioning. Rats unilaterally lesioned in the SNc with 7.5µg lactacystin were treated with either chronic drug vehicle, 3mg/kg or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining and cell quantification. Immunopositive microglia within the ipsilateral SNc were counted, and estimated total numbers were compared between treatment groups. Data are shown as mean total cell number $\pm$ SEM and were analysed by one-way ANOVA.

A: A large number of OX-6+ microglia were found in the ipsilateral SNc of drug vehicle treated animals ($n=8$) following lactacystin lesioning. Although chronic treatment with 3mg/kg DCPG ($n=6$) induced a slight reduction in the number of OX-6+ microglia in the ipsilateral SNc, this was not found to be a significant effect. Chronic treatment with 15mg/kg DCPG ($n=5$) demonstrated no significant effect on OX-6+ microglial numbers in the ipsilateral SNc ($F_{(2,15)}=1.92$, $p=0.18$).

B: A very large number of CD68+ phagocytic microglia were found in the ipsilateral SNc of drug vehicle treated animals ($n=8$) following lactacystin lesioning. Chronic treatment with 3 and 15mg/kg DCPG ($n=7$ /group) demonstrated no effect on CD68+ microglial numbers in the ipsilateral SNc ($F_{(2,19)}=0.00$, $p=0.99$).

3.3.2 Chronic systemic DCPG treatment in 2.5µg lactacystin lesioned rats

3.3.2.1 Effects of chronic peripheral DCPG on behavioural deficits

Due to the high degree of dopaminergic neuronal loss detected following lesioning with 7.5µg lactacystin by 19 days post-lesioning, a lower concentration of 2.5µg lactacystin was used for unilateral lesioning to create a partial lesion model which may unmask any neuroprotective effects of DCPG. Only the higher 15mg/kg dose of DCPG was used in this study due to the limited amount of drug available and the lack of neuroprotection by DCPG administration in rats lesioned with 7.5µg of lactacystin. Additionally, a larger battery of behavioural tests was introduced to regularly assess any changes in motor symptoms in response to lesioning and drug treatment, in order to detect subtler changes in motor deficits.

Prior to lesioning, all animals demonstrated an equal use of both forelimbs at baseline in the vertical cylinder test for forelimb use asymmetry ($48.7 \pm 1.4\%$ contralateral forelimb use, $n=11$) (Figure 3.12A). Following unilateral lesioning of the SNc with 2.5µg lactacystin, two-way ANOVA analysis of treatment groups showed a significant effect of lesioning over time (Figure 3.12 A, ($F_{(3,27)}=3.07$ $p=0.044$) but no effect of 15mg/kg DCPG treatment on forelimb-use asymmetry ($F_{(1,9)}=0.08$ $p=0.78$ ns). The negative area under the curve (AUC) analysis similarly showed no significant effect of 15mg/kg DCPG treatment following 2.5µg lactacystin lesioning (Figure 3.12 B, unpaired t-test $p=0.23$).

Vertical wall exploration of the cylinder was assessed independently in the same tests as a more sensitive marker of forelimb use asymmetry. At baseline, animals displayed an approximately equal use of both forelimbs for exploration of the cylinder wall ($52.5 \pm 6.3\%$ contralateral forelimb use; Fig 3.13A). Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 3.13 A, ($F_{(3,27)}=11.16$ $p<0.0001$) but no effect of chronic 15mg/kg DCPG treatment on forelimb-use asymmetry for exploration of the cylinder wall ($F_{(1,9)}=0.01$ $p=0.91$). Bonferroni post-hoc tests demonstrated a significant effect of lesioning over time from baseline in both treatment groups (vehicle-treated animals: day 0 vs. days 7 and 14 $p<0.05$, 15mg/kg DCPG-treated group: day 0 vs. day 7 $p<0.001$, day 0 vs. day 14 $p<0.01$, day 0 vs. day 18 $p<0.01$). Negative area under the curve (AUC) analysis similarly showed no significant effect of 15mg/kg DCPG treatment following 2.5µg lactacystin lesioning (Figure 3.13B, unpaired t-test $p=0.35$ ns).

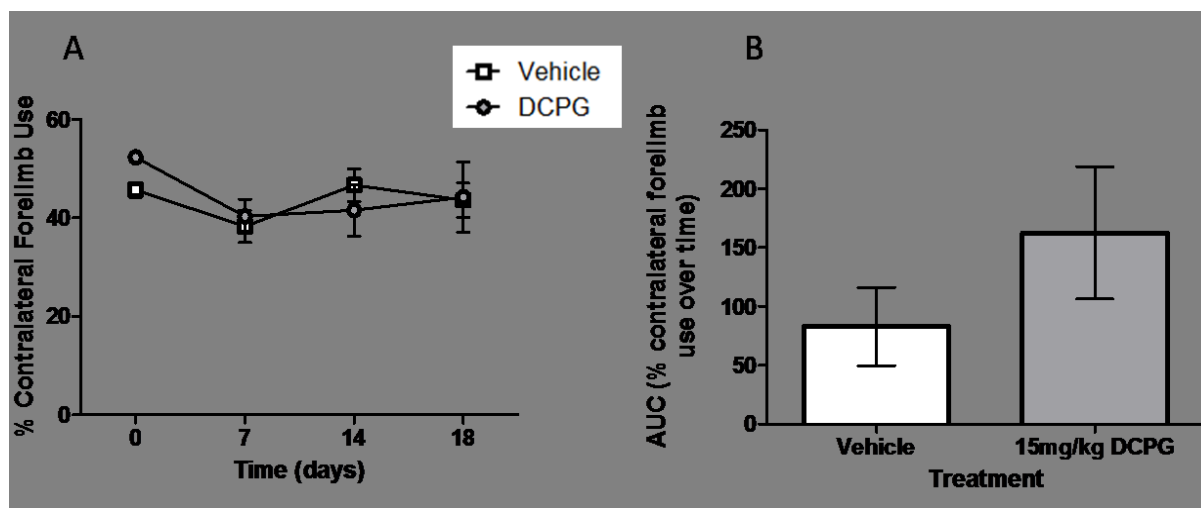


Figure 3.12: Effect of chronic drug vehicle or DCPG treatment on forelimb-use asymmetry in the vertical cylinder test. Rats were unilaterally lesioned in the SNc with 2.5 μ g lactacystin and treated with drug vehicle (white bars, n=6) or 15mg/kg DCPG (grey bars, n=5) daily from day 4 post-surgery for 14 days. Rats were tested in a vertical cylinder to determine forelimb-use asymmetry prior to surgery at baseline (day 0), 7 days, 14 days and 18 days after lesioning surgery. Data are shown as % contralateral forelimb use (push-off, cylinder wall exploration and landing combined) $\pm$ SEM.

A: There was a significant effect of lesion ($F_{(3,27)}=3.07$ $p=0.044$) but not DCPG treatment ($F_{(1,9)}=0.08$ $p=0.78$ ns) on contralateral forelimb use when comparing the two treatment groups (two-way ANOVA).

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using an unpaired t-test ($p=0.23$). AUC units are expressed as time x % contralateral forelimb use.

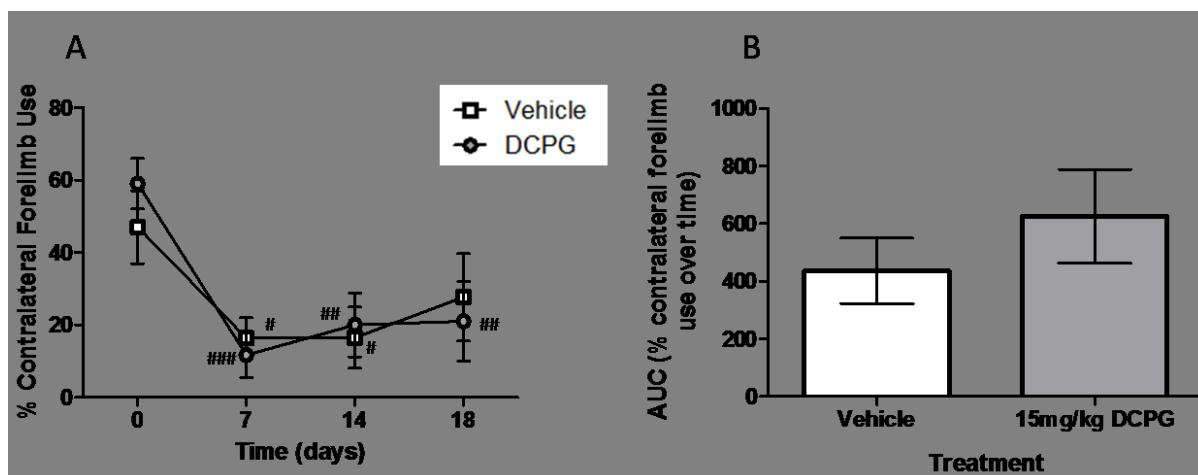


Figure 3.13: Effect of chronic drug vehicle or DCPG treatment on forelimb-use asymmetry during wall exploration of the vertical cylinder. Rats were unilaterally lesioned in the SNc with 2.5 μ g lactacystin and treated with drug vehicle (white bars, n=6) or 15mg/kg DCPG (grey bars, n=5) daily from day 4 post-surgery for 14 days. Rats were tested in a vertical cylinder to determine forelimb use asymmetry prior to surgery at baseline (day 0), 7 days, 14 days and 18 days after lesioning surgery. Data are shown as % contralateral forelimb use (cylinder wall exploration alone) $\pm$ SEM.

A: There was a significant effect of lesion ($F_{(3,27)}=11.16$ $p<0.0001$) but not DCPG treatment ($F_{(1,9)}=0.01$ $p=0.91$) on contralateral forelimb use when comparing the two treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7 and 14 compared to baseline in the vehicle-treated group ($^{\#}p<0.05$), and at days 7, 14 and 18 compared to baseline in the DCPG-treated group ($^{\#}p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$).

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using an unpaired t-test ($p=0.35$). AUC units are expressed as time x % contralateral forelimb use.

Following unilateral lactacystin lesioning, it was observed that 7.5µg lactacystin lesioned animals demonstrated a contraversive bias in spontaneous circling for up to 10 days post-surgery. Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 3.14 A, ($F_{(3,27)}=5.0p=0.006$) but no effect of chronic 15mg/kg DCPG treatment on spontaneous rotational asymmetry($F_{(1,9)}=0.12p=0.73$ ns). Bonferroni post-hoc tests demonstrated a significant effect of lesioning at day 7 in the vehicle-treated group (day 0 vs. day 7 $p<0.01$) but no significant effect of lesioning was detected in the DCPG-treated group. Area under the curve (AUC) analysis similarly showed no significant effect of 15mg/kg DCPG treatment following 2.5µg lactacystin lesioning on spontaneous rotational asymmetry (Figure 3.14 B, unpaired t-test $p=0.63$ ns).

Prior to lesioning, animals successfully stepped $103.3 \pm 2.7\%$ of total ipsilateral forelimb steps in the adjusted stepping test for forelimb akinesia. Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 3.15A, ($F_{(3,27)}=6.24p=0.0023$) but no effect of chronic 15mg/kg DCPG treatment on contralateral forelimb akinesia in the stepping test ($F_{(1,9)}=0.05p=0.82$). Bonferroni post-hoc tests demonstrated a significant effect of lesioning at days 14 and 18 in the vehicle-treated group (day 0 vs. days 14 and 18 $p<0.05$) and at day 18 in the DCPG-treated group compared to baseline (day 0 vs. day 18 $p<0.05$). Negative area under the curve (AUC) analysis similarly showed no significant effect of 15mg/kg DCPG treatment following 2.5µg lactacystin lesioning on contralateral forelimb akinesia in the stepping test (Figure 3.15 B, unpaired t-test $p=0.55$). Prior to lesioning, animals successfully placed their contralateral forelimb 100% of the time relative to total ipsilateral placements in the vibrissae-evoked forelimb placing test. Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 3.16 A, ($F_{(3,27)}=11.60$ $p=0.0001$) but no effect of chronic 15mg/kg DCPG treatment on vibrissae-evoked contralateral forelimb placements ($F_{(1,9)}=1.15p=0.31$ ns). Bonferroni post-hoc tests demonstrated a significant effect of lesioning of the SNc with 2.5µg lactacystin at days 7 and 14 in the vehicle-treated group (day 0 vs. day 7 $p<0.05$, and day 0 vs. day 14 $p<0.01$) and at days 7, 14 and 18 in the DCPG-treated group compared to baseline (day 0 vs. day 7 $p<0.05$, day 0 vs. days 14 and 18 $p<0.01$). Negative area under the curve (AUC) analysis similarly showed no significant effect of 15mg/kg DCPG treatment following 2.5µg lactacystin lesioning on contralateral forelimb placements (Figure 3.16 B, unpaired t-test $p=0.49$). Rotational asymmetry was investigated at the conclusion of the experiment (day 18 post lesioning) utilising amphetamine induced rotational behaviour as an indirect measure of lesion magnitude. Drug vehicle treatment daily for 14 days starting 4 days post unilateral SNc lesioning with 2.5µg lactacystin was associated with a marked induction of circling behaviour following amphetamine administration on day 18 of the study. Similar to the effects of DCPG in the battery of behavioural tests discussed above, an unpaired t-test confirmed that administration of 15mg/kg DCPG failed to

attenuate the circling behaviour induced after amphetamine administration (net mean ipsiversive rotations/5 minutes for drug vehicle-treated animals: 32.3 ± 12.8 , 15mg/kg-DCPG: 33.8 ± 15.4) (Figure 3.17, $p=0.94$ ns).

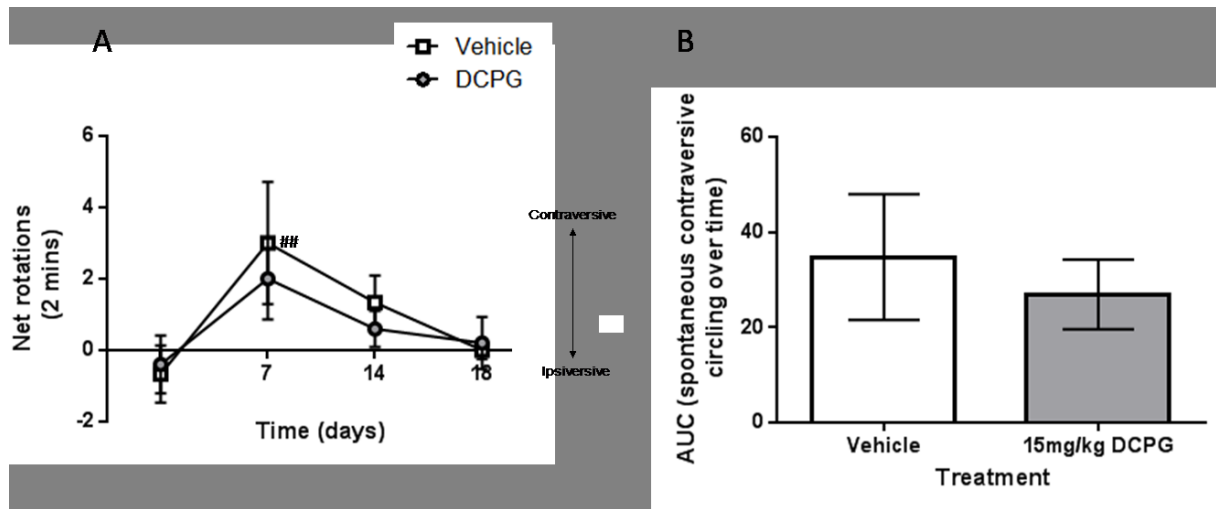


Figure 3.14: Effect of chronic drug vehicle or DCPG treatment on spontaneous circling behaviour. 2.5µg lactacystin-lesioned rats treated with vehicle (n=6) or 15mg/kg DCPG (n=5) from day 4 post-surgery for 14 days were tested for spontaneous circling in a Perspex circling bowl over a 2-minute period prior to surgery at baseline (day 0), 7 days, 14 days and 18 days after lesioning surgery. Data are shown as net 360° rotations ± SEM, with positive values signifying a contraversive bias in rotations, and negative values representing an ipsiversive bias.

A: There was a significant effect of lesion ($F_{(3,27)}=5.0$ $p=0.006$) but not DCPG treatment ($F_{(1,9)}=0.12$ $p=0.73$) on spontaneous circling behaviour when comparing the two treatment groups using two-way ANOVA. Bonferroni post-hoc tests revealed a significant effect of lesion at day 7 compared to baseline in the vehicle-treated group ($^{##}p<0.01$).

B: No difference between treatment groups was revealed when comparing area under curve of spontaneous circling over time using an unpaired t-test ($p=0.63$). AUC units are expressed as time x spontaneous contraversive rotations.

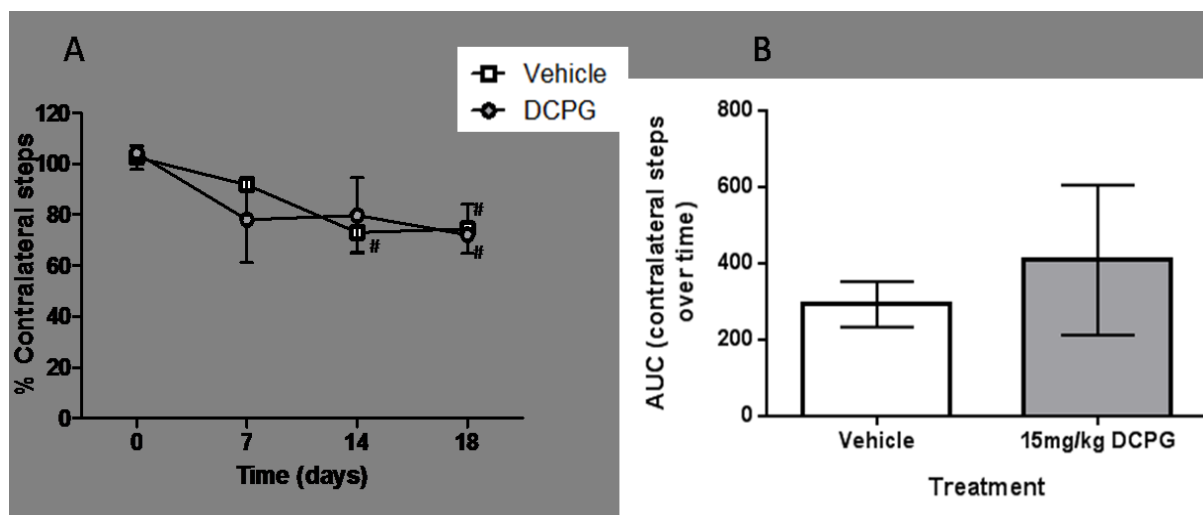


Figure 3.15: Effect of chronic drug vehicle or DCPG treatment on forelimb akinesia in the stepping test. 2.5µg lactacystin-lesioned rats treated with vehicle (n=6) or 15mg/kg DCPG (n=5) from day 4 post-surgery for 14 days were tested for forelimb akinesia in the adjusted stepping test. Testing was carried out prior to surgery at baseline (day 0), 7, 14 and 18 days after lesioning surgery. Data are shown as % contralateral steps relative to number of ipsilateral steps taken $\pm$ SEM.

A: There was a significant effect of lesion ($F_{(3,27)}=6.24$, $p=0.0023$) but not DCPG treatment ($F_{(1,9)}=0.05$, $p=0.82$) on contralateral steps when comparing the two treatment groups using two-way ANOVA. Bonferroni post-hoc tests revealed a significant effect of lesion at days 14 and 18 compared to baseline in the vehicle-treated group ($^{\#}p<0.05$), and at day 18 compared to baseline in the DCPG-treated group ($^{\#}p<0.05$).

B: No difference between treatment groups was revealed when comparing area under curve of contralateral steps over time using an unpaired t-test ($p=0.55$). AUC units are expressed as time x % contralateral steps.

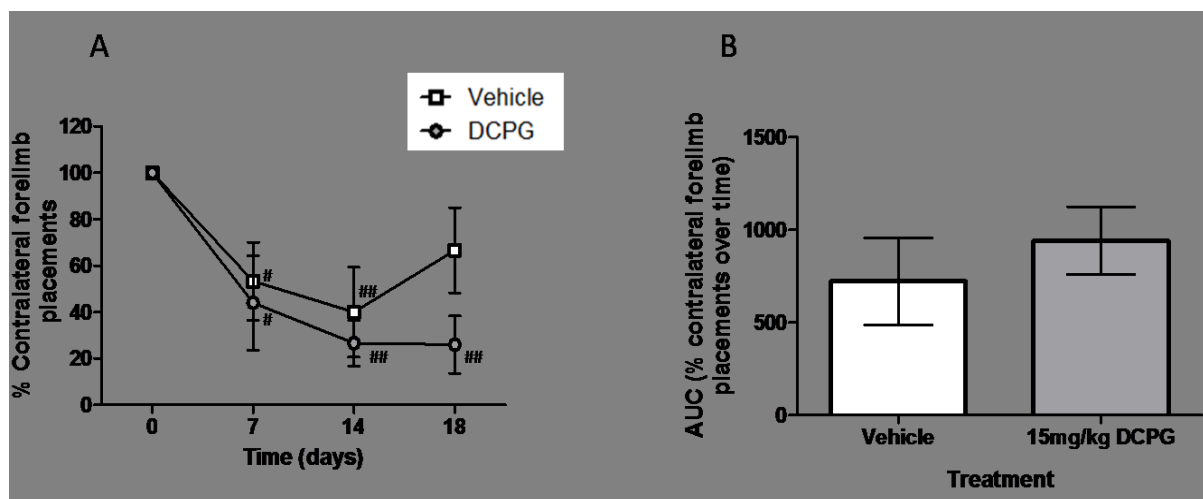


Figure 3.16: Effect of chronic drug vehicle or DCPG treatment on vibrissae-evoked forelimb placement. 2.5µg lactacystin-lesioned rats were treated with drug vehicle (n=6) or 15mg/kg DCPG (n=5) from day 4 post-surgery for 14 days and tested for evoked forelimb placement on a flat surface in response to vibrissae stimulation. Testing was carried out prior to surgery at baseline (day 0), 7, 14 and 18 days after lesioning surgery. Data are shown as % contralateral forelimb placements relative to total ipsilateral placements (total 5 per side) ± SEM.

A: There was a significant effect of lesion ($F_{(3,27)}=11.60$ $p=0.0001$) but not DCPG treatment ($F_{(1,9)}=1.15$ $p=0.31$) on contralateral forelimb placements following vibrissae stimulation when comparing the two treatment groups using two-way ANOVA. Bonferroni post-hoc tests revealed a significant effect of lesion at days 7 and 14 compared to baseline in the vehicle-treated group ($^{\#}p<0.05$, $^{##}p<0.01$), and at days 7, 14 and 18 compared to baseline in the DCPG-treated group ($^{\#}p<0.05$, $^{##}p<0.01$).

B: No difference between treatment groups was revealed when comparing area under curve of contralateral steps over time using an unpaired t-test ($p=0.49$). AUC units are expressed as time x % contralateral forelimb placements.

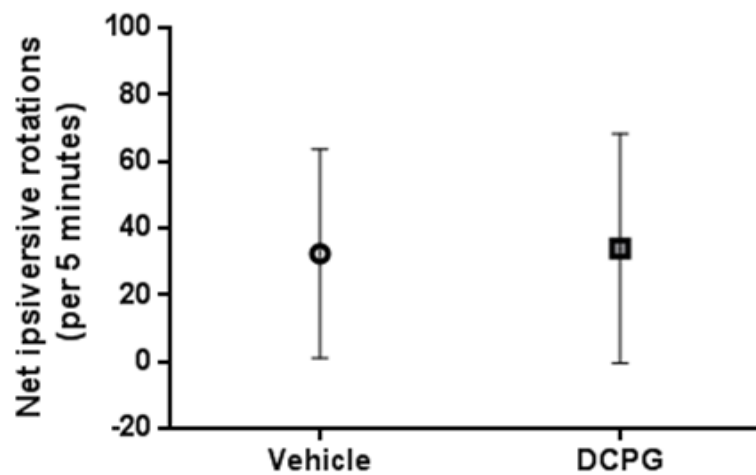


Figure 3.17: Effect of chronic DCPG treatment on rotational asymmetry at day 18 post lactacystin lesioning. Rats unilaterally lesioned in the SNc with 2.5 μ g lactacystin were treated with 5mg/kg amphetamine (i.p.) at the conclusion of the experiment, 18 days after lactacystin lesioning, to assess rotational asymmetry as an indication of lesion magnitude. Net ipsiversive rotations were measured 30 minutes after amphetamine administration for 30 minutes in 5-minute time-bins. Animals treated with 15mg/kg (n=5) DCPG from day 4 post-lesioning for 14 days showed no significant reduction in rotational asymmetry from drug vehicle treated animals (n=6) when analysed with an unpaired t-test. Data are shown as net mean ipsiversive rotations per 5 minutes $\pm$ SEM.

3.3.2.2 Effects of chronic peripheral DCPG administration on dopaminergic neuronal survival and microglial activation in the 2.5µg lactacystin lesioned SNc

In 2.5µg lactacystin lesioned animals, the SNc of the unlesioned hemisphere contained approximately 8,500 TH+ dopaminergic neurons, which is comparable to previous studies (Baquet et al. 2009, Jackson-Lewis et al. 2000, Nair-Roberts et al. 2008)(Figure 3.18 & 3.19). Administration of 15mg/kg DCPG i.p. for 14 days starting from 4 days post lesioning did not affect the numbers of TH+ dopaminergic neurons in the unlesioned SNc (Figure 3.19 A). Numbers of Nissl+ neurons detected in the unlesioned SNc was slightly higher at ~10,000, reflecting the previously described fact that not all neurons in the SNc are dopaminergic. Similarly, DCPG administration at 15mg/kg daily for 14 days did not affect the numbers of Nissl+ neurons in the unlesioned SNc (Figure 3.19 B).

Two-way ANOVA analysis revealed an extremely significant effect of lesioning with 2.5µg lactacystin on TH+ neurons in the ipsilateral SNc by day 19 for both treatment groups (Figure 3.18 and 3.19 A, $F_{(1,9)}=196.30$ $p<0.0001$), but no effect of 15mg/kg DCPG treatment on TH+ neuronal estimates in the ipsilateral SNc ($F_{(1,9)}=0.40$ $p=0.54$ ns). Similarly, two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning with 2.5µg lactacystin on Nissl+ neurons in the ipsilateral SNc by day 19 for both treatment groups (Figure 3.19 B, $F_{(1,9)}=249.72$ $p<0.0001$), but no effect of 15mg/kg DCPG treatment on Nissl+ neuronal estimates in the ipsilateral SNc ($F_{(1,9)}=0.76$ $p=0.40$). In the animals receiving drug vehicle treatment for 14 days starting 4 days post-surgery, Bonferroni multiple comparisons tests showed lesioning of the left SNc with 2.5µg lactacystin resulted in a highly significant ($p<0.0001$) and severe neuronal loss, as assessed by both TH+ and Nissl+ estimates, in the ipsilateral, lesioned SNc compared to the contralateral, unlesioned SNc at day 19 post-lesioning (Figure 3.18 & 3.19). Bonferroni multiple comparisons tests similarly showed lesioning of the left SNc with 2.5µg lactacystin resulted in a highly significant ($p<0.0001$) and severe TH+ and Nissl+ neuronal loss in animals administered 15mg/kg DCPG daily for 14 days starting 4 days post-surgery (Figure 3.18 & 3.19). No significant neuroprotection of TH+ dopaminergic neurons by DCPG against lactacystin toxicity was detected in the ipsilateral lesioned SNc (Figure 3.19 A, vehicle: 1187 ± 197 vs 15mg/kg DCPG: 1983 ± 410 TH+ cells remaining in the lesioned SNc). Similarly, no significant neuroprotection of Nissl+ neurons by DCPG against lactacystin toxicity was detected in the ipsilateral lesioned SNc (Figure 3.19 B, vehicle: 1525 ± 276 vs 15mg/kg: 2654 ± 451 Nissl+ cells remaining in the lesioned SNc).

Comparing percentage cell loss in the lesioned relative to the unlesioned SNc revealed no clear difference between drug vehicle and DCPG-treated animals as illustrated in Figure 3.18 for both TH+ (C) and Nissl+ (D) cell count estimates. Percentage loss of TH+ neurons after drug vehicle treatment was $86 \pm 1.9\%$ compared to $76.3 \pm 5.2\%$ for 15mg/kg DCPG-treated animals. Similarly, the percentage

loss of Nissl+ neurons following drug vehicle treatment was $85.56 \pm 2.02\%$ compared to $74 \pm 5.1\%$ following 15mg/kg DCPG treatment.

Following unilateral lesioning of the SNc with 2.5µg lactacystin, OX-6+ microglia were detected in the lesioned SNc, but no OX-6+ microglia were detected in the contralateral, unlesioned SNc of animals treated with drug vehicle, as illustrated in Figure 3.20. This finding was not affected by treatment for 14 days with 15mg/kg DCPG from day 4 post lactacystin lesioning. Following lactacystin lesioning, a large increase in OX-6+ immunoreactivity was seen in the lesioned SNc in drug vehicle treated animals (4171 ± 1161 OX-6+ microglia, $n=6$). Treatment with 15mg/kg DCPG from day 4 post-lesioning for 14 days did not significantly affect the numbers of OX-6+ microglia in the lesioned SNc when analysed with an unpaired t-test (Figure 3.22 A: 4464 ± 1109 OX-6+ microglia, $p=0.86$ ns, $n=5$).

Similar to OX-6+ microglia, CD68+ phagocytes were detected in the 2.5µg lactacystin lesioned SNc at day 19 post lesioning, but no CD68+ phagocytes were seen in the contralateral, unlesioned SNc in animals treated with drug vehicle, as illustrated in Figure 3.21. This asymmetric positive immunoreactivity was not affected by treatment for 14 days with 15mg/kg DCPG from day 4 post lactacystin lesioning. In the animals receiving drug vehicle treatment for 14 days starting 4 days post-surgery, lesioning of the left SNc with 2.5µg lactacystin resulted in 10939 ± 1900 CD68+ phagocytes located within the lesioned SNc (Figure 3.22 B). Administration of DCPG at 15mg/kg daily for 14 days starting 4 days post-surgery resulted in no significant attenuation in numbers of CD68+ phagocytes in the lesioned SNc at day 19 post-lesioning when analysed with an unpaired t-test (Figure 3.22 B: 15mg/kg DCPG-treated animals: 10050 ± 2259 CD68+ phagocytes, $p=0.77$ ns).

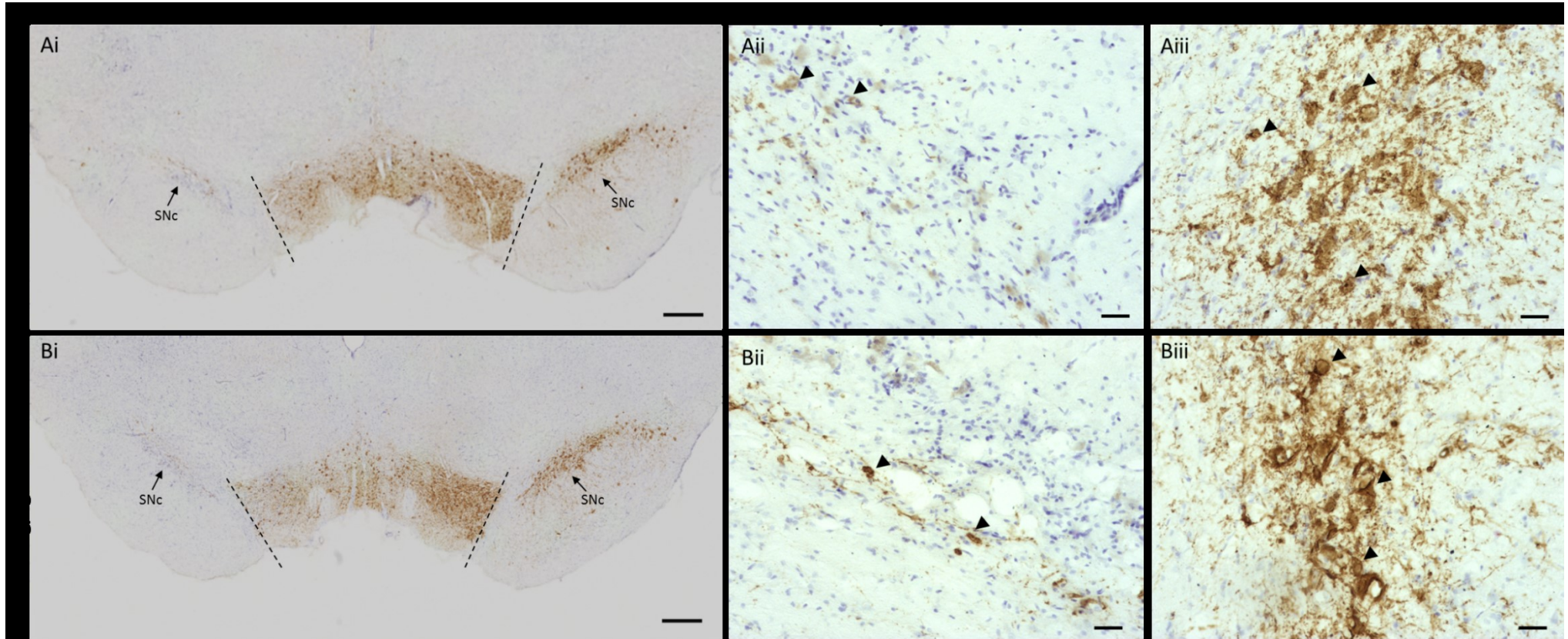


Figure 3.18: Effect of chronic DCPG treatment on TH+ dopaminergic neurons in the SNc of 2.5µg lactacystin-lesioned rats at day 19 post-lesioning. Representative photomicrographs of TH+ immunostaining. Rats unilaterally lesioned in the SNc with 2.5µg lactacystin were treated with either drug vehicle or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining. An extensive loss of TH+ dopaminergic neurons (brown neuronal staining) was seen in the ipsilateral SNc compared to the contralateral SNc of drug vehicle treated animals (Ai-iii). Similarly, an extensive loss of TH+ dopaminergic neurons was seen in the ipsilateral SNc compared to the contralateral SNc of lactacystin lesioned animals treated daily with 15mg/kg DCPG (Bi-iii). Arrows in Ai and Bi denote the SNc in the ipsilateral lesioned and contralateral unlesioned hemisphere. Arrowheads in magnified images denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100µm. High magnification images taken at 20x magnification, scale bar: 30µm.

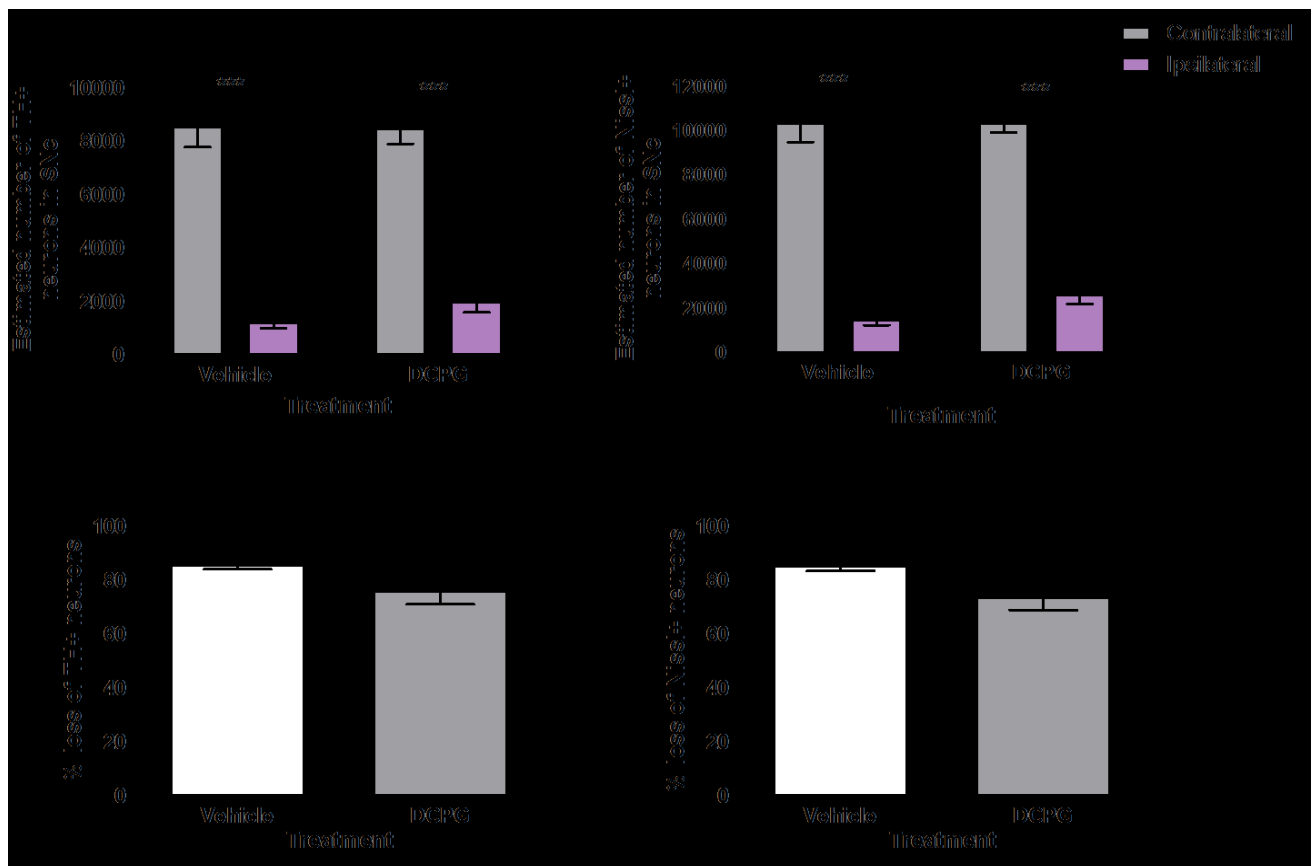


Figure 3.19: Effect of chronic DCPG treatment on the total number of TH+ dopaminergic and Nissl+ neurons in the SNc of 2.5µg lactacystin-lesioned rats at day 19 post-lesioning. Rats unilaterally lesioned in the SNc with 2.5µg lactacystin were treated with either drug vehicle or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining and cell quantification. Total neuronal cell numbers in the SNc were estimated in both the ipsilateral lesioned and contralateral unlesioned hemispheres of each 2.5µg lactacystin-lesioned rat and compared. For all comparisons, drug vehicle treated: n=6, 15mg/kg DCPG treated: n=5.

A: A significant reduction in the number of TH+ dopaminergic neurons was seen in the ipsilateral SNc compared to the contralateral SNc in all both treatment groups, but no effect of DCPG treatment on ipsilateral TH+ dopaminergic neurons was found. Data are shown as estimated total cell number $\pm$ SEM and analysed with a two-way ANOVA, *** p <0.001 ipsilateral vs. contralateral SNc

B: A significant reduction in the number of Nissl+ neurons was seen in the ipsilateral SNc compared to the contralateral SNc in both treatment groups, with no effect of DCPG treatment on ipsilateral Nissl+ neurons. Data are shown as estimated total cell number $\pm$ SEM and analysed with a two-way ANOVA, *** p <0.001 ipsilateral vs. contralateral SNc.

C: The percentage loss of TH+ dopaminergic neurons in the ipsilateral SNc relative to the contralateral SNc at day 19 demonstrated no effect of DCPG on protection of ipsilateral TH+ dopaminergic neurons. Data are shown as % cell loss relative to the contralateral SNc $\pm$ SEM.

D: The percentage loss of Nissl+ neurons in the ipsilateral SNc relative to the contralateral SNc at day 19 demonstrated no effect of DCPG on protection of ipsilateral Nissl+ neurons. Data are shown as % cell loss relative to the contralateral SNc $\pm$ SEM.

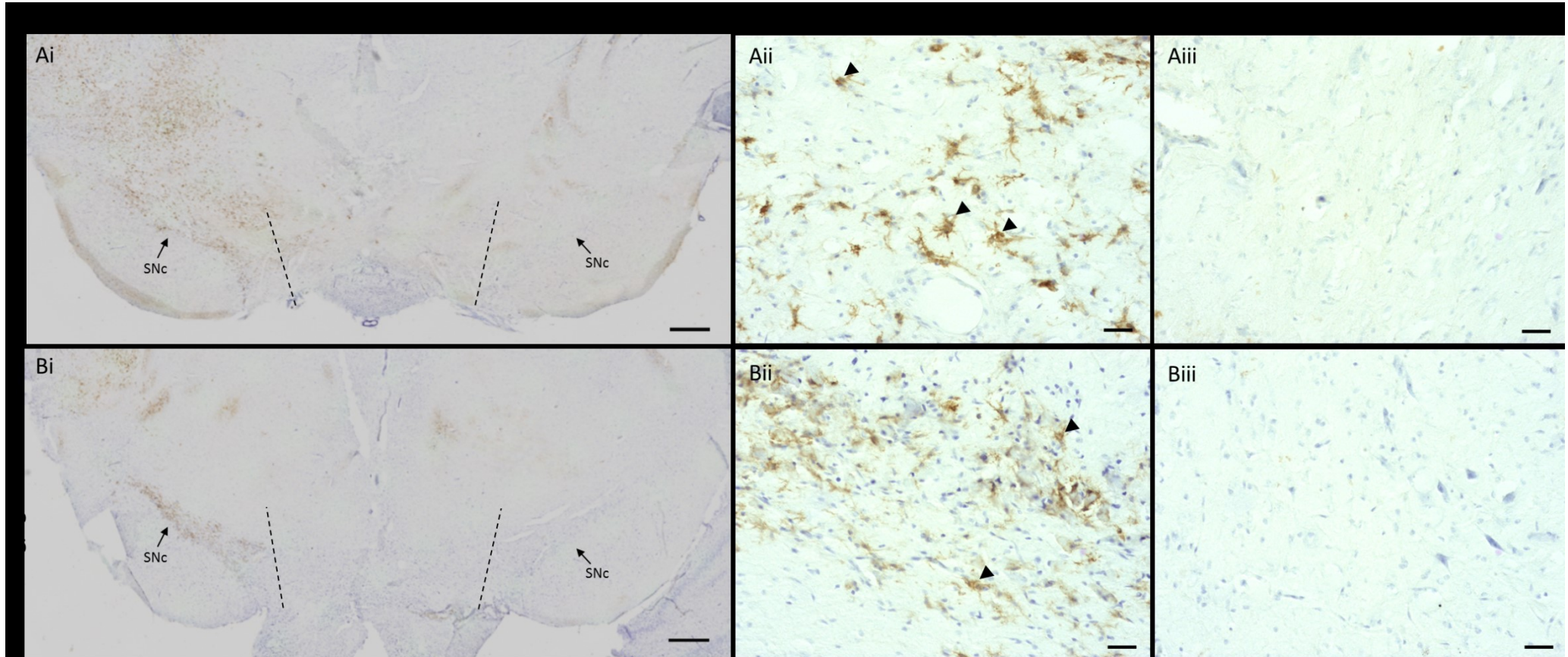


Figure 3.20: Effect of chronic DCPG treatment on OX-6+ microglia in the SNc of 2.5µg lactacystin-lesioned rats at day 19 post-lesioning. Representative photomicrographs of OX-6+ immunostaining. Rats unilaterally lesioned in the SNc with 2.5µg lactacystin were treated with either drug vehicle or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining. Lactacystin lesioning resulted in OX-6+ immunostaining (brown cell staining) in the ipsilateral SNc (Aii and Bii) that was not visible in the contralateral SNc (Aiii and Biii), regardless of treatment. No significant difference in OX-6+ immunostaining in the ipsilateral SNc was detected between drug vehicle treated animals (Ai, n=6) and 15mg/kg (Bi, n=5) DCPG treated animals. Arrows in Ai and Bi denote the SNc in the ipsilateral lesioned and contralateral unlesioned hemisphere. Arrowheads in magnified images (Aii and Bii) denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100µm. High magnification images taken at 20x magnification, scale bar: 30µm.

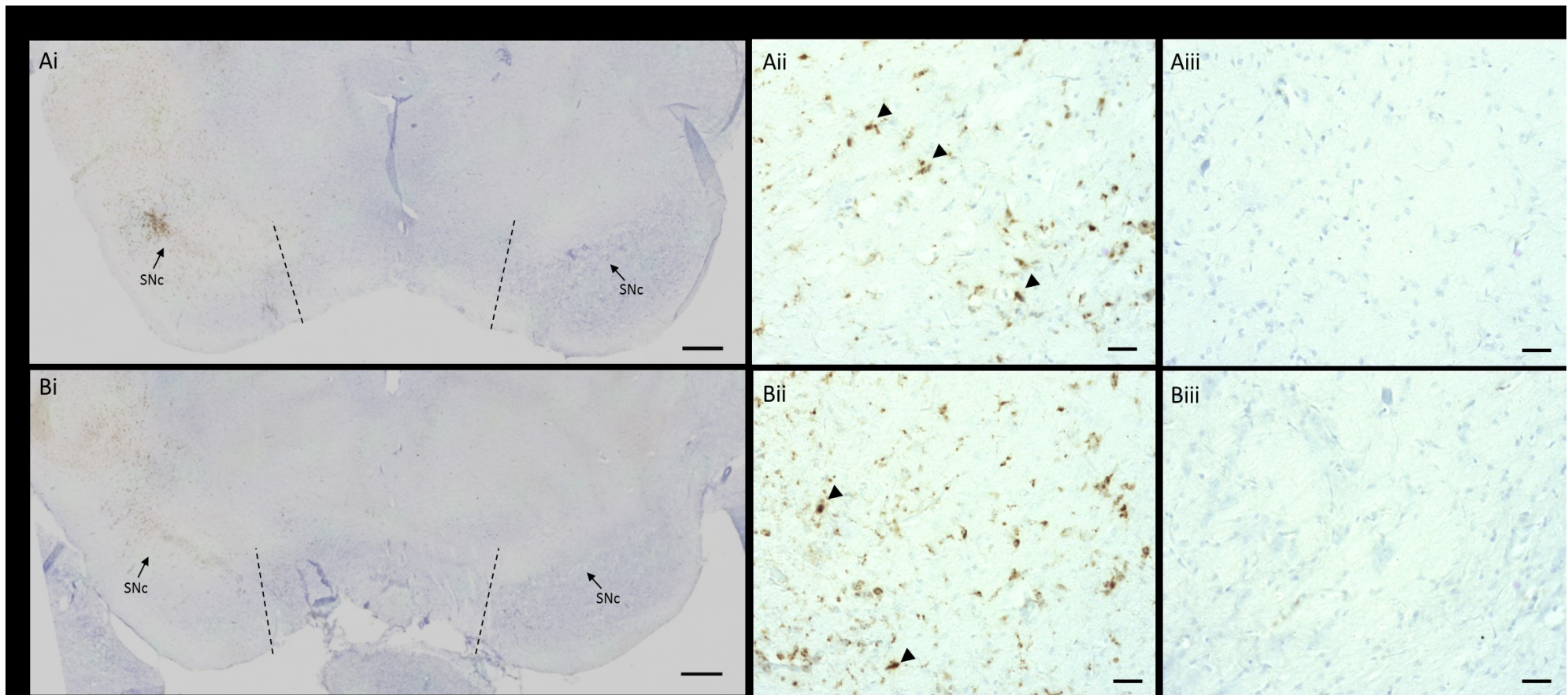


Figure 3.21: Effect of chronic DCPG treatment on CD68+ macrophages in the SNc of 2.5µg lactacystin-lesioned rats at day 19 post-lesioning. Representative photomicrographs of CD68+ immunostaining. Rats unilaterally lesioned in the SNc with 2.5µg lactacystin were treated with either drug vehicle or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining. Lactacystin lesioning resulted in CD68+ immunostaining (brown cell staining) in the ipsilateral SNc (Aii and Bii) that was not visible in the contralateral SNc (Aiii and Biii), regardless of treatment. No significant difference in OX-6+ immunostaining in the ipsilateral SNc was detected between drug vehicle treated animals (Ai, n=6) and 15mg/kg (Bi, n=5) DCPG treated animals. Arrows in Ai and Bi denote the SNc in the ipsilateral lesioned and contralateral unlesioned hemisphere. Arrowheads in magnified images (Aii and Bii) denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100µm. High magnification images taken at 20x magnification, scale bar: 30µm.

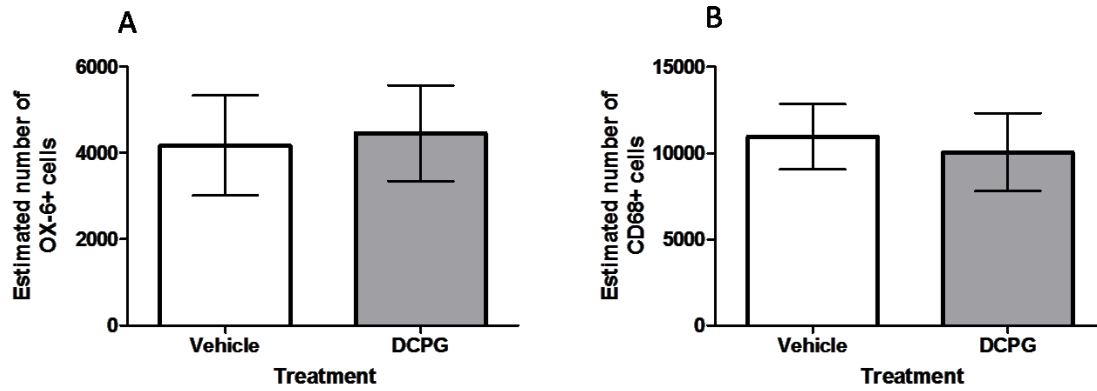


Figure 3.22: Effect of chronic DCPG treatment on microglia in the SNc of 2.5µg lactacystin-lesioned rats at day 19 post-lesioning. Rats unilaterally lesioned in the SNc with 2.5µg lactacystin were treated with either chronic drug vehicle or 15mg/kg DCPG i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining and cell quantification. Immunopositive microglia within the ipsilateral SNc were counted, and estimated total numbers were compared between treatment groups. Data are shown as mean total cell number $\pm$ SEM.

A: A large number of OX-6+ microglia were found in the ipsilateral SNc of drug vehicle treated animals (n=6) following lactacystin lesioning. Chronic treatment with 15mg/kg DCPG (n=5) demonstrated no significant effect on OX-6+ microglial numbers in the ipsilateral SNc when analysed with an unpaired t-test.

B: A very large number of CD68+ phagocytic microglia was found in the ipsilateral SNc of drug vehicle treated animals (n=6) following lactacystin lesioning. Chronic treatment with 15mg/kg DCPG (n=5) demonstrated no effect on CD68+ phagocytic microglial numbers in the ipsilateral SNc when analysed with an unpaired t-test.

3.3.3 Acute systemic DCPG treatment in high concentration (7.5µg) lactacystin-lesioned rats

3.3.3.1 Effects of single peripheral administration of DCPG on behavioural deficits

Acute testing of 3mg/kg and 15mg/kg DCPG was carried out using a battery of behavioural tests in 7.5µg lactacystin lesioned rats. Following unilateral lesioning of the SNc with 7.5µg lactacystin, rats were left for 21 days to allow measurable behavioural deficits to develop. At day 14 post-lesioning, animals were assessed for rotational asymmetry following amphetamine administration, and those that rotated on average <1 net ipsiversive rotation per minute were discounted from the study as poorly lesioned (n=3). Animals that would not rear in the vertical cylinder test (n=3) were also discounted from analysis. From day 21 post-lesioning onwards, animals (n=7 per group) were acutely treated with a single i.p. administration of 3mg/kg or 15mg/kg of DCPG or drug vehicle and behaviourally assessed. Animals demonstrated a strong forelimb use asymmetry and contralateral forelimb akinesia >21 days after unilateral lactacystin lesioning during baseline assessments. As behavioural assessments were carried out at different time points after lesioning to allow wash out of test drugs (i.e. day 22, day 28, day 34), behavioural deficits gradually increased over the testing period, necessitating separate baseline measurements prior to each test, and data was normalised to a percentage of mean baseline scores and analysed with two-way ANOVA.

In the vertical cylinder test, drug vehicle treated animals demonstrated no change in forelimb use asymmetry from baseline for either method of analysis (Figure 3.23 A and B), indicating that repeated testing (over 2 days) in the cylinder had no effect on behaviour. No significant attenuation of forelimb use asymmetry from baseline was observed following acute administration of 3 or 15mg/kg DCPG where all parameters of the test were assessed (push-off, wall exploration and landing) (Figure 3.23 A: 3mg/kg DCPG: $101 \pm 3.4\%$ of baseline, 15mg/kg DCPG: $101 \pm 7\%$ of baseline) ($F_{(2,36)}=0.02$, $p=0.97$ ns). Similarly, no significant attenuation of forelimb use asymmetry from baseline was observed following acute administration of 3 or 15mg/kg DCPG where exploration of the cylinder wall alone was assessed (Figure 3.23 B: 3mg/kg DCPG: $95.9 \pm 2.1\%$ of baseline, 15mg/kg DCPG: $98.4 \pm 7.4\%$ of baseline) ($F_{(2,36)}=0.07$, $p=0.93$ ns). In the adjusted stepping test, drug vehicle treated animals demonstrated no change in forelimb akinesia from baseline, indicating no effect of repeat testing on behaviour in this test. No significant reduction in forelimb akinesia from baseline was seen following 3 or 15mg/kg DCPG administration (Figure 3.23 C: 3mg/kg DCPG: $114.3 \pm 15.2\%$ of baseline, 15mg/kg DCPG: $105.7 \pm 15\%$ of baseline) ($F_{(2,36)}=0.22$, $p=0.79$ ns). Finally, in the vibrissae-evoked forelimb placement test, no effect of repeat testing on behaviour was seen in drug vehicle treated animals (Figure 3.23 D). Again, no significant improvement in contralateral forelimb placement was observed from baseline following acute drug vehicle or DCPG treatment (Figure 3.23 D: 3mg/kg DCPG: 100% of baseline, 15mg/kg DCPG: 85.7% of baseline) ($F_{(2,36)}=1.04$, $p=0.36$ ns).

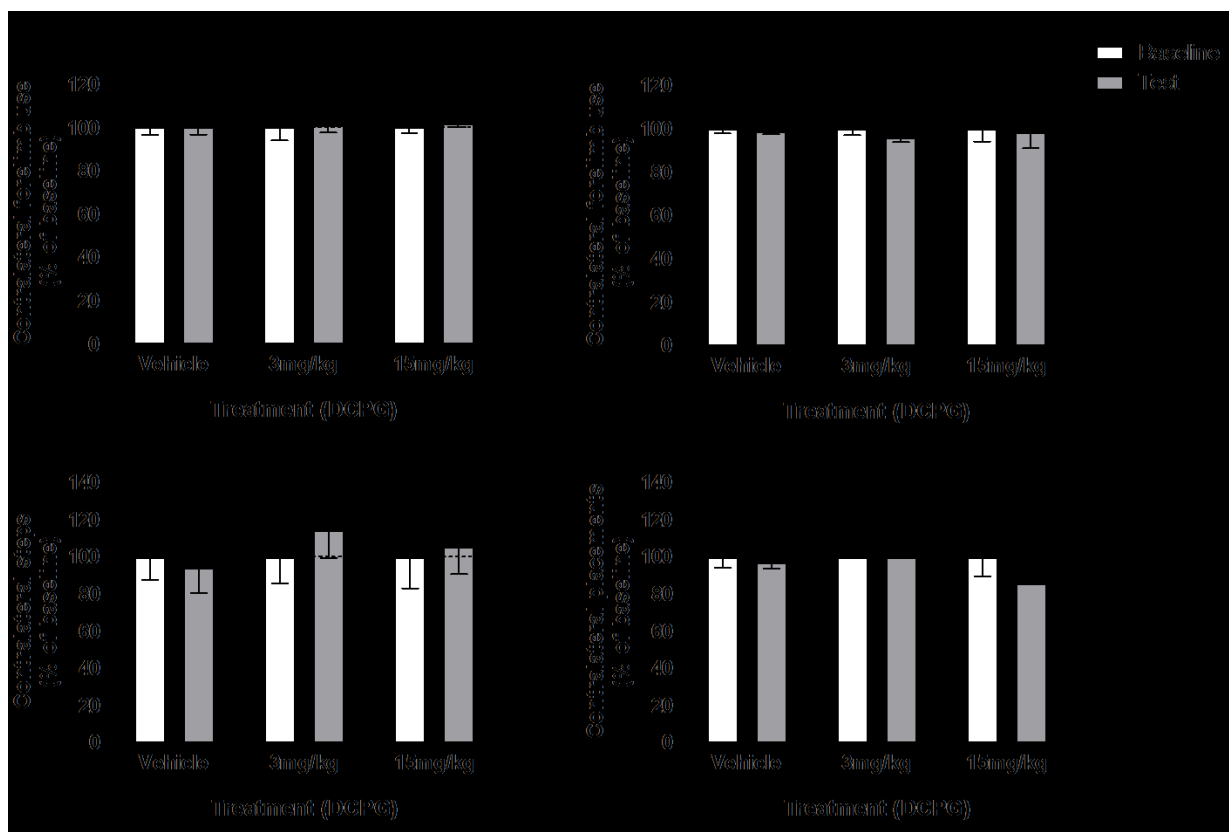


Figure 3.23: Effect of single DCPG administration on forelimb-use asymmetry, forelimb akinesia and forelimb motor initiation. Lactacystin-lesioned rats were assessed in the vertical cylinder test to determine forelimb-use asymmetry, the adjusted stepping test to assess forelimb akinesia and in the vibrissae-evoked forelimb placement test to assess forelimb motor initiation at both baseline (1 day prior to treatment, represented by white bars), and on the following ‘test’ day (represented by grey bars), 30 minutes after drug vehicle or DCPG (3mg/kg, or 15mg/kg) treatment. Data was analysed with two-way ANOVA and is displayed as percentage of baseline score ± SEM, n=7 per treatment group.

A: Rats showed no significant change in contralateral forelimb use from baseline following administration of 3 or 15mg/kg DCPG when assessing forelimb use in the vertical cylinder for push-off, cylinder exploration and landing ($F_{(2,36)}=0.02p=0.97$).

B: Rats showed no significant change in contralateral forelimb use for wall exploration of the vertical cylinder from baseline following administration of 3 or 15mg/kg DCPG ($F_{(2,36)}=0.07p=0.93$).

C: Rats showed no significant change in the number of contralateral steps from baseline following administration of 3 or 15mg/kg DCPG ($F_{(2,36)}=0.22p=0.79$).

D: Rats showed no significant change in number of successful vibrissae-evoked contralateral forelimb placements from baseline following administration of 3 or 15mg/kg DCPG ($F_{(2,36)}=1.04p=0.36$).

3.4 Discussion

The aim of this chapter was to elucidate the value of modulating the mGlu8 receptor in PD using the selective and brain permeable mGlu8 receptor agonist DCPG in the lactacystin model. Acute dosing with DCPG was assessed to see whether it was able to alleviate the motor deficits in the lactacystin PD model, whilst chronic delayed-start dosing with DCPG was assessed to see whether DCPG was neuroprotective against the toxic effects of lactacystin. The overall hypothesis of this Chapter was that activation of the mGlu8 receptor with the DCPG could provide neuroprotection and symptomatic relief in the lactacystin rat model of PD. However, DCPG was unable to provide detectable neuroprotection or neuroprotection-driven symptomatic improvement following delayed-start chronic systemic administration for 14 days in the lactacystin model of PD at doses of 3mg/kg and 15mg/kg. Additionally, acute administration of DCPG was unable to provide antiparkinsonian activity in the same model of PD at the same doses.

3.4.1 Chronic peripheral DCPG administration provides no histological or functional neuroprotection in lactacystin-lesioned animals

As expected, motor deficits in the drug vehicle-treated 7.5µg lactacystin-lesioned animals were more severe than in the drug vehicle-treated 2.5µg lactacystin-lesioned rats, limiting assessment of motor deficit improvement in the 2.5µg lactacystin-lesioned rats despite the introduction of additional behavioural tests. Collectively, behavioural tests employed in this chapter to assess motor deficits demonstrated no robust effect of chronic peripheral DCPG treatment from day 4 post-lesioning at either 3mg/kg or 15mg/kg doses in either high or low concentration (7.5µg or 2.5µg) lactacystin-lesioned rats, indicating no neuroprotection-driven symptomatic improvement by delayed-start chronic DCPG treatment.

Supporting this, dopaminergic cell counts indicate that chronic systemic treatment with DCPG provides no neuroprotection of SNc dopaminergic neurons against lactacystin toxicity in rats unilaterally lesioned with 2.5µg or 7.5µg lactacystin. Total neuronal estimates from Nissl+ cell counts revealed a comparable loss to that of TH+ dopaminergic neurons for all groups, indicating that dopaminergic neurons are killed by lactacystin rather than downregulating expression of the TH enzyme. A large variability in numbers of OX-6+ and CD68+ microglia was seen between rats within the same treatment group, suggesting the inflammatory reaction to lactacystin lesioning varies greatly between individuals. As expected, the concentration of lactacystin used for lesioning did affect the number of OX-6+ microglia in the SNc, with ~4000 OX-6+ cells detected in the ipsilateral SNc of 2.5µg lactacystin-lesioned drug vehicle treated animals compared to ~7000 OX-6+ cells in the ipsilateral SNc of 7.5µg lactacystin-lesioned drug vehicle treated animals. However, no change in numbers of OX-

6+ cells were seen in response to prolonged DCPG treatment for either 2.5µg or 7.5µg lactacystin-lesioned animals. Similarly, no change in numbers of CD68+ cells were seen in response to prolonged DCPG treatment for either 2.5µg or 7.5µg lactacystin-lesioned animals. Collectively, these findings suggest that chronic systemic DCPG treatment has no effect on microglial activation in the SNc in the lactacystin model. The number of CD68+ macrophages was demonstrated to be higher than the number of OX-6+ activated microglia in the SNc, likely due to the recruitment of CD68+ macrophages into the brain from the periphery by signals from activated microglia, a process that occurs in PD (Mosley *et al.* 2012). As recruited phagocytic CD4+ and CD8+ T cells do not express the MHC class II antigen, they would not stain positive for OX-6 but would be CD68+, explaining the difference in numbers of these cell populations in the SNc following lesioning.

As mGlu8 receptors are found on activated microglia and astrocytes, targeting of receptors on these cell types was predicted to result in anti-inflammatory or other beneficial mechanisms such as a reduction in proinflammatory cytokine release. However, this did not appear to happen in the present studies, as no neuroprotection following chronic DCPG administration was detected and no change in numbers of activated microglia were seen in the lactacystin model. Previous work in our lab indicated systemic administration of DCPG for 10 days was neuroprotective in the 6-OHDA model at 15mg/kg, and interestingly, this was associated with a significant reduction in OX-6+ immunostaining in the SNc. However, animals were pre-treated for 1 day prior to lesioning and for a further 9 days post-lesioning (Chan, H., unpublished observation, 2010), suggesting that beginning DCPG treatment prior to lesioning may provide a more robust neuroprotective and anti-inflammatory effect. Additionally, a loss of only ~70% of nigral dopaminergic neurons occurred in response to 6-OHDA lesioning of the MFB, compared to the 85-95% loss of dopaminergic neurons seen following lactacystin lesioning of the SNc in this study. This may explain why no neuroprotection was seen in the present delayed start to treatment study, and also why no change in microglial activation was observed; it might be that DCPG is able to prevent or reduce the initiation of damaging inflammation if given prior to the introduction of a toxin, rather than attenuating it after the inflammatory cascade has begun. As the neurodegenerative process was already established in the lactacystin model when DCPG was introduced at day 4 post-lesioning, any potentially neuroprotective mechanisms that might have had an effect earlier may have been overwhelmed. It may also be that the systemic doses used in the present study limited DCPG's efficacy in the lactacystin model; however, much higher doses were not possible due to their previously demonstrated weight-loss effect in animals at 30mg/kg (Chan, H., unpublished observation, 2010).

3.4.2 Motor deficits are not reversed by acute peripheral DCPG administration in 7.5µg lactacystin-lesioned animals

Although no neuroprotection-driven improvements in motor deficits were demonstrated following chronic treatment with 3 or 15mg/kg DCPG in the previous study, it was important to determine if acute treatment with DCPG could provide symptomatic relief. However, no improvement in forelimb-use asymmetry was apparent in the vertical cylinder test following DCPG treatment at either dose, and analysing forelimb-use for wall exploration alone similarly showed no change from baseline. Similarly, no change in contralateral forelimb akinesia was detected following DCPG administration in the adjusted stepping test or the vibrissae-evoked forelimb placement test. Collectively, these tests demonstrate no effect of acute administration of DCPG on motor deficits, suggesting that DCPG is not effective as an antiparkinsonian agent at these doses in the lactacystin model.

DCPG's apparent lack of antiparkinsonian activity in the lactacystin model contradicts the findings of Johnson et al. (2013), where DCPG was found to improve forelimb asymmetry in the unilateral 6-OHDA model. The disparity between the present study's findings and the findings of Johnson and colleagues may be the result of focal vs. peripheral administration of DCPG; Johnson and colleagues injected DCPG i.c.v, meaning it was present at much higher concentrations within the brain whilst avoiding the potential side effects caused by higher doses in the body. In order to achieve the same brain concentrations (10nM) in the present study as Jones et al. achieved, it is likely that very high doses of DCPG would have to be given peripherally, which is problematic due to the aforementioned peripheral side effect. Additionally, differences in the type of toxin used and the site of lesioning may also be responsible for the disparity seen between these studies; 6-OHDA selectively kills dopaminergic neurons via OS, and when injected into the MFB causes less extensive dopaminergic cell loss from the SNc compared to when injected directly into the SNc (see Introduction section 1.3.2). Lactacystin is a non-selective proteasome inhibitor that can cause dysfunction of other cell populations, perhaps worsening the pathology, and when injected directly into the SNc causes extensive cell loss and a more severe model of PD. The severity of the lactacystin model in this case could potentially limit the detection of a moderately effective antiparkinsonian treatment, although Johnson et al. did not quantify the loss of nigral dopaminergic neurons, only the loss of striatal dopamine (>95%), so the models cannot be directly compared here as striatal dopamine levels decrease prior to dopaminergic neuronal cell loss (Deumens et al., 2001). Furthermore, the assessment and analysis of forelimb asymmetry by Johnson et al. in the 6-OHDA model differed slightly from that used in this study; exposure to a conical-shaped vertical cylinder was assessed for 10 minutes, almost twice as long as in the present study, and a composite measure of forelimb usage for wall contacts and landing (but not pushing-off) was calculated as in Lundblad *et al.* (2004). This method

of analysis resulted in more surface contacts to assess, perhaps allowing smaller changes in behaviour to be distinguished.

Given its presynaptic localisation at strategic points in the affected BG pathways, particularly at the corticostriatal, striatopallidal and subthalamonigral synapses, it is surprising that mGlu8 receptor activation with DCPG failed to effectively modulate abnormal transmission in the indirect pathway to provide antiparkinsonian effects in the lactacystin model. However, it is hard to determine exactly where DCPG may be functionally active in the BG, as studies have ruled out its efficacy at the striatopallidal and subthalamonigral synapses (Beurrier et al. 2009, Lopez et al. 2007, Broadstock et al. 2012, Valenti et al. 2005, Valenti et al. 2003) , suggesting the moderate efficacy of DCPG in the 6-OHDA model seen by Johnson et al. (2013) may be due to its effects in the striatum. As manipulation of corticostriatal, striatopallidal and subthalamonigral pathways is highly unlikely to affect the pathological processes involved in lactacystin toxicity, and mGlu8 receptor activation is not thought to reduce excitatory transmission at the STN:SNc synapse (which may be responsible for further excitotoxic cell death in PD) (Valenti et al., 2005), modulation of synaptic transmission within the BG by DCPG is unlikely to occur in this model.

The contradictory findings of DCPG's efficacy on motor deficits in models of PD may be due to its effects at other receptor types at high concentrations. Thomas et al. (2001) demonstrated that DCPG has a biphasic concentration-response curve on neonatal rat spinal cord, displaying both high and low affinity components. The high affinity component (which DCPG has around a 300-fold higher potency for) is believed to be the mGlu8 receptor; however, the low affinity component is yet to be identified. Indeed, intranigral injection of DCPG was found to be effective at reversing reserpine-induced akinesia in rats; however this effect was not blocked by a group III receptor antagonist, suggesting another receptor was mediating the antiparkinsonian effects in the SNr (Broadstock et al. 2012). It has recently been suggested that DCPG may activate mGlu2 receptors at high concentrations (Mercier *et al.* 2013), which may explain its antiparkinsonian effects in the SNr (Johnson *et al.* 2011). This complicates DCPG's action when given at high concentrations, particularly when it is focally administered into the brain at >submicromolar concentrations.

3.5 Conclusions

Collectively, results in this chapter indicate that delayed-start chronic peripheral DCPG administration provides no neuroprotection-driven attenuation of motor deficits or histological neuroprotection in lactacystin-lesioned animals. Additionally, acutely administered DCPG demonstrates no robust antiparkinsonian effects, indicating DCPG cannot modulate BG pathways sufficiently to attenuate motor deficits in the lactacystin model. DCPG is unable to reduce numbers of inflammatory cells or

modulate signalling in the lactacystin model, but may be effective in other models of PD. Interestingly, DCPG was found to be much more effective as an agonist on cloned human mGlu8 receptors than on native rat mGlu8 receptors, with around 40 fold lower potency on rat mGlu8(Thomas et al. 2001), suggesting DCPG may perhaps provide much more of an antiparkinsonian or neuroprotective effect in humans or other species than it does in rats. Also, it may be that DCPG is unable to provide robust neuroprotective effects in the lactacystin model due to the delay in administering the treatment, and questions the clinical value of preclinical studies demonstrating neuroprotective effects of compounds when treatment prior to the induction of neurodegeneration is used. Further investigation into the effects of targeting the mGlu8 receptor in PD are necessary to understand its potential neuroprotective mechanisms, and to determine if it is a valid target for neuroprotective and antiparkinsonian therapies in humans. However, studies are limited at present by the lack of mGlu8 receptor selective compounds.

Chapter 4: Anti-inflammatory effects of the mGlu8 receptor agonist DCPG *in vivo* in the LPS model of Parkinson's disease and *in vitro* on primary microglia

4.1 Introduction

In normal physiological conditions, microglia display a resting, ramified morphology, constantly surveying their environment (Davalos *et al.* 2005b, Nimmerjahn *et al.* 2005). However, in pathological conditions or when activated in response to an environmental change such as the introduction of LPS, a component of gram-negative bacteria cell walls, they adopt an amoeboid morphology, shifting their phenotype for an effective immune response. Microglial phenotype is proposed to be much more highly plastic and dynamic than previously thought, encompassing a broad range of activation states that are context dependent. Although activated microglia can adopt an anti-inflammatory (M2-like) phenotype with beneficial properties such as clearing pathogens, debris and toxins, releasing neurotrophic factors and playing a role in repair and neurogenesis, they can also adopt a pro-inflammatory (M1-like) phenotype with a neurotoxic impact, depending on the activating conditions (Kreutzberg 1996, Nimmerjahn *et al.* 2005, Takeuchi & Suzumura 2014). In neurodegenerative disease, microglia exist in an activation state that can amplify excitotoxic damage and exacerbate neurodegeneration by releasing glutamate, reactive oxygen and nitrogen species and neurotoxic pro-inflammatory molecules such as cytokines including TNF α and IL-1 β , as well as increasing BBB permeability and recruiting peripheral immune cells, perpetuating the pro-inflammatory state (Barger & Basile 2001, Mosley *et al.* 2012, Barger *et al.* 2007, Cunningham *et al.* 2005, Hirsch *et al.* 2003, Parker *et al.* 2002). Additionally, damaged neurons may induce further microglial activation via the release of microglial activating compounds such as matrix metalloproteinase-3 (MMP-3) or ATP, propagating and sustaining the vicious cycle of neuroinflammation and neurodegeneration (Streit *et al.* 1999, Davalos *et al.* 2005a, Kim *et al.* 2005, Block *et al.* 2007). Increased levels of pro-inflammatory cytokines can be detected in the brain and CSF of PD patients, particularly TNF α , IL-1 β and IL-6 (Blum-Degen *et al.* 1995, Mogi *et al.* 1994). These cytokines can be directly toxic to neurons, for example TNF α can bind to receptors on dopaminergic neurons and induce apoptosis (Baud & Karin 2001). Cytokines can also induce indirect cytotoxic effects through the up-regulation of microglial iNOS, leading to the production of NO which can be extremely damaging to neurons and induce cell death (Chao *et al.* 1992, Gibbons & Dragunow 2006, Hirsch *et al.* 2003, Hunot *et al.* 1996, Liu *et al.* 2002). Furthermore, cell surface exposure of an 'eat me' signal, for example phosphatidylserine externalisation usually displayed prior to apoptosis in response to cellular stressors such as OS or ATP depletion, may lead to premature phagocytosis of potentially viable cells by activated microglia, further contributing to the degenerative process. These cells might otherwise have remained alive or been rescuable, particularly as phosphatidylserine externalisation is reversible and does not always lead to apoptosis (Marinova-Mutafchieva *et al.* 2009, Neher *et al.* 2011).

Microglial toxicity to dopaminergic neurons is well documented in a number of cell culture studies and animal models of PD (Gao *et al.* 2002a, Gao *et al.* 2002b, Liu & Hong 2003). Indeed, inhibiting microglial activation has been demonstrated to be neuroprotective in rodent models of PD (Jackson-Lewis *et al.* 2002, Song *et al.* 2013), and blocking phagocytosis may prevent some forms of inflammatory-mediated neurodegeneration (Neher *et al.* 2012, Neher *et al.* 2011). Additionally, suppressing the activity of the cytokines TNF α and IL-1 β with neutralising antibodies reduces LPS-induced dopaminergic cell loss *in vitro*, supporting their pivotal role in mediating dopaminergic neuronal loss (Gayle *et al.* 2002).

Targeting activated microglia via mGlu receptors could provide therapeutic benefits by reducing inflammatory damage to neurons. Indeed, some mGlu receptor ligands demonstrating neuroprotective properties have reduced the innate inflammatory response in models of PD (Betts *et al.* 2012, Chan *et al.* 2010). When stimulated with LPS, microglial activation is attenuated by group III mGlu receptor agonists L-AP4 and RS-PPG in culture (Taylor *et al.* 2003). Furthermore, L-AP4 and RS-PPG treatment reduced microglial-induced neurotoxicity following activation with LPS, an effect attributed to mGlu receptor-mediated modulation of neurotoxin production by microglia.

Group III mGlu receptor activation has also been shown to promote a more neurotrophic, anti-inflammatory phenotype in microglia. Neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell derived neurotrophic factor (GDNF) are involved in the enhancement of neuronal growth and maintenance, as well as protecting against neuronal damage and promoting recovery (Connor & Dragunow 1998). In support of this, Liang and colleagues recently demonstrated that activation of microglial group III mGlu receptors by glutamate itself results in the release of neurotrophic factors via increased iCa²⁺ release and subsequent activation of the PKC pathway. They proposed that this is a self-regulating process to limit microglial neurotoxicity under conditions of excess glutamate seen in disease pathology (Liang *et al.* 2010). McMullan and colleagues have suggested that activation of group II and III mGlu receptors encourages microglial phenotype to swap from a pro-inflammatory activated state towards an activated yet beneficial phenotype, and that this may be modulated by a reduction in cyclic AMP signalling (McMullan *et al.* 2012). Collectively, these findings imply that targeting group III mGlu receptors may reduce or alter the microglial activation state and is likely to have multiple beneficial consequences on the progression of PD pathology.

LPS is particularly useful for studying the effects of the innate inflammatory reaction on cell death in PD due to the sensitivity of dopaminergic nigral neurons to inflammatory damage. Hence, the LPS model is effective for testing novel anti-inflammatory and potentially neuroprotective therapies on these neuroinflammatory causes of cell death. Although LPS itself does not directly cause neuronal

cell death, it is well established that intranigral injection of LPS *in vivo* induces an innate immune response and results in a progressive and preferential loss of dopaminergic neurons in the SNc, confirming that microglial activation can induce cell death (Castaño et al. 1998, Herrera et al. 2000, Liu & Bing 2011). Extensive microglial activation peaks soon after LPS injection into the nigra (1-2 days), with selective inflammatory-induced neurodegeneration of dopaminergic neurons in the SNc beginning after 2-3 days (Liu 2006, Castano et al., 1998, Liu et al., 2000). Additionally, dysfunctional motor coordination and a decreased motor speed have both been demonstrated in centrally LPS-lesioned rodents (Tanaka et al. 2013, Zhou et al. 2012), although this seems to be dependent on the concentration of LPS used and the time course of the experiment, as some studies have found no behavioural deficits develop (Ariza *et al.* 2010). Interestingly, the LPS model has more recently been shown to reproduce other aspects of PD pathology such as α -synuclein aggregation (Tanaka et al. 2013). Microglial cell cultures can also be activated with LPS, allowing the isolation of, and investigation into specific aspects of microglial behaviour that would be difficult to measure *in vivo*. The level of microglial activation induced by LPS can be quantified through measurement of cytotoxic molecules released into the culture medium such as NO and TNF α as an indicator of pro-inflammatory activation.

Although targeting of group III mGlu receptors on microglia is thought to modulate their activity, it is still unclear which receptor subtype/s may be responsible for this. Supra-nigral treatment in rats with the mGlu4 receptor PAM VU0155041 was not only protective against 6-OHDA-induced neuronal cell loss but it also caused a significant reduction in microglial activation, suggesting a strong anti-inflammatory mechanism of mGlu4 receptor activation (Betts et al. 2012). However, as VU0155041 was applied locally and prior to 6-OHDA lesioning, it is hard to determine whether the reduced microglial response was due to attenuated inflammation or through direct protective actions of mGlu4 receptor activation on neurons; if there is less cell death and debris to clear, there will likely be a corresponding reduction in microglial activation. Studies with delayed administration of ligands following lesioning are needed to resolve this question. Expression of the mGlu8 receptor is up-regulated on activated microglia in MS lesions in patient tissue (Geurts et al. 2005) as well as being strongly detected on primary microglia from rat pups (Taylor et al. 2003). However, the effects of mGlu8 receptor activation on microglia is yet to be explored. The localisation of the mGlu8 receptor on microglia, particularly activated microglia, may provide a useful target to attenuate the detrimental effects or enhance the beneficial effects of microglial activation. DCPG may protect dopaminergic neurons from cell death by reducing the innate inflammatory response triggered by the injection of LPS or may provide additional neuroprotection against lesioning via beneficial anti-inflammatory mechanisms.

The hypothesis for these studies was that activation of the mGlu8 receptor with DCPG can provide protection of SNc dopaminergic neurons against inflammatory-mediated damage via attenuation of microglial pro-inflammatory activation. In order to further investigate the potential neuroprotective mechanisms of mGlu8 receptor activation with DCPG previously detected in the 6-OHDA model in our lab previously, the aim of this Chapter is to investigate the ability of DCPG to modulate inflammation, which is thought to contribute to the progression of PD. The effects of chronic DCPG treatment on inflammatory-mediated neurodegeneration will be investigated in the LPS model of PD by assessing neuroprotection of nigral dopaminergic neurons and any functional motor improvements, and possible attenuation of the microglial response which may contribute to its previously demonstrated neuroprotective action. Additionally, in order to further study DCPG's impact on the inflammatory response and its possible underlying mechanisms of action, the ability of DCPG to attenuate LPS-induced activation of primary microglial cultures will be investigated *in vitro*.

4.2 Experimental design

4.2.1 Chronic systemic DCPG treatment in LPS-lesioned rats: behavioural and neuroprotective effects

To establish if 15mg/kg DCPG could provide neuroprotection through modulation of the innate inflammatory response *in vivo* and further investigate the neuroprotective mechanisms and behavioural effects of the mGlu8 receptor agonist DCPG, the LPS model of PD was used. Male Sprague-Dawley rats (240-290g n=16) were unilaterally lesioned in the SNc with 5µg LPS at day 0 to induce inflammatory-mediated cell death in the SNc (see Methods section 2.1.3 and experimental design, Figure 4.1). LPS has been shown to induce measurable motor deficits, although symptoms are dependent on the extent of the nigrostriatal lesion (see section 1.3.2). This dose of LPS (5µg) was selected to induce a moderate nigrostriatal lesion that would develop fairly rapidly and was expected to induce some measurable motor deficits by day 8, based on the degree of dopaminergic cell loss anticipated (>70%) (Gang et al. 2004). Following lesioning surgery, animals were allowed to recover for 24 hours, with systemic treatment beginning on day 1. Animals were randomly assigned to two treatment groups; 15mg/kg DCPG (n=8) and vehicle (n=8). Drug or vehicle treatment was carried out before midday from day 1 post-lesioning due to the rapid innate inflammatory response, and continued daily for 7 days, as illustrated in Figure 4.1. Forelimb use asymmetry was regularly assessed using the vertical cylinder test (see method in 2.1.5.1) to detect changes in motor symptoms in response to lesioning and drug treatment. Performance in the vertical cylinder test was assessed prior to lesioning surgery to ascertain a baseline score. Animals were then re-tested behaviourally at intervals on days 4 and 8 post-surgery to monitor deficit development and detect possible neuroprotective effects of DCPG in this model. DCPG was administered i.p. immediately after behavioural testing so that the drug did not directly impact on behaviour, and any functional neuroprotection-driven behavioural changes could be detected. On day 8, at the conclusion of the experiment and following vertical cylinder testing, amphetamine-induced rotations (see section 2.1.5.2) were carried out to assess rotational asymmetry and determine the extent of the lesion and potential neuroprotection. On day 9, brains were removed and fixed for histological processing and immunohistochemical analysis. Correct needle placement was verified after Nissl counterstaining of cryostat cut sections using the rat atlas to guide assessment (section 2.2.2.6). Where lesioning was not in the correct place, animals were discounted from analysis (see Table 2.1 for lesion success rate). Staining of dopaminergic neurons was carried out on serial sections throughout the SNc utilising TH immunohistochemistry with Nissl counterstaining (section 2.2.2.3) followed by stereological quantification. Additionally, immunolabelling of activated microglia was performed using OX-6 and immunolabelling of phagocytic macrophages was carried out using CD68 on serial sections throughout the SNc, followed by stereological quantification (see method in 2.2.3).

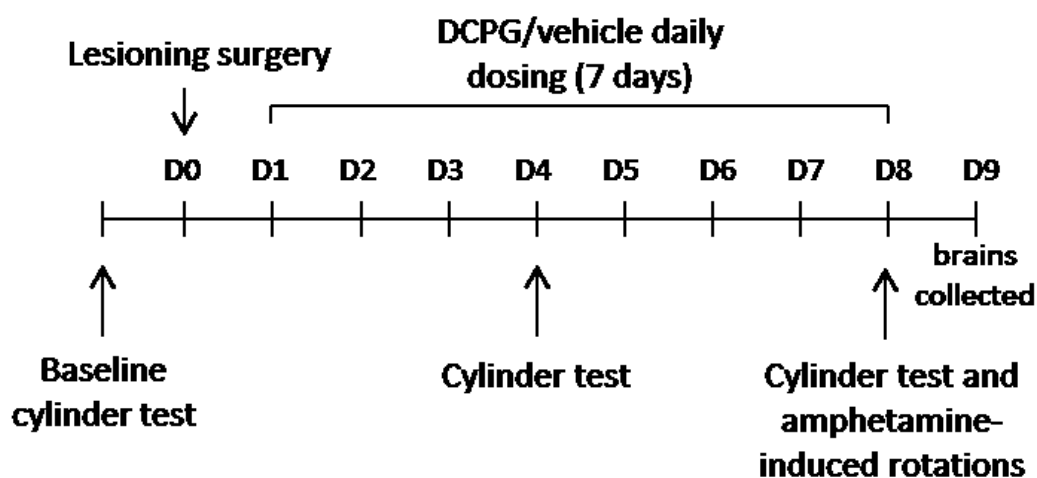


Figure 4.1: Schematic of experimental design for chronic DCPG treatment in LPS-lesioned animals.

Rats were unilaterally lesioned with 5 μ g LPS at day 0, and peripherally administered either vehicle or DCPG (15mg/kg) i.p. once daily from day 1 post-lesioning for 7 days (D1-D7). Baseline forelimb asymmetry was assessed with the vertical cylinder test prior to lesioning and again at days 4 and 8 post-surgery to monitor deficit development and detect any neuroprotective effects of DCPG in this model. Amphetamine-induced rotations were carried out at day 8 to assess rotational asymmetry and determine the extent of the lesion and detect any functional neuroprotection. Brains were removed for histological processing on day 9.

4.2.2 DCPG treatment of primary microglial cultures

Primary rat microglia were isolated from pup brains at P7 using the Percoll gradient separation technique as outlined in section 2.3.1.3 of Methods. Cells were plated in 24 well plates at 1×10^5 cells/well and left to re-establish for 24 hours before treatment began. Prior to testing DCPG's effect on microglial activation, isolated primary cultures were first verified for their purity by fluorescent immunocytochemical staining for the microglial CD11b protein using the anti-OX-42 antibody (see section 2.2.2.3). Additionally, co-staining for the mGlu8 receptor was verified to ensure it was expressed on these primary microglia, as well as specific staining to show no contamination of the cultures with astrocytes or neurons using GFAP and NeuN antibodies (see Table 2.2 and Figure 4.11). In order to optimise the LPS-induced activation of primary microglia, initial experiments were carried out exposing microglia to a range of LPS concentrations in medium for 18 hours in order to determine an appropriate sub-maximal level of activation. This time point (18h) was chosen based on previous findings within our lab that indicated 18h of LPS treatment causes robust microglial activation, and was also based on literature that demonstrates 18 hours is a midway point between the peak expression of the proinflammatory cytokine TNF α and NO synthesis (Byrnes *et al.* 2009, Zujovic *et al.* 2000). Following 18h incubation with LPS, NO levels were quantified in culture medium using the Griess assay to detect nitrite levels (as an index of NO production), and TNF α levels in medium were detected with ELISAs in order to measure the level of activation induced by incubation with varying LPS concentrations, as described in Methods section 2.3.1.7 and illustrated in Figure 4.2 A. A concentration of 125ng/ml LPS was subsequently chosen to activate microglia based on its ability to produce a measurable, reproducible and robust sub-maximal level of activation.

In order to test DCPG's potential to modify the inflammatory response to LPS, primary microglia were isolated and allowed to re-establish for 24h in 24 well plates as previously described, and culture medium was collected from resting cells in order to quantify nitrite levels to ensure cells were not activated prior to commencement of the experiment. Cells were pre-treated with a range of DCPG concentrations in medium for 1 hour prior to the addition of 125ng/ml LPS directly into the medium to induce activation. Pre-treatment with DCPG was carried out to ensure maximal effect of the compound prior to microglial activation. Concentrations of DCPG ranged from 1nM-100 μ M, and were selected to determine a dose-response curve, including doses above and below the EC₅₀ of DCPG (31nM) for the mGlu8 receptor (Jantas *et al.* 2014, Thomas *et al.* 2001). Microglial activation was assessed at the experimental end point of 18h post LPS activation (19h post DCPG treatment) as illustrated in Figure 4.2 B. Cell culture medium was collected and nitrite and TNF α levels were quantified to detect any effect of DCPG treatment on microglial activation. Additionally, the neutral red assay to quantify cell viability was performed directly on remaining cells to detect possible

cytotoxic or proliferative effects of DCPG treatment, as described in Methods section 2.3.1.5. Additionally, the neutral red assay to quantify cell viability was performed directly on remaining cells to detect possible cytotoxic or proliferative effects of DCPG treatment, as described in Methods section 2.3.1.5.

4.2.3 Preparation of drug solution for peripheral administration

For *in vivo* studies, DCPG solution was made daily in sterile saline from fresh compound as described in Methods section 2.1.4. Where a small amount of drug solution remained after dosing, it was kept at -20°C overnight and mixed with fresh compound for the following day's dosing. This was to minimise wastage of the compound and was not believed to affect its efficacy. For *in vitro* studies, DCPG stock was dissolved in saline and added to cell culture medium at a 100x dilution.

4.2.4 Data and Statistical analysis

All raw data was analysed in Microsoft Excel and statistical analysis was performed in Graphpad Prism Software Version 5 (San Diego, CA, USA). Behavioural tests were analysed according to the methods outlined in 2.1.5. Forelimb use asymmetry is shown as percentage forelimb use in the vertical cylinder test. To evaluate the effect of chronic DCPG treatment on motor behaviour in the LPS model, comparisons between treatment groups were carried out with two-way repeated measures ANOVA followed by Bonferroni post correction. Additionally, the negative area under the curve (AUC) of contralateral forelimb use was calculated for each treatment group over time to detect the effect of drug treatment, and comparisons between groups were carried out with unpaired t-tests. Amphetamine-induced rotational asymmetry was analysed as net mean ipsiversive rotations per 5 minutes (over a 30-minute time period) and comparisons between treatment groups were carried out with unpaired t-tests. Cell counts were analysed using two-way ANOVA to compare total cell numbers between hemispheres. Data regarding cell loss is also represented as percentage cell loss $\pm$ SEM relative to the unlesioned, contralateral hemisphere.

For primary cell culture experiments, treatment conditions were replicated in triplicate in 24 well plates. Two independent primary cell culture experiments were carried out, and mean values from each experiment were calculated. Unfortunately, time and financial constraints limited further independent experiments. As only two independent experiments were carried out, no statistics could be performed on the data as SEM could not be calculated, but data is shown as mean $\pm$ range to visually compare all treatment conditions to the vehicle treated condition.

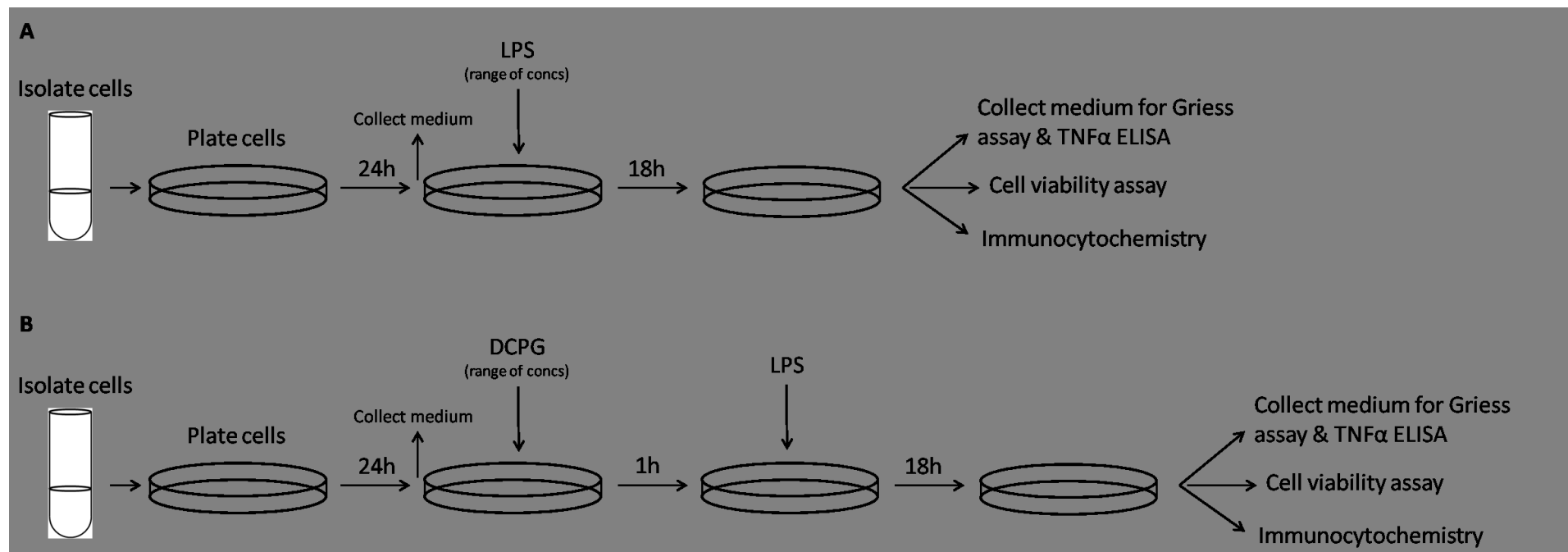


Figure 4.2: Schematic of experimental design for primary microglial cell culture. Primary microglia were isolated from rat pups at p7 and plated for 24 hours to recover before treatment began. A: Optimisation of LPS treatment took place initially to optimise experimental parameters. Following isolation and plating for 24 hours for recovery, cells were exposed to a range of LPS doses for 18 hours in order to ascertain the dose needed for sub-maximal microglial activation. At the experimental endpoint, culture medium from 24 well plates was collected for quantification of nitrite levels using the Griess assay and TNFα levels using ELISA, and viability of treated cells was quantified. Immunocytochemical verification of microglial purity and mGlu8 receptor expression was carried out on fixed cells. B: Following isolation and plating for 24 hours for recovery, cells were pre-treated with a range of DCPG doses (1nM-100μM) in culture medium for 1 hour prior to addition of the previously established dose of 125ng/ml LPS into the medium. After 18 hours, cell culture medium was collected for quantification of nitrite levels using the Griess assay and TNFα levels using ELISA, and viability of treated cells were quantified, as well as immunocytochemical verification of microglial purity.

4.3 Results

4.3.1 Chronic systemic DCPG treatment in LPS-lesioned rats

4.3.1.1 Effects of chronic peripheral DCPG on behavioural deficits

Prior to lesioning, all animals demonstrated an equal use of both forelimbs at baseline in the vertical cylinder test for forelimb use asymmetry (Figure 4.3A: $48.8 \pm 1\%$). Two-way ANOVA analysis of contralateral forelimb use demonstrated no significant effect of treatment ($F_{(1,8)}=2.26$, $p=0.17$) or lesion ($F_{(2,16)}=1.42$, $p=0.27$) as illustrated in Figure 4.3 A. Unpaired t-test analysis of the negative area under curve (AUC) to detect the effect of treatment over time demonstrated no significant effect of treatment overall ($p=0.1029$), although a downward trend was visible in the DCPG treated group suggesting some slight positive effect of treatment on forelimb use asymmetry (Figure 4.3 B).

Wall exploration of the cylinder was assessed independently in the same test as a more sensitive marker of forelimb use asymmetry. At baseline, animals displayed an approximately equal use of both forelimbs for exploration of the vertical cylinder wall, although animals in the DCPG-treated group demonstrated a slight preference for use of the contralateral forelimb (Figure 4.4 A: drug vehicle treated animals: $51 \pm 2.9\%$ vs. DCPG treated animals: $68 \pm 6\%$ contralateral forelimb use at baseline), although this was not found to differ significantly from the drug vehicle treated group in Bonferroni post hoc analysis following two-way ANOVA, as discussed below (Figure 4.4 C). Interestingly, two-way ANOVA analysis of contralateral forelimb use for exploration demonstrated a significant effect of treatment ($F_{(1,8)}=18.17$, $p=0.0028$) but not lesion ($F_{(2,16)}=0.61$, $p=0.55$), however Bonferroni post-hoc tests showed no significant difference at any single time point, as illustrated in Figure 4.4 A. The lack of an effect of lesion over time suggests that $5\mu\text{g}$ LPS did not cause significant behavioural deficits over this time period. Furthermore, unpaired t-test analysis of the AUC to detect the effect of treatment over time demonstrated no significant effect of treatment overall ($p=0.7695$) suggesting no overall effect of DCPG treatment on forelimb use asymmetry during exploration of the cylinder (Figure 4.4 B).

Rotational asymmetry was investigated at the conclusion of the experiment (day 8 post lesioning) utilising amphetamine induced rotational behaviour as an indirect measure of lesion magnitude. Daily drug vehicle treatment i.p. for 7 days starting 1-day post unilateral SNc lesioning with $5\mu\text{g}$ LPS was associated with a slight induction of circling behaviour following amphetamine administration on day 8 of the study. Administration of 15mg/kg DCPG i.p. daily for 7 days starting on day 1 post lesioning failed to significantly attenuate the circling behaviour induced after amphetamine administration (Figure 4.5: net mean ipsiversive rotations/5 minutes for drug vehicle-treated animals: 22.9 ± 12.6 , 15mg/kg -DCPG treated animals: 9.3 ± 3.6 , unpaired t-test: $p=0.32$, ns).

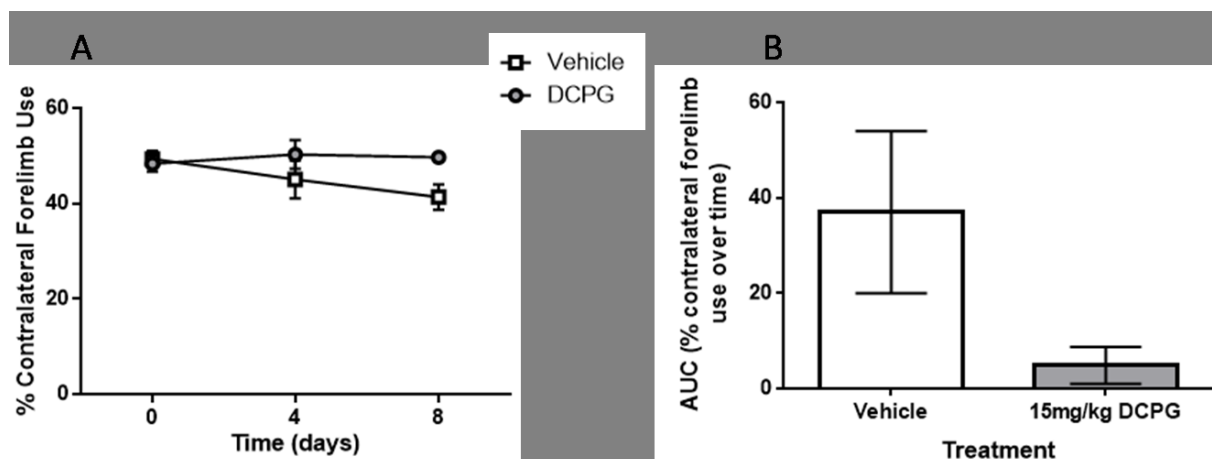


Figure 4.3: Effect of chronic DCPG treatment on forelimb-use asymmetry in the vertical cylinder test.

Rats unilaterally lesioned in the SNc with 5 μ g LPS and treated daily with drug vehicle (n=5) or 15mg/kg DCPG (n=5) i.p. from day 1 post-surgery for 7 days were tested in a vertical cylinder to determine forelimb-use asymmetry during rearing prior to surgery at baseline (day 0) and at 4 and 8 days after lesioning surgery. Data are shown as mean % contralateral forelimb use (push-off, cylinder wall exploration and landing combined) $\pm$ SEM.

A: There was no significant effect of lesion ($F_{(2,16)}=1.42$, $p=0.27$) or of DCPG treatment ($F_{(1,8)}=2.26$, $p=0.17$) on contralateral forelimb use when comparing the two treatment groups over time using a two-way ANOVA.

B: No significant difference between treatment groups was found when comparing negative area under curve of contralateral forelimb use over time using an unpaired t-test ($p=0.10$), although a downward trend was visible. AUC units are expressed as time x % contralateral forelimb use.

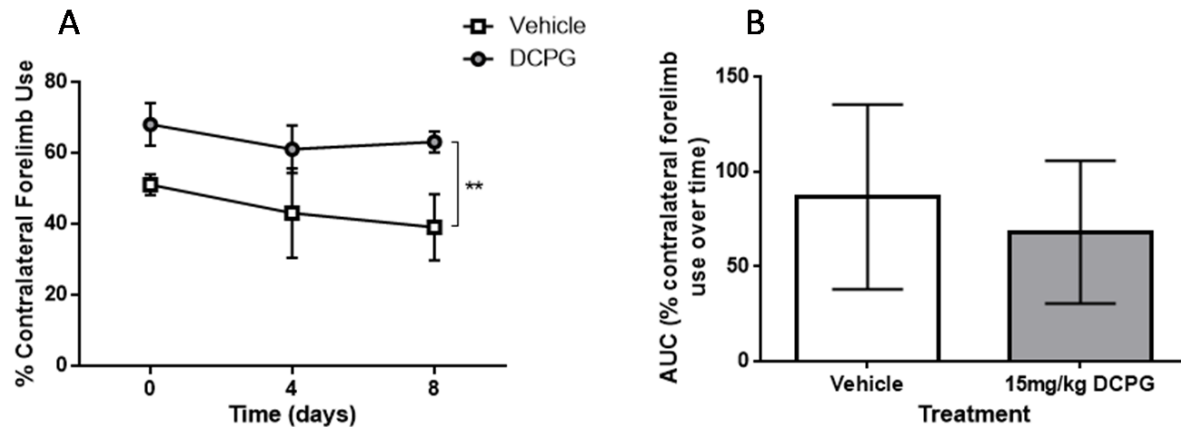


Figure 4.4: Effect of chronic DCPG treatment on forelimb-use asymmetry during wall exploration of the vertical cylinder. Rats unilaterally lesioned in the SNc with 5 μ g LPS and treated daily with drug vehicle or 15mg/kg DCPG i.p. from day 1 post-surgery for 7 days were tested in a vertical cylinder to determine forelimb-use asymmetry during exploration of the cylinder wall prior to surgery at baseline (day 0) and at 4 and 8 days after lesioning surgery. Data are shown as mean % contralateral forelimb use (wall exploration alone) $\pm$ SEM.

A: There was a significant effect of treatment ($F_{(1,8)}=18.17$, $p=0.0028$) but not of lesion ($F_{(2,16)}=0.61$, $p=0.55$) on contralateral forelimb use when comparing the two treatment groups over time using a two-way ANOVA. DCPG-treated animals demonstrated a contralateral forelimb-use bias for wall exploration throughout the experiment explaining the overall treatment effect, $**p<0.01$, and Bonferroni post-hoc tests showed no significant difference at any single time point.

B: No significant difference between treatment groups was found when comparing negative area under curve of contralateral forelimb use over time using an unpaired t-test ($p=0.76$). AUC units are expressed as time x % contralateral forelimb use.

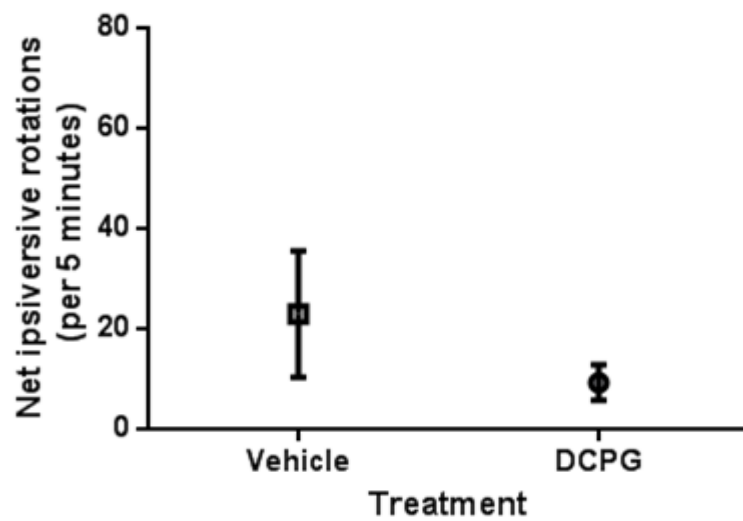


Figure 4.5: Effect of chronic DCPG treatment on rotational asymmetry at day 8 post LPS lesioning.

Rats unilaterally lesioned in the SNc with 5 μ g LPS were treated with 5mg/kg amphetamine (i.p.) at the conclusion of the experiment, 8 days after lactacystin lesioning, to assess rotational asymmetry as an indication of lesion magnitude. Net ipsiversive rotations were measured 30 minutes after amphetamine administration for 30 minutes in 5-minute time-bins. Animals treated with 15mg/kg DCPG i.p. from day 1 post-lesioning for 7 days showed no significant reduction in rotational asymmetry from drug vehicle treated animals. Data are shown as net mean ipsiversive rotations per 5 minutes $\pm$ SEM and analysed with an unpaired t-test ($p=0.32$).

4.3.1.2 Effects of chronic peripheral DCPG administration on neuronal survival and microglial activation in the LPS lesioned SNc

The SNc of the unlesioned hemisphere in drug vehicle treated animals contained approximately 10,000 TH+ dopaminergic neurons, which is similar to other estimates (Baquet et al. 2009, Jackson-Lewis et al. 2000, Nair-Roberts et al. 2008). Administration of 15mg/kg DCPG i.p. for 7 days starting 1-day post lesioning did not affect the numbers of TH+ dopaminergic neurons in the unlesioned SNc (Figure 4.6 and 4.7 A). Numbers of Nissl+ neurons detected in the unlesioned SNc was slightly higher at ~12,000, reflecting the additional non-dopaminergic neuronal populations in the SNc. Again, DCPG administration at 15mg/kg daily for 7 days did not affect the numbers of Nissl+ neurons in the contralateral SNc (Figure 4.6 and 4.7 B). Two-way ANOVA analysis revealed a significant effect of lesion ($F_{(1,16)}=7.35$ $p=0.015$) on TH+ neurons in the ipsilateral hemisphere at 9 days following unilateral lesioning of the SNc with 5µg LPS in both treatment groups overall, although Bonferroni post hoc analysis did not reveal a significant loss of TH+ neurons in the ipsilateral hemisphere in drug vehicle- or DCPG-treated rats (Figure 4.7 A). No significant effect of treatment on TH+ neurons in the ipsilateral SNc was detected ($F_{(1,16)}=1.58$ $p=0.22$), although a slight trend towards neuroprotection in the 15mg/kg DCPG-treated group is apparent (Figure 4.7 A). Similarly, two-way ANOVA analysis revealed a significant effect of lesion ($F_{(1,16)}=7.69$ $p=0.013$) on Nissl+ neurons in the ipsilateral hemisphere at 9 days following unilateral lesioning of the SNc with 5µg LPS in both treatment groups overall, with Bonferroni's multiple comparisons tests revealing a significant effect of lesion in the vehicle-treated group ($p<0.05$)(Figure 4.7 B). However, no significant effect of treatment on Nissl+ neurons in the ipsilateral SNc was detected overall ($F_{(1,16)}=0.97$ $p=0.33$), although again a slight trend towards neuroprotection in the 15mg/kg DCPG-treated group is apparent (Figure 4.7 B)..

Normalisation of cell counts to percentage loss in the ipsilateral relative to the contralateral SNc revealed a slight difference between vehicle and 15mg/kg DCPG-treated groups, as illustrated in Figure 4.7 for both TH+ (C) and Nissl+ (D) cell count estimates. Percentage loss of TH+ dopaminergic neurons after drug vehicle treatment was $52.6 \pm 8.6\%$ compared to $28.3 \pm 3.7\%$ following 15mg/kg DCPG treatment (vehicle vs. 15mg/kg), demonstrating a slight trend towards neuroprotection of TH+ neurons against LPS lesioning following 15mg/kg DCPG treatment ($24.26 \pm 9.37\%$ difference). The percentage loss of Nissl+ neurons mirrored that of TH+ neurons in the ipsilateral SNc, with $53.1 \pm 8.8\%$ Nissl+ neuronal loss following vehicle treatment compared to $25.8 \pm 3.8\%$ Nissl+ neuronal loss following 15mg/kg DCPG treatment, supporting the slight trend towards neuroprotection ($27.33 \pm 9.58\%$ difference) following 15mg/kg DCPG treatment.

Following unilateral lesioning of the SNc with 5µg LPS, OX-6+ microglia were observed in the lesioned SNc, but no OX-6+ immunostaining was observed in the contralateral, unlesioned SNc in

animals treated with drug vehicle, as illustrated in Figure 4.8. The lack of OX-6 staining in the contralateral SNc was not affected by treatment for 7 days with 15mg/kg DCPG from day 1 post lactacystin lesioning (Figure 4.8). Lactacystin lesioning was associated with a large increase in OX-6+ immunoreactivity in the lesioned SNc compared to a total absence of OX-6+ in the unlesioned SNc in drug vehicle treated animals (Figure 4.10 A: 1852 ± 1161 OX-6+ microglia). Treatment with 15mg/kg DCPG from day 1 post-lesioning for 7 days did not significantly attenuate the numbers of OX-6+ microglia in the lesioned SNc as analysed by an unpaired t-test (Figure 4.10 A: 1003 ± 374 OX-6+ microglia, $p=0.46$).

Following unilateral lesioning of the SNc with 5µg LPS, CD68+ macrophages were detected in the lesioned SNc, but no CD68+ macrophages were seen in the contralateral, unlesioned SNc in animals treated with drug vehicle, as illustrated in Figure 4.9. Treatment with 15mg/kg DCPG from day 1 post LPS lesioning for 7 days did not affect the absence of CD68+ staining in the unlesioned SNc, as illustrated in Figure 4.9. In the animals receiving drug vehicle treatment, lesioning of the left SNc with LPS resulted in high numbers (12368 ± 2009) of CD-68+ macrophages located within the lesioned SNc compared to their total absence in the unlesioned SNc. Administration of 15mg/kg DCPG daily for 7 days starting 1-day post-surgery resulted in no significant attenuation in numbers of CD68+ macrophages in the lesioned SNc at day 9 post-lesioning as analysed by an unpaired t-test (Figure 4.9 and 4.10 B: 15mg/kg DCPG-treated animals: 8329 ± 3367 CD-68+ macrophages, $p=0.47$).

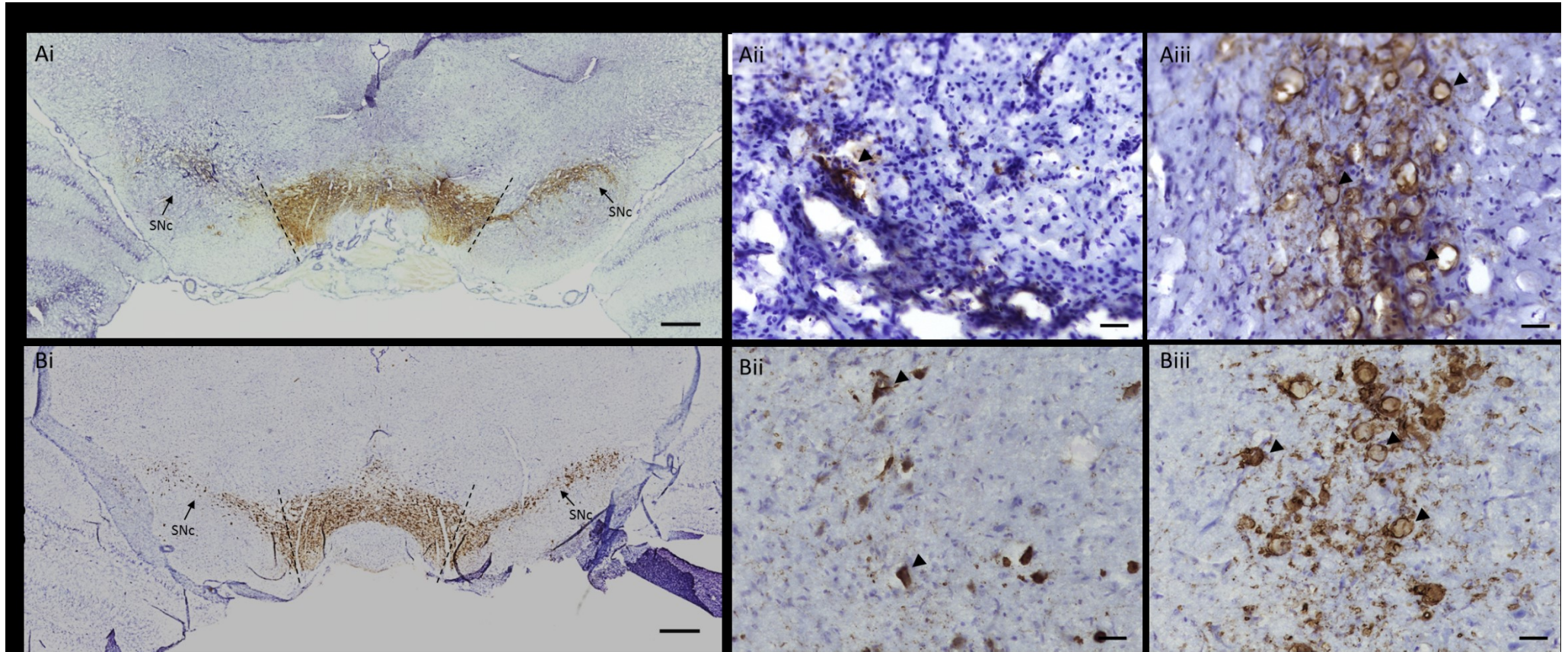


Figure 4.6: Effect of chronic DCPG treatment on TH+ dopaminergic neurons in the SNc of LPS-lesioned rats at day 9 post-lesioning. Representative photomicrographs of TH+ immunostaining. Rats unilaterally lesioned in the SNc with 5 μ g LPS were treated with either drug vehicle (Ai) or 15mg/kg DCPG (Bi) i.p. daily from day 1 post-surgery for 7 days. At day 9 post-lesioning, brains were removed and processed for immunohistochemical staining. A significant loss of TH+ dopaminergic neurons (brown neuronal staining) was seen in the ipsilateral SNc (Aii) compared to the contralateral SNc (Aiii) of drug vehicle treated animals. In comparison, TH+ cell loss was slightly attenuated in the ipsilateral SNc of LPS lesioned animals treated daily with DCPG (Bii-Biii). Arrows in Ai and Bi denote the SNc in the ipsilateral lesioned and contralateral unlesioned hemisphere. Arrowheads in magnified images denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100 μ m. High magnification images taken at 20x magnification, scale bar: 30 μ m.

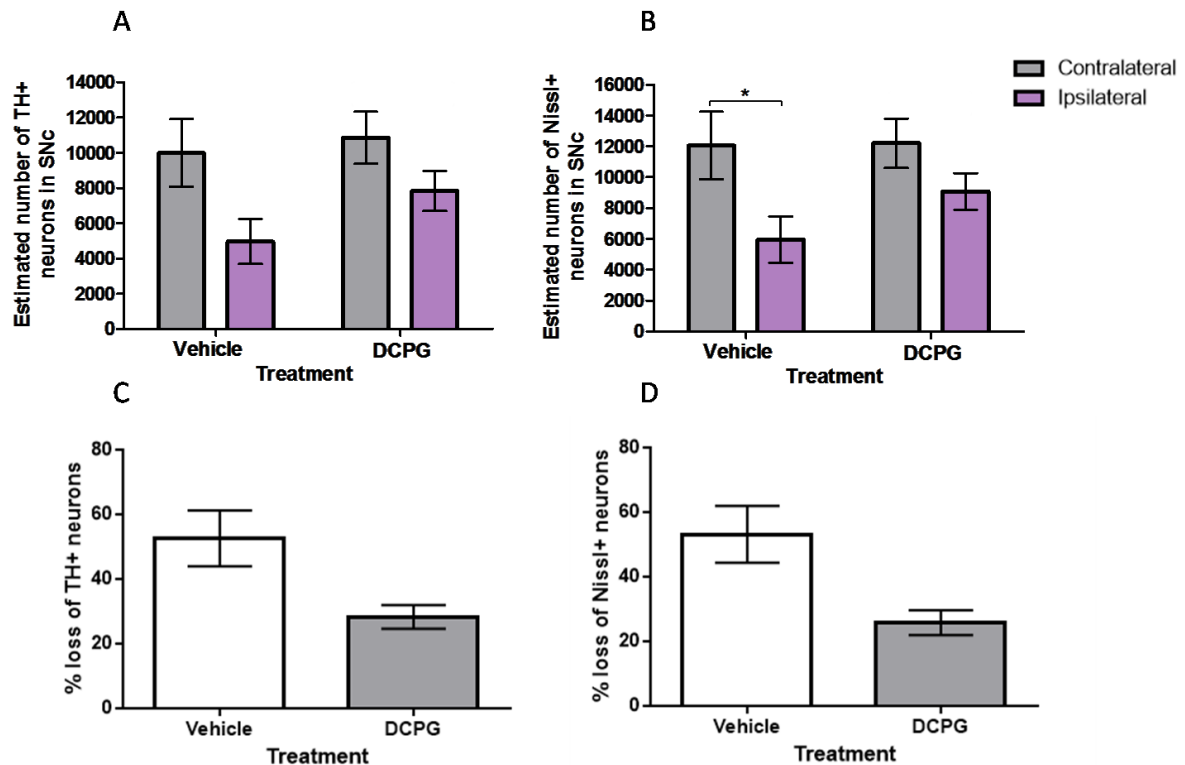


Figure 4.7: Effect of chronic DCPG treatment on the total number of TH+ dopaminergic and Nissl+ neurons in the SNc of LPS-lesioned rats at day 9 post-lesioning. Rats unilaterally lesioned in the SNc with 5µg LPS were treated with either drug vehicle or 15mg/kg DCPG i.p. daily from day 1 post-lesioning surgery for 7 days. At day 9 post-lesioning, brains were removed and processed for immunohistochemical staining and cell quantification. Total neuronal cell numbers in the SNc were estimated in both the ipsilateral lesioned and contralateral unlesioned hemispheres of each rat and compared. For all comparisons, drug vehicle treated: n=5, and 15mg/kg DCPG treated: n=5.

A: No significant reduction in the number of TH+ dopaminergic neurons was seen in the ipsilateral SNc compared to the contralateral SNc in either treatment group following lesioning ($F_{(1,16)}=7.35$ $p=0.015$). No significant effect of treatment on TH+ neuronal estimates in the ipsilateral SNc was detected at day 9 following lesioning with LPS ($F_{(1,16)}=1.58$ $p=0.22$), however a slight trend towards neuroprotection in the ipsilateral SNc is apparent following DCPG treatment. Data are shown as estimated total cell number $\pm$ SEM and analysed with a two-way ANOVA.

B: A significant reduction in the number of Nissl+ neurons in the ipsilateral SNc was detected following lesioning in drug vehicle-treated animals ($F_{(1,16)}=7.69$ $p=0.013$, * $p<0.05$). No significant effect of treatment on Nissl+ neuronal estimates in the ipsilateral SNc was detected at day 9 following lesioning with LPS ($F_{(1,16)}=0.97$ $p=0.33$), however a slight trend towards neuroprotection in the ipsilateral SNc is apparent following DCPG treatment. Data are shown as estimated total cell number $\pm$ SEM and analysed with a two-way ANOVA and Bonferroni's multiple comparisons test.

C: Comparing percentage loss of TH+ neurons at day 9 demonstrated a slight trend towards attenuation of TH+ dopaminergic neuronal loss following chronic 15mg/kg DCPG treatment. Data are shown as mean % ipsilateral SNc TH+ cell loss relative to the contralateral SNc $\pm$ SEM.

D: Comparing percentage loss of Nissl+ neurons at day 9 similarly demonstrated a slight trend towards attenuation of Nissl+ dopaminergic neuronal loss following chronic 15mg/kg DCPG treatment. Data are shown as mean % ipsilateral SNc Nissl+ cell loss relative to the contralateral SNc $\pm$ SEM.

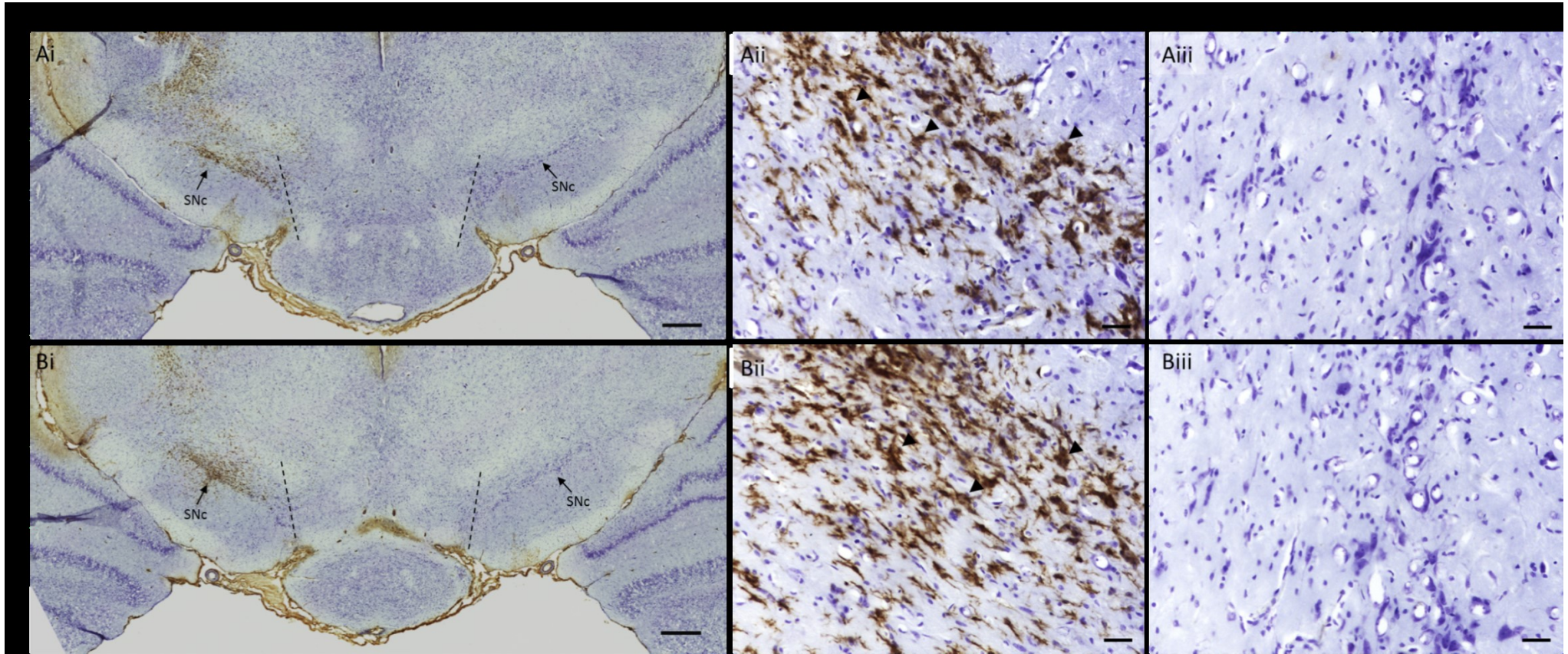


Figure 4.8: Effect of chronic DCPG treatment on OX-6+ microglia in the SNc of LPS-lesioned rats at day 9 post-lesioning. Representative photomicrographs of OX-6+ immunostaining. Rats unilaterally lesioned in the SNc with 5 μ g LPS were treated with either drug vehicle (Ai) or 15mg/kg DCPG (Bi) i.p. daily from day 1 post-surgery for 7 days. At day 9 post-lesioning, brains were removed and processed for immunohistochemical staining. LPS lesioning resulted in OX-6+ immunostaining (brown cell staining) in the ipsilateral SNc (A and Bii) that was not visible in the contralateral SNc (A and Biii), regardless of treatment. No significant difference in OX-6+ immunostaining in the ipsilateral SNc was detected between drug vehicle treated animals and 15mg/kg DCPG treated animals. Arrows denote the SNc in the ipsilateral lesioned hemisphere and the contralateral unlesioned hemisphere. Arrowheads in magnified images denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100 μ m. High magnification images taken at 20x magnification, scale bar: 30 μ m.

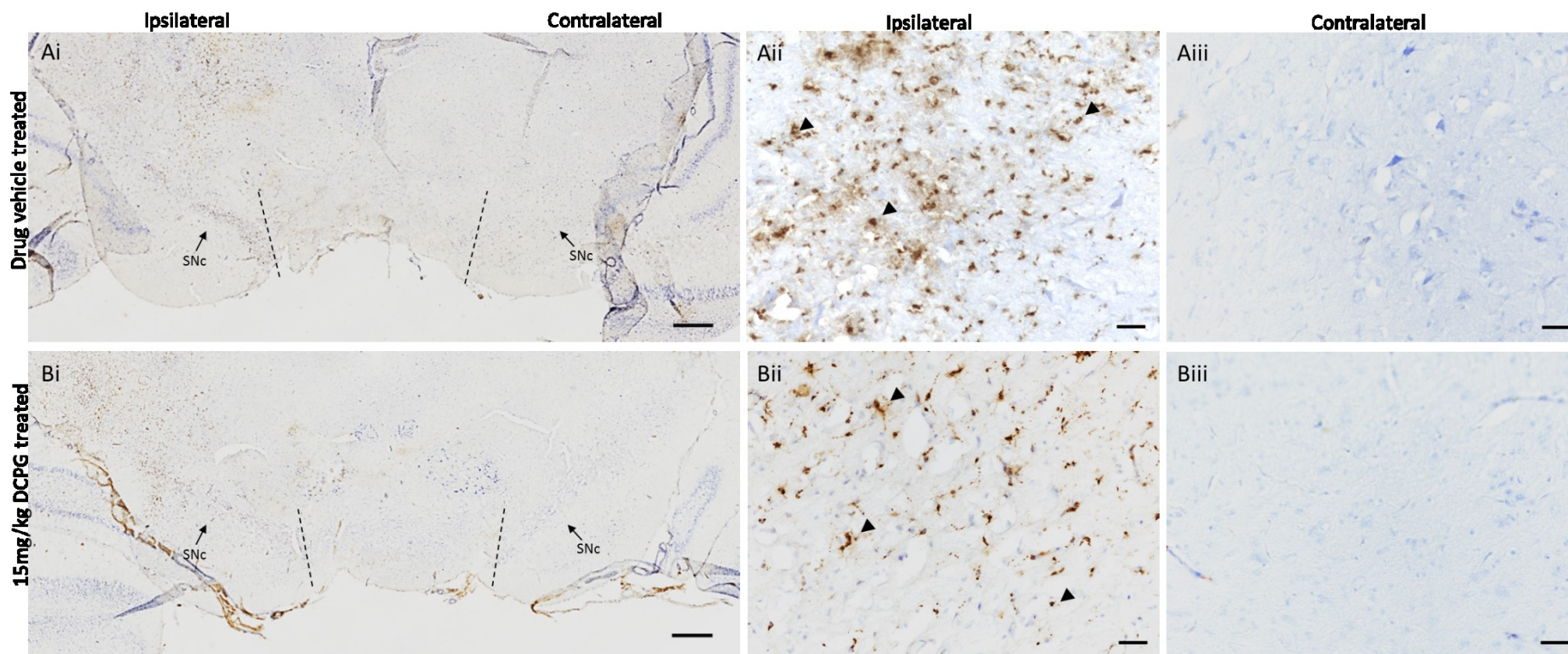


Figure 4.9: Effect of chronic DCPG treatment on CD68+ macrophages in the SNc of LPS-lesioned rats at day 9 post-lesioning. Representative photomicrographs of CD68+ immunostaining. Rats unilaterally lesioned in the SNc with 5 μ g LPS were treated with either drug vehicle (Ai) or 15mg/kg DCPG (Bi) i.p. daily from day 1 post-surgery for 7 days. At day 9 post-lesioning, brains were removed and processed for immunohistochemical staining. LPS lesioning resulted in CD68+ immunostaining (brown cell staining) in the ipsilateral SNc (A and Bii) that was not visible in the contralateral SNc (A and Biii), regardless of treatment. No significant difference in CD68+ immunostaining in the ipsilateral SNc was detected between drug vehicle-treated animals and 15mg/kg DCPG treated animals. Arrows denote the SNc in the ipsilateral lesioned hemisphere and the contralateral unlesioned hemisphere. Arrowheads in magnified images denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100 μ m. High magnification images taken at 20x magnification, scale bar: 30 μ m.

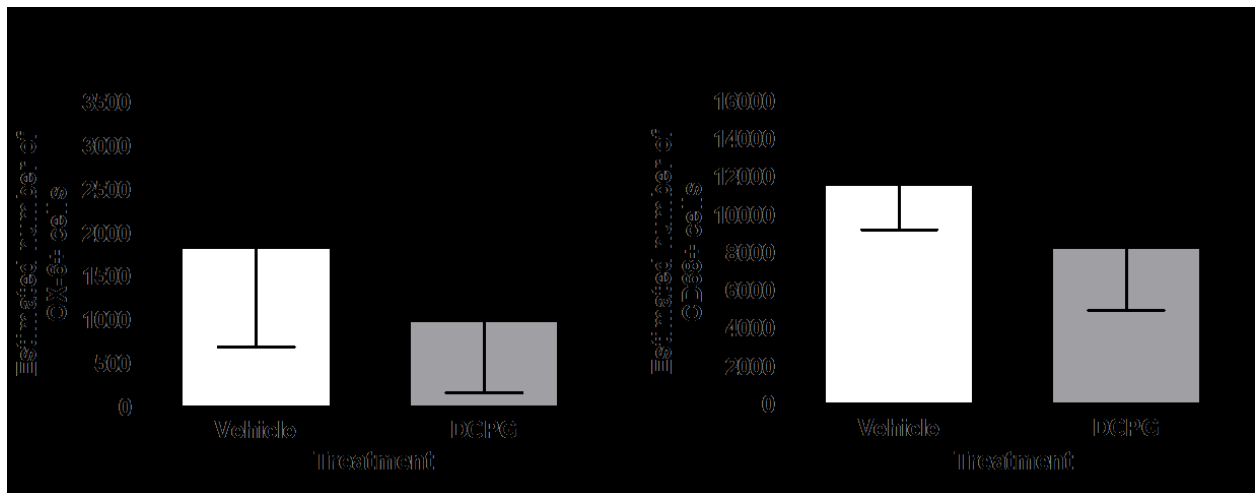


Figure 4.10: Effect of chronic DCPG treatment on microglia and macrophages in the ipsilateral SNc of LPS-lesioned rats at day 9 post-lesioning. Rats unilaterally lesioned in the SNc with 5 μ g LPS were either treated chronically with drug vehicle or 15mg/kg DCPG i.p. daily from day 1 post-surgery for 7 days. At day 9 post-lesioning, brains were removed and processed for immunohistochemical staining and cell quantification. Immunopositive microglia within the ipsilateral SNc were counted, and estimated total numbers were compared between treatment groups. Data are shown as mean total cell number $\pm$ SEM and analysis performed using unpaired t-tests.

A: OX-6+ microglia were found in the ipsilateral SNc of drug vehicle treated animals (n=5) following LPS lesioning. Chronic treatment with 15mg/kg DCPG (n=5) demonstrated no significant effect on OX-6+ microglial numbers in the ipsilateral SNc ($p=0.46$).

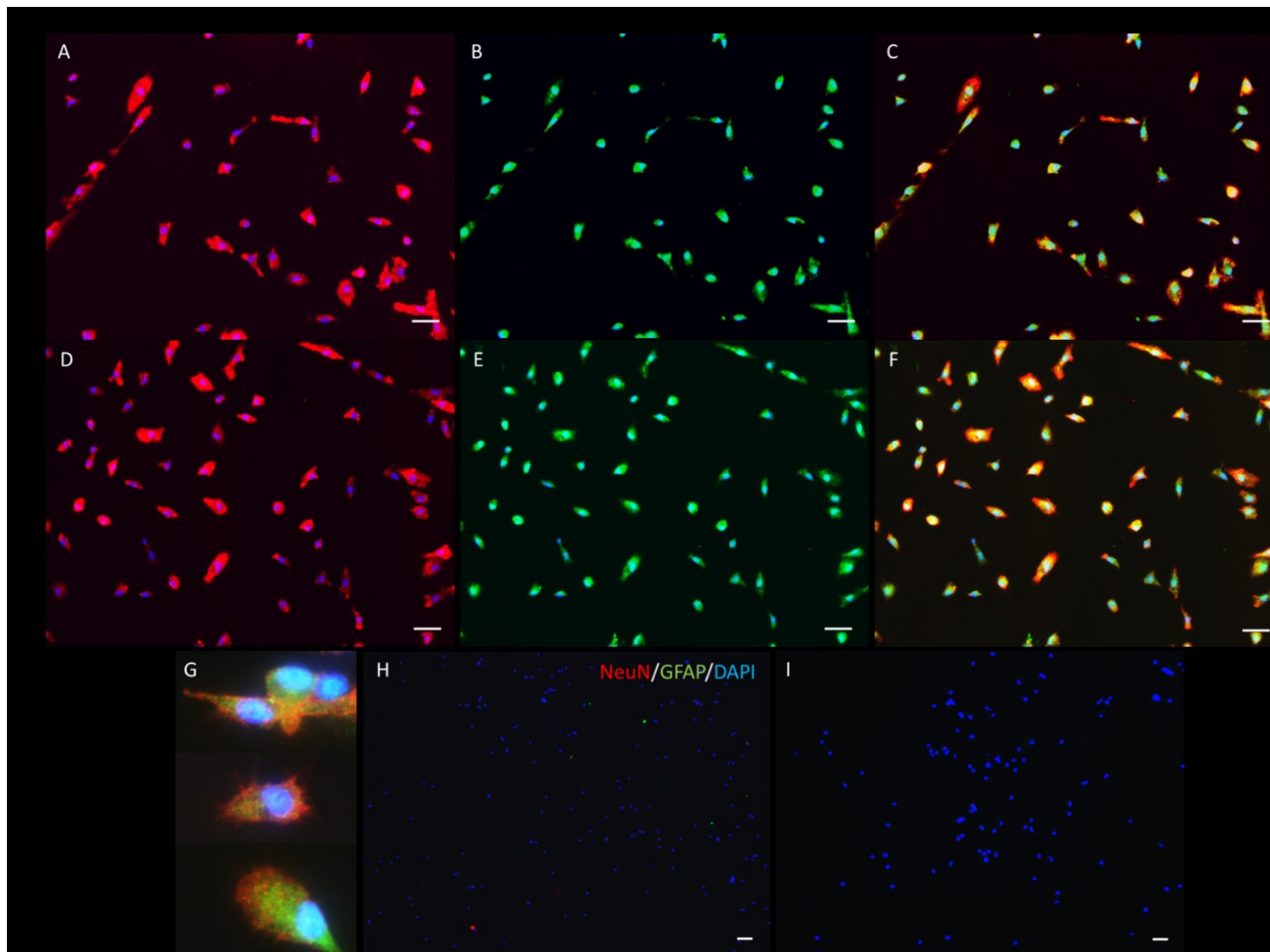
B: A large number of CD68+ macrophages were found in the ipsilateral SNc of drug vehicle treated animals (n=5) following LPS lesioning. Chronic treatment with 15mg/kg DCPG (n=5) demonstrated no effect on CD68+ microglial numbers in the ipsilateral SNc ($p=0.47$).

4.3.2 Activation of primary microglia and treatment with DCPG

4.3.2.1 Verifying the purity of microglial cell cultures and expression of the mGlu8 receptor

Immunofluorescent staining of untreated primary microglial cells 24 hours after isolation revealed no contamination by OX42 negative non-microglial cells, as illustrated in Figure 4.11. Cells activated with 125ng/ml LPS showed similar levels of OX42 positive staining with no OX42 negative cells visible (Figure 4.11 C). Specificity of OX42+ staining was confirmed by exclusion of the primary antibody (Figure 4.11 I). Interestingly, the intensity of OX42+ staining varied between cells, with some cells showing low but detectable levels of OX42+ staining and others much higher stain intensities. All OX42+ microglia showed positive co-staining for the mGlu8 receptor (green staining in Figure 4.11), with both resting cells and cells activated by LPS (125ng/ml) demonstrating positive staining for the mGlu8 receptor (Figure 4.11 B and E). The specificity of mGlu8 receptor staining was confirmed by exclusion of the primary antibody (Figure 4.11 I). Additional staining to detect neurons (NeuN) and astrocytes (GFAP) revealed no staining of these cell types as shown in Figure 4.11 H, confirming the purity of primary microglial cell cultures.

Figure 4.11 (next page): Confirming purity of isolation of primary microglial cells and their expression of the mGlu8 receptor. Rat primary microglia were isolated from pup brains at P7 and plated in 24 well plates (on glass coverslips) at a density of 1×10^5 before being left to re-establish for 24 hours. Cells were then left untreated or treated with LPS (125ng/ml) for 18 hours in order to induce activation. Cells were subsequently fixed and stained with immunofluorescence. All visually checked isolated primary cells in culture stained positive for OX42, a pan microglial marker (red), confirming their purity. OX42+ staining was present in untreated cells (A) and cells activated with 125ng/ml LPS (D). Primary microglia also stained positive for the mGlu8 receptor (green) in untreated (B) and LPS treated (E) conditions. Composite images (C, F and G) demonstrate the cellular staining of the mGlu8 receptor and nucleus counterstained with DAPI (blue) within OX42+ microglial cells (red). No positive staining for neurons (NeuN, red) or astrocytes (GFAP, green) was apparent in cultures (H) and specificity of staining was confirmed by excluding the primary antibodies (I, OX42 negative (red), mGlu8 receptor negative (green), DAPI+ staining all nuclei (blue)). High magnification images (G) taken at 20x magnification, low magnification images taken at 2x and 4x magnification. Scale bar: 30µm.



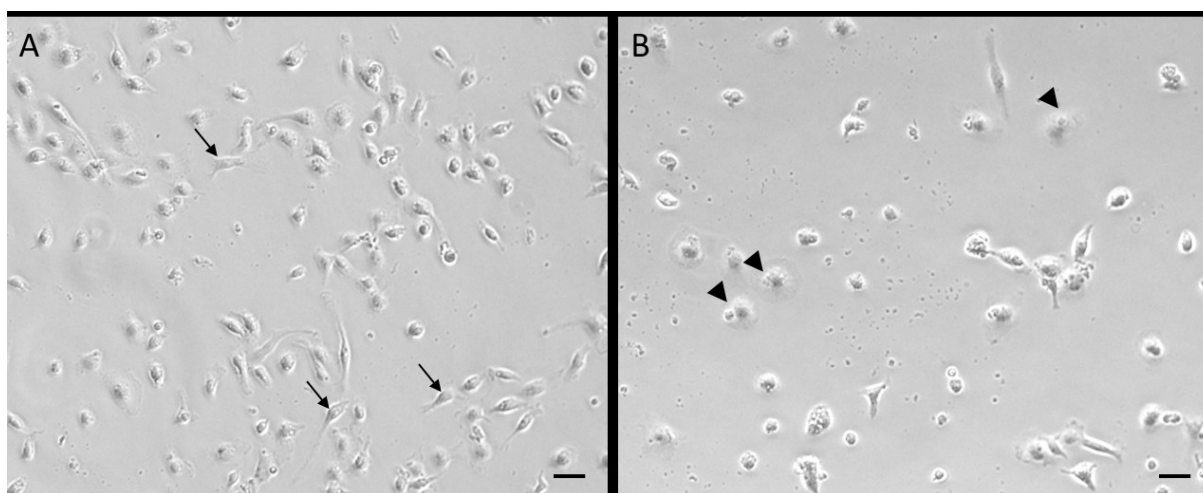


Figure 4.12: Primary microglial cell morphology at rest and in response to LPS activation. Rat primary microglia were isolated from pup brains at P7 and plated on 24 well plates at a density of 1×10^5 before being left to re-establish for 24 hours. Cells were then treated with LPS (125ng/ml) for 18 hours in order to induce activation. (A) Untreated primary microglia show a characteristic resting, ramified morphology, with compact cell bodies and thin processes sampling the environment. Black arrows demonstrate representative cells. (B) In response to LPS activation, microglia adopt an activated morphology, with swollen cell bodies and retracted processes. Some cells demonstrate an amoeboid, phagocytic morphology, as shown by black arrowheads. Images taken at 10x magnification. Scale bar: 25 μ m.

4.3.2.2 Optimising the concentration of LPS

After isolation from rat pup brains at P7, cells were plated in 24 well plates and left to re-establish for 24 hours. Cells were verified to be resting prior to stimulation with LPS through morphological assessment (see Figure 4.12 A) and quantification of nitrite levels (as an index of NO production) in collected culture medium using the Griess assay. Levels of NO production by microglia after plating and prior to LPS stimulation were very low ($0.36 \pm 0.17 \mu\text{M}$ nitrite) confirming no activation of cells prior to LPS stimulation. Cells were incubated with vehicle (control) or a range of LPS concentrations for 18 hours to induce microglial activation (Figure 4.12 B). Two independent primary cell culture experiments were carried out, and mean values from each experiment were calculated, so no statistics could be performed on the data as SEM could not be calculated, but data is shown as mean $\pm$ range to visually compare all treatment conditions to the vehicle treated condition.

Collected cell culture medium was analysed for levels of nitrite and TNF α , as a measure of microglial activation. Results show incubation of cells with $>62.5 \text{ ng/ml}$ LPS induced an increase in NO production compared to control (Figure 4.13 A) by primary microglia. Similarly, incubation of cells with $>62.5 \text{ ng/ml}$ LPS induced an increase in TNF α production compared to control (Figure 4.13 B) by primary microglia. No appreciable change in cell viability from control conditions was detected in response to increasing concentrations of LPS, although a dip in TNF α levels at 250ng/ml LPS may be due to the slightly lower cell viability seen in this condition (250ng/ml: $67 \pm 20\%$ of control) (Figure 4.13 C). As most concentrations of LPS tested here induced a significant and measurable activation of

microglia, 125ng/ml LPS was selected for subsequent testing of DPCG. This concentration was chosen due to its ability to produce a measurable, reproducible and robust sub-maximal level of activation ($0.37 \pm 0.3\mu\text{M}$ nitrite and $0.097 \pm 0.092\text{ng/ml}$ TNF α for vehicle-treated cells vs. $3.08 \pm 0.18\mu\text{M}$ nitrite and $0.41 \pm 0.033\text{ng/ml}$ TNF α for 125ng/ml LPS-treated cells).

4.3.2.3 Effect of DCPG treatment on LPS-induced primary microglial activation

Primary microglia were incubated with a range of DCPG concentrations (1nM-100 μM) for 1h prior to addition of the previously optimised condition of 125ng/ml LPS to induce activation. Well conditions were repeated in triplicate. As expected, LPS-treated cells in the drug vehicle condition demonstrated an increase in NO and TNF α production compared to untreated (no LPS) control cells (Figure 4.14 A and B,). However, levels of nitrite and TNF α in culture medium were not attenuated by pre-treatment with any concentration of DCPG when compared to drug vehicle treated cells, indicating no effect of DCPG on LPS-induced microglial activation. At the end of the experiment (18h post-LPS), any potential effect of DPCG on cell viability was assessed. DCPG had no appreciable effect on cell viability (Figure 4.14 C), indicating no cytotoxic or proliferative effects of DCPG on primary microglia at doses up to 100 μM .

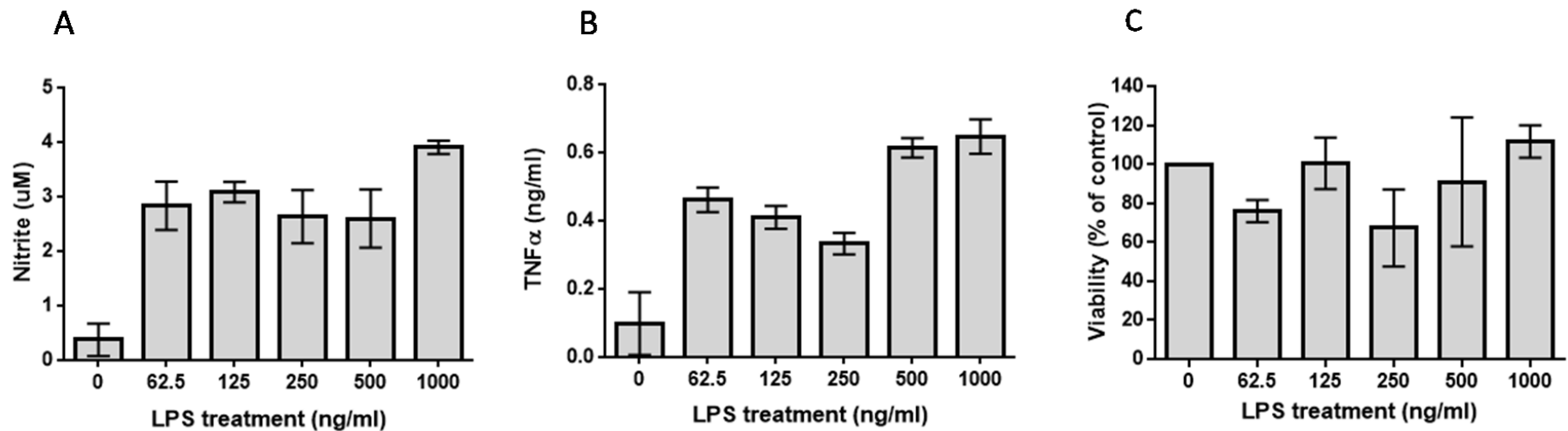


Figure 4.13: Optimisation of LPS concentration for microglial activation. Rat primary microglia were isolated from pup brains at P7 and plated on 24 well plates at a density of 1×10^5 before being left to re-establish for 24 hours. Cells were then incubated with a range of LPS concentrations for 18 hours in order to induce activation. After 18h incubation with LPS, cell culture medium was collected for assessment of microglial activation, and cell viability was assessed directly on remaining cells. Data is shown as mean $\pm$ range of conditions in triplicate from two independent experiments.

A: An increase in nitrite levels (indicating NO production) compared to control (0ng/ml LPS) was apparent in response to incubation with all concentrations of LPS tested as measured by culture medium nitrite levels in the Griess assay. Data is shown as mean nitrite concentration $\pm$ range.

B: An increase in TNF α levels compared to control (0ng/ml LPS) was apparent in response to incubation with all concentrations of LPS as measured by culture medium levels in an ELISA. Data is shown as mean TNF α concentration $\pm$ range.

C: No change in cell viability, as assessed in the Neutral Red assay, was apparent following incubation with any concentration of LPS. Data is shown as percentage viability of control $\pm$ range.

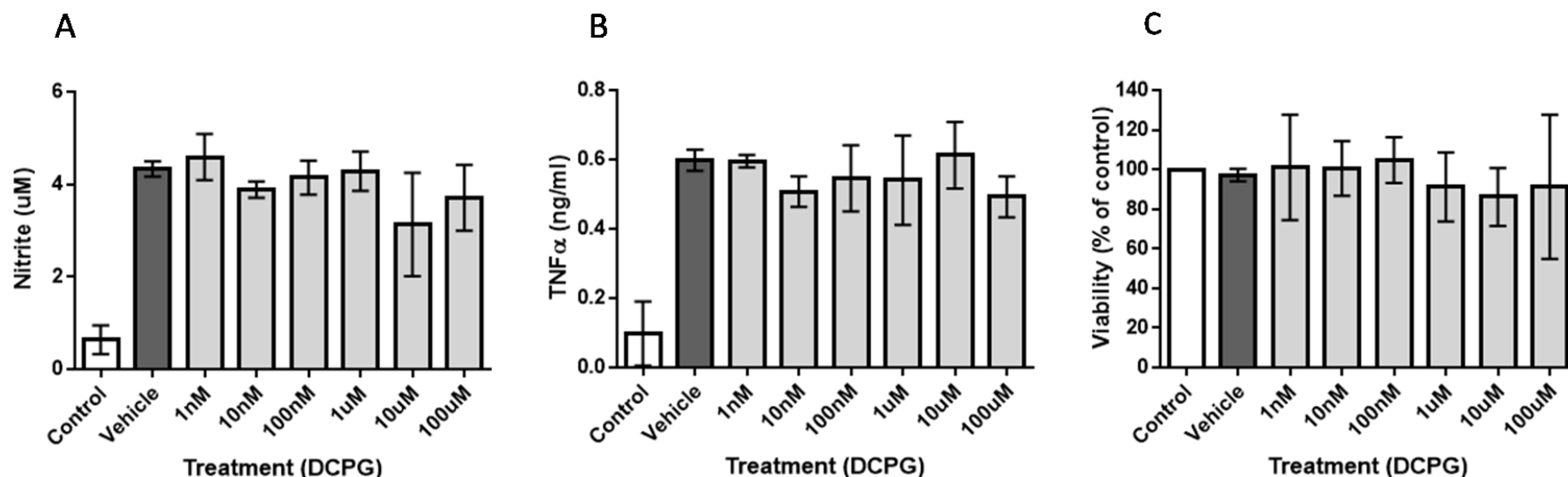


Figure 4.14: Effects of DCPG treatment on LPS-induced primary microglial activation. Rat primary microglia were isolated from rat pup brains at P7 and plated on 24 well plates at a density of 1×10^5 before being left to re-establish for 24 hours. Primary microglia were pre-incubated with a range of DCPG concentrations for 1 hour prior to addition of 125ng/ml LPS to the medium for a further 18 hours incubation in order to induce activation. After 18 hours incubation with LPS (19 hours with DCPG), cell culture medium was collected for assessment of microglial activation, and cell viability was assessed directly on remaining cells. Data is shown as mean $\pm$ range of conditions in triplicate from two independent experiments.

A: Drug vehicle-treated cells demonstrated a visible increase in nitrite levels (indicating NO production) compared to control (0ng/ml LPS) in response to incubation with LPS, as measured by culture medium nitrite levels in the Griess assay. However, no attenuation of LPS-induced NO production was apparent in response to DCPG treatment at any concentration compared to the vehicle condition. Data is shown as mean nitrite concentration $\pm$ range.

B: Drug vehicle-treated cells demonstrated a visible increase in TNFα levels compared to control (0ng/ml LPS) in response to incubation with LPS, as measured by culture medium levels in an ELISA. However, no significant attenuation of LPS-induced TNFα production was apparent in response to DCPG treatment at any concentration compared to the vehicle condition. Data is shown as mean TNFα concentration $\pm$ range.

C: No change in cell viability, as assessed in the Neutral Red assay, was apparent following incubation with any concentration of DCPG. Data is shown as percentage viability of untreated control $\pm$ range.

4.4 Discussion

The hypothesis for this study was that activation of the mGlu8 receptor with DCPG can provide protection of SNc dopaminergic neurons against inflammatory-mediated damage via attenuation of microglial pro-inflammatory activation. The experiments described in this Chapter therefore aimed to examine the ability of DCPG to provide neuroprotection *in vivo* against inflammatory-induced neurodegeneration by LPS, and to further examine its ability to attenuate the microglial activation induced by LPS, which may contribute to its previously demonstrated neuroprotective action in the 6-OHDA model. Delayed-start chronic treatment with DCPG was assessed to determine whether mGlu8 receptor activation is neuroprotective against inflammatory-induced neurodegeneration in the LPS model of PD. DCPG's ability to modify microglial activation was further investigated *in vitro* using primary microglial cultures activated with LPS.

4.4.1 Chronic peripheral DCPG administration demonstrates a slightly neuroprotective trend in LPS-lesioned animals

No significant decline in contralateral forelimb use was observed over time in drug vehicle-treated animals, indicating unilateral lesioning with 5µg LPS does not induce robust motor deficits by day 8 post-lesioning. Similarly, no significant change in contralateral forelimb use from baseline was observed in unilaterally LPS-lesioned animals chronically treated with DCPG; however, the lack of forelimb use asymmetry in the drug vehicle-treated animals limits assessment of any neuroprotection-driven attenuation of motor deficits in DCPG-treated animals. A limited rotational asymmetry was seen at day 8 post-lesioning in response to amphetamine administration; however, no attenuation of rotational asymmetry was apparent following chronic DCPG treatment, suggesting no functional neuroprotection occurred, although there was much variance within treatment groups which limited comparisons. This variance in behaviour and the lack of a statistically significant effect may be due to the underpowering of the study, as a sample size of 5 animals per treatment group were included in the final analysis, as discussed below. As most animals showed limited rotational asymmetry by day 8 post-lesioning, it is hard to evaluate any potential functional neuroprotection by DCPG treatment at this stage. It was unclear from previous literature whether the present experimental parameters would induce overt behavioural deficits. It is likely that the exposure period to LPS induced microglial activation was insufficient to induce a nigrostriatal lesion large enough to cause distinct and measurable motor deficits. Indeed, dysfunctional motor coordination, decreased motor speed and rotational asymmetry have all been demonstrated in LPS-lesioned rodents, albeit with a lower concentration of LPS, but only after a longer period of time (>15 days with 2µg LPS) (Tanaka et al. 2013, Zhou et al. 2012). The present experiment was designed based on the findings of Gang et al

(2004), who demonstrated a 70% dopaminergic neuronal loss 7 days after lesioning of the SNc with 5µg LPS; however, they did not investigate corresponding motor changes. As approximately 50% of dopaminergic neurons in the SNc are lost before symptoms appear, it was expected that animals would demonstrate some measurable motor deficits by day 8 post-lesioning, assuming the degree of SNc dopaminergic neuronal loss demonstrated by Gang et al. was replicated. Importantly, in the present study it seems that intranigral injection of 5µg LPS did not cause sufficient dopaminergic neuronal cell loss after 8 days (~53% in vehicle-treated rats) to produce marked behavioural deficits, limiting detection of motor improvements in response to DCPG in this model.

As the LPS model is a purely inflammation-driven model of neurodegeneration, it provided a way to test the neuroprotective efficacy of DCPG on the inflammatory causes of cell death in PD. Pathological examination of the animals at day 9 revealed that chronic systemic treatment with 15mg/kg DCPG provides a tendency towards neuroprotection of dopaminergic neurons of the SNc of rats unilaterally lesioned with LPS, with a 23% chance of randomly observing an effect of this size. A hint of protection of $\sim 24 \pm 9.4\%$ of dopaminergic neurons was seen following delayed-start chronic administration of 15mg/kg DCPG. This finding suggests that activation of the mGlu8 receptor with 15mg/kg DCPG may attenuate some aspect of neuroinflammatory damage in order to provide some slight neuroprotection. A similar tendency towards neuroprotection by DCPG was seen in Nissl+ neuronal counts, and the degree of Nissl+ cell loss indicates that dopaminergic neurons were killed due to the innate inflammatory reaction in response to LPS, rather than existing in an impaired state with down-regulation of the TH enzyme. Importantly, the lack of statistical significance detected in this study is likely due to the variability between animals combined with the limited efficacy of DCPG, and therefore it is possible that the study was underpowered in this case. As no studies had previously been carried out on this model in our lab, the same sample size used for the lactacystin experiments was aimed for (n=7), although despite lesioning 8 per group initially, a sample size of 5 animals per treatment group were included in the final analysis. Retrospective analysis of this study suggests that a 55% neuroprotection of TH+ neurons by DCPG would have had to occur to be statistically detected in the present study. A minimum sample size of 17 animals per treatment group would be required to detect a moderate 25% neuroprotection of TH+ neurons by DCPG (as indicated by the 24% protection data in this study, Figure 4.7 C), providing >80% power and a significance level of 5%.

Notably, the degree of dopaminergic cell loss at day 9 in vehicle-treated control animals following intranigral lesioning with 5µg LPS was slightly lower in the present study (53% loss) than that seen by Gang et al. (2004) of around 70% loss by day 7 following intranigral lesioning with 5µg LPS, suggesting a slightly slower rate of degeneration in the present study than predicted. However, this disparity in the degree of dopaminergic neuronal loss may be due to differences in cell quantification

methods; dopaminergic neuron quantification by Gang et al. was semi-quantitative, utilising a high power image analysis system to determine cell numbers by ratio coverage, and sampling a far smaller portion of the SNc than used in this thesis (Gang et al. 2004). It is highly unlikely that this was as accurate an estimation of cell loss as that achieved in the present study with stereology performed on serial sections throughout the nigra, particularly as numbers of dopaminergic neurons vary across different levels of the SNc, likely making sampling a smaller portion of the SNc less accurate.

4.4.2 DCPG does not attenuate microglial activation

The tendency towards neuroprotection observed following chronic administration of 15mg/kg DCPG was not associated with a reduction in numbers of OX-6+ activated microglia or CD68+ macrophages within the lesioned SNc. Additionally, a large variability in numbers of OX-6+ and CD68+ cells was seen between rats within the same treatment group, suggesting the innate inflammatory response to LPS lesioning varies greatly between individuals. The number of CD68+ macrophages in the SNc was estimated to be much higher than the number of OX-6+ activated microglia, despite only fully activated phagocytic microglia expressing the CD68 antigen. However, this is likely due to the recruitment of CD68+ macrophages into the brain from the periphery by signals (such as TNF α release) from activated microglia, a process that is thought to occur in PD (Mosley et al. 2012). As phagocytic CD4+ and CD8+ T cells do not express the MHC class II antigen, they would not stain positive for OX-6, explaining the difference in numbers of these cell populations in the SNc in the present study. Collectively, these findings suggest that the slight trend towards neuroprotection observed following chronic systemic DCPG treatment is not due to a reduction in microglial activation in the SNc.

In vivo, primary microglia isolated at P7 were confirmed to express the mGlu8 receptor both when resting and when activated by LPS, supporting the findings of Taylor and colleagues (Taylor et al. 2003) and confirming the presence of this receptor for potential activation by DCPG. However, results showed that activation of the mGlu8 receptor with concentrations of DCPG up to 100 μ M failed to reduce the release of two key markers of microglial activation, NO and TNF α . As the EC₅₀ of DCPG at the mGlu8 receptor is 31nM, it is likely that it was adequately activated by the higher concentrations of DCPG used here.

It is interesting that, as discussed in Chapter 3, previous work in our lab indicated systemic administration of DCPG for 10 days was neuroprotective in the 6-OHDA model at 15mg/kg, and that this was associated with a significant reduction in OX-6+ immunostaining in the SNc (Chan, H., unpublished observation, 2010). It was therefore thought that perhaps DCPG was able to reduce the initiation of inflammation (as measured by activated microglia) if given prior to the introduction of a toxin, rather than attenuating it after the inflammatory cascade had begun, as 6-OHDA lesioned

animals were pre-treated with DCPG for 1 day prior to lesioning. However, the *in vitro* evidence found here suggests that this is unlikely, as no reduction in primary microglial activation to LPS was observed following 1-hour pre-treatment with DCPG; if DCPG were able to switch microglial phenotype, a corresponding decrease in NO and TNF α levels would be expected. Nevertheless, as only a limited amount of culture medium was available for assessment from each treatment condition, investigation into changes in other pro-inflammatory or anti-inflammatory mediators was not possible. Recent research suggests that interleukin-1 (IL-1 β) mediates much of the dopaminergic neurotoxicity in response to LPS (Tanaka et al. 2013), suggesting that the effects of mGlu8 receptor activation on this cytokine may warrant further investigation. Previous studies broadly investigating group III mGlu receptor activation in response to excess glutamate have demonstrated an enhancement of other microglial mechanisms such as a reduction in glutamate release and an increase in neurotrophic factor release (Liang et al. 2010, McMullan et al. 2012). However, these changes would likely only occur with an obvious change in microglial phenotype, which was not observed in the present study. Taylor et al. (2003) demonstrated that the broad group III mGlu receptor agonists L-AP4 and RS-PPG can reduce microglial activation and the associated neurotoxicity *in vitro*, so the lack of attenuation of microglial activation by DCPG in the present study may indicate that another group III mGlu receptor subtype other than mGlu8, likely the mGlu4 receptor as rat microglia do not express mGlu7, is responsible for the effects on microglia seen in those studies. However, as DCPG provided a slight neuroprotective trend *in vivo* against LPS-induced inflammatory damage in the present study, it is possible that DCPG can protect against some aspect of neuroinflammatory damage to provide neuroprotection. Since no measurable change in microglial activation was observed in response to DCPG, the putative neuroprotective effects of mGlu8 receptor activation by DCPG could instead be attributed to changes in the way that neurons survive the toxic effects of microglial activation, for example, by directly decreasing their responsiveness to cytotoxic factors released by activated microglia such as glutamate, NO and cytokines, or by reducing the levels of cytotoxic factors that neurons are exposed to via indirect mechanisms.

As impaired glutamate uptake is observed in models of PD, and excess glutamate in the synaptic cleft is excitotoxic to neurons, enhancement of astrocytic glutamate reuptake could provide protection against excitotoxicity. Astrocytic glutamate transporters such as GLAST and GLT-1 are vital for homeostatic control of extracellular glutamate levels and normal glutamatergic neurotransmission (Anderson & Swanson 2000). Interestingly, activation of the mGlu3 receptor with the group II agonist (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) positively modulates glutamate transporter protein expression in human cultured astrocytes (Aronica et al. 2003), and GLAST and GLT-1 protein levels increase after treatment with the group II mGlu receptor agonist 4-amino-2,4-

pyrrolidinedicarboxylic acid (APDC)(Beller et al. 2011). It is feasible that the up-regulation of astrocytic glutamate transporters seen following group II mGlu receptor activation might occur following group III mGlu receptor activation too, as they similarly couple to Gi/o and stimulate MAPK and PI-3-K signalling pathways, although there are currently no publications demonstrating this effect on glutamate transporters by group III mGlu receptors. Although the mGlu8 receptor subtype is not detectable on primary cultures of rat and mouse astrocytes under normal conditions (Besong et al. 2002, Janssens & Lesage 2001, Phillips *et al.* 1998), it has been detected on reactive astrocytes(Tang & Lee 2001, Geurts et al. 2005, Byrnes et al. 2009), so it is possible that DCPG could enhance glutamate uptake by activation of the mGlu8 receptor on reactive astrocytes. Activation of astrocytic mGlu8 receptors may lead to enhanced glutamate uptake via activation of MAPK signalling or an increase in antioxidant levels such as reduced glutathione (GSH). The combination of NO with ROS (particularly superoxide) leads to production of peroxynitrite, which can potently inhibit GLAST and GLT-1, thus reducing glutamate transporter activity, which hinders glutamate uptake by astrocytes(Blanc *et al.* 1998, Piani *et al.* 1992, Miralles *et al.* 2001, Trotti *et al.* 1996). Pre-treatment of primary rat astrocyte cultures with L-AP4 has been shown to attenuate LPS-induced astroglial neurotoxicity, likely due to the restoration of the endogenous antioxidant GSH to normal levels following mGlu receptor group III activation, which reverses the impairment in glutamate uptake (Zhou et al. 2006). L-AP4 similarly restores astrocyte-mediated glutamate uptake in vitro following MPTP administration and is consequently neuroprotective (Yao et al. 2005). Additionally, the group III mGlu receptor agonist L-SOP has been shown to increase GSH levels and reduce ROS in the SN following unilateral 6-OHDA lesioning of the nigra in rats (Gu et al. 2003). It is therefore plausible that activation of astrocytic mGlu8 receptors may lead to increased clearance of extracellular ROS and glutamate following LPS administration, resulting in neuroprotection.

Activation of mGlu8 receptors directly on neurons may additionally protect against cytokine and OS-induced cell death by activating anti-apoptotic and intracellular cell survival signalling pathways. Indeed, there is evidence suggesting activation of group III mGlu receptors by L-AP4 is neuroprotective via activation of the PI-3-K and MAPK signalling pathways (Iacovelli et al. 2002) which may decrease ROS levels and regulate OS via formation of antioxidant systems (Spillson & Russell 2003), and may additionally provide neuroprotection through stabilisation of microtubules (Jiang et al. 2006). Activation of mGlu8 receptors may also stimulate astrocytes to produce neurotrophic and growth factors such as transforming growth factor- β (TGF- β) or BDNF, which protect against neuronal damage and promote recovery (Connor & Dragunow 1998). Production of neurotrophic factors by reactive astrocytes contribute to the neuroprotective effects of group II mGlu receptor activation, a

mechanism that again could feasibly be extended to group III receptor activation (Bruno *et al.* 1998, Matarredona *et al.* 2001).

4.5 Conclusions

In conclusion, results described in this chapter indicate that delayed-start chronic peripheral DCPG administration provides a suggestion of histological neuroprotection against LPS-induced neuroinflammatory damage in the SNc, although further higher powered studies are needed to confirm this. Importantly, DCPG does not attenuate numbers of activated microglia or macrophages in the lesioned SNc *in vivo*, nor does it reduce LPS-induced activation of primary microglia in culture as measured by TNF α and NO production. This indicates that mGlu8 receptor activation does not attenuate microglial activation, but, if it is providing true neuroprotection of dopaminergic neurons, it is likely via a different mechanism of action than predicted, such as by reducing extracellular levels of glutamate and ROS via restoration of antioxidant levels and enhancement of glutamate reuptake by astrocytes, by inducing production of neurotrophic factors by astrocytes, or by activating cell survival pathways in neurons directly. Further investigation into the effects of targeting the mGlu8 receptor in PD are necessary to understand its possible underlying neuroprotective mechanisms, and to determine if it is a valid target for neuroprotective and antiparkinsonian therapies in humans. However, studies are limited at present by the lack of other mGlu8 receptor selective compounds available, so further investigation, perhaps using mGlu8 receptor antagonists such as (*RS*)- α -methylserine-*O*-phosphate (MAP4) or (1*R*,3*R*,4*S*)-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-II) in conjunction with DCPG to see if they block its effects, would be helpful to further characterise the role of mGlu8 receptors in PD.

Chapter 5: The effects of acute and chronic administration of the mGlu4 receptor PAM VU0364770 *in vivo* on motor deficits and neuroprotection in the lactacystin model of Parkinson's disease

5.1 Introduction

The mGlu4 receptor is the most promising therapeutically of all group III mGlu receptors for the treatment of PD so far examined. Early research utilising agonists broadly targeting group III mGlu receptors such as L-AP4 and L-SOP concluded that the neuroprotective and antiparkinsonian activity of these compounds was mediated predominantly via the mGlu4 receptor (MacInnes et al. 2004, Marino et al. 2003, Valenti et al. 2003). This has encouraged further investigation into the efficacy of specifically targeting the mGlu4 receptor in PD. The mGlu4 receptor is located in key areas of the BG circuitry, particularly at the corticostriatal, striatopallidal and subthalamonigral synapses (SNc and SNr) (Duty 2010, Valenti et al. 2005). Agonists and PAMs targeting the mGlu4 receptor are considered to be particularly useful as 'disease-dependent drugs' as they act on the overactive indirect pathway of the BG in PD (Marino et al. 2003).

Several compounds specifically targeting the mGlu4 receptor have demonstrated symptomatic improvements in animal models of PD by reducing activity at striatopallidal and subthalamonigral synapses, normalising signalling within the indirect pathway of the BG (Marino et al. 2003, Valenti et al. 2005, Valenti et al. 2003). This receptor subtype received increased interest following the discovery that PHCCC, the first group III subtype-selective PAM with partial selectivity for the mGlu4 receptor, showed efficacy in models of PD. PHCCC reversed reserpine-induced akinesia in rats when administered into cerebral ventricular (i.c.v), intranigral (into SNr) and systemically (Battaglia et al. 2006, Broadstock et al. 2012, Marino et al. 2003), indicating the mGlu4 receptor is involved in inhibition of excess glutamate release at the SNr. PHCCC has also been shown to protect the nigrostriatal pathway in animal models of PD when infused into the GPe and when given systemically (Battaglia et al. 2006). This effect is thought to be mediated solely through the mGlu4 receptor, as no neuroprotection occurred in mGlu4 knockout MPTP-treated mice administered PHCCC, although adaptive changes in other mGlu receptors may have compensated (Battaglia et al. 2006). PHCCC has proved an extremely useful tool for investigating the therapeutic role of targeting the mGlu4 receptor in PD, however, its group I antagonistic properties complicate the unravelling of its mechanism of action and its poor BBB permeability and physicochemical properties limit its potential clinical use.

Designing orthosteric agonists to target specific subtypes of mGlu receptors has proved particularly difficult due to the high level of site conservation between and particularly within mGlu receptor subclass groups; agonists often have off-target activity on other mGlu receptors because of this. Additionally, classical orthosteric agonists have suffered from difficulties crossing the BBB due to their highly hydrophilic nature (Amalric et al. 2013), so more lipophilic ligands targeting the same receptors but with novel mechanisms of action have been pursued in recent years. Allosteric sites are

more distinct between specific subtypes within this class of receptors, and high throughput screening techniques have been utilised more recently to discover novel PAMs selective for the mGlu4 receptor with improved selectivity, potency and pharmacokinetic properties. PAMs are regulatory molecules that cannot exert their effect without the presence of an orthosteric ligand, potentiating the natural ligand's effects and maintaining physiological patterns of activation (Conn et al. 2009). They are predicted to have a superior safety profile as they prevent constant receptor activation, which may be responsible for adverse side effects (Célanire & Campo 2012).

In recent years, several mGlu4 receptor selective PAMs and agonists with improved properties have been developed. LSP1-2111 and LSP4-2022 are novel, systemically active mGlu4 receptor selective orthosteric agonists with proven antiparkinsonian properties (Beurrier et al. 2009, Cajina *et al.* 2013, Goudet et al. 2012), but are not yet commercially available. VU0155041, a mixed allosteric agonist/PAM at the mGlu4 receptor, has been shown to dose-dependently reverse both reserpine-induced akinesia and haloperidol-induced catalepsy in rats (Niswender et al. 2008a) and reduces motor deficits in unilateral 6-OHDA-lesioned rats when injected supra-nigrally (Betts et al. 2012). Additionally, central administration of VU0155041 provides around 40% neuroprotection against dopaminergic neuronal loss in unilaterally 6-OHDA-lesioned rats coinciding with a significant reduction in microglial activation, suggesting an anti-inflammatory mechanism of mGlu4 receptor activation (Betts et al. 2012). Unfortunately, VU0155041 is limited by its poor BBB permeability and its weak potency at mGlu4, limiting its therapeutic value (Célanire & Campo 2012). The mGlu4 receptor PAMs VU0364770, Lu AF21934 and ADX88178 are also brain penetrant and are effective antiparkinsonian agents when systemically administered in rodent models of PD (Bennouar et al. 2013, Jones et al. 2012, Le Poul et al. 2012). However, Lu AF21934 and ADX88178 were only effective in models of drug-induced catalepsy, with no effect on 6-OHDA induced forelimb akinesia when administered alone (Bennouar et al. 2013, Le Poul et al. 2012). In contrast, the selective mGlu4 receptor PAM VU0364770 has been shown to be effective in reversing both haloperidol-induced catalepsy and reserpine-induced akinesia, and is moderately effective in the 6-OHDA forelimb akinesia model (Jones et al. 2012).

Developed at Vanderbilt University as an improvement on previous similar compounds, VU0364770 (ML282) has been made commercially available as a novel probe for further evaluation (Engers *et al.* 2013, Lindsley & Hopkins 2012). VU0364770 is a highly selective, potent PAM of the mGlu4 receptor and is also a weak agonist at the mGlu6 receptor and weak antagonist at the mGlu5 receptor, as well as having some monoamine oxidase (MAO) inhibitory action. These additional binding properties could potentially enhance the action of this mGlu4 receptor PAM; activation of the mGlu6 receptor is thought to be neuroprotective, although it is mainly found in the retina (Nakajima

et al. 1993), inhibition of the mGlu5 receptor may also be neuroprotective through a reduction of excess excitation (Aguirre *et al.* 2001, Battaglia 2004) and MAO-A and B inhibition (Engers et al., 2013) could enhance dopamine levels in the striatum, improving parkinsonian symptoms. Compared to other mGlu4 receptor PAMs currently available, VU0364770 (see Figure 5.1) has a favourable pharmacokinetic profile, and although it is quickly cleared from systemic circulation, it has excellent CNS exposure with a brain to plasma ratio of 1.4 over 6 hours when given at a 10mg/kg dose subcutaneously (s.c.) (Jones et al. 2012). VU0364770 has the best *in vivo* efficacy of all mGlu4 PAMs to date (Engers et al. 2013) and thus far, it is the only mGlu4 PAM to show efficacy when administered alone in models of PD (Jones et al., 2012). VU0364770 was chosen for the present study due to its potency and selectivity for the mGlu4 receptor, its improved pharmacokinetic properties over similar compounds and its reported *in vivo* efficacy in reducing forelimb akinesia. It is also currently the only systemically active selective mGlu4 receptor PAM that is commercially available to purchase.

The hypothesis of these studies was that activation of the mGlu4 receptor with the mGlu4 selective PAM VU0364770 can provide neuroprotection and symptomatic relief in the lactacystin rat model of PD. The aim of this chapter is to investigate the value of targeting the mGlu4 receptor with VU0364770 in the lactacystin model of PD, in order to assess its effectiveness as a potential novel treatment. To date, no published studies have examined the putative neuroprotective effects of VU0364770. According to the anatomical distribution of the mGlu4 receptor within the BG circuitry and the functional effects of potentiating glutamate's activity at this receptor, VU0364770 could theoretically provide protection against further excitotoxic damage in the SNc via a reduction in excess glutamatergic signalling from the STN. Additionally, the localisation of the mGlu4 receptor on microglia may provide additional neuroprotection against lesioning via anti-inflammatory mechanisms. In this chapter, neuroprotection studies were carried out in the lactacystin model with 10mg/kg and 30mg/kg VU0364770 to determine if these doses could provide neuroprotection, particularly as these doses previously provided symptomatic relief in haloperidol-treated animals, and a good brain exposure is seen at a 10mg/kg dose (Jones et al. 2012). Using two doses allowed investigation into a possible dose-dependent relationship. The expense of the compound when given systemically in prolonged studies limited the use of much higher dosages.

Most neuroprotection studies begin drug administration prior to toxic insult; however, this is not particularly informative if a clinical therapy is given at a stage where disease progression and damage is already established. As in Chapter 3 with DCPG, the neuroprotective effect of chronic systemic administration of VU0364770 was investigated using a delayed-start study design in rats unilaterally lesioned with 7.5µg and 2.5µg of lactacystin in order to test VU0364770's neuroprotective

effects in models with different degrees of neurodegeneration (see section 2.2.4) using both behavioural assessment and histological analysis.

Additionally, possible acute symptomatic improvement through the measurement of motor deficits in response to escalating single doses of VU0364770 was investigated. VU0364770 has been found to reduce forelimb asymmetry at 100mg/kg in rats unilaterally lesioned with 6-OHDA in the MFB, and reverses haloperidol-induced catalepsy in rats at 10, 30 and 56.6mg/kg doses (Jones et al. 2012). However, the effectiveness of this compound on reducing motor deficits in the lactacystin model of PD is yet to be established. In this chapter, the effects of acute administration of VU0364770 on motor behaviour were investigated in the lactacystin model at doses of 10mg/kg, 30mg/kg and 100mg/kg, to determine if the antiparkinsonian effects reported by Jones and colleagues (2012) could be replicated in this model. A battery of behavioural tests were utilised in this study with an aim to detect more subtle behavioural changes.

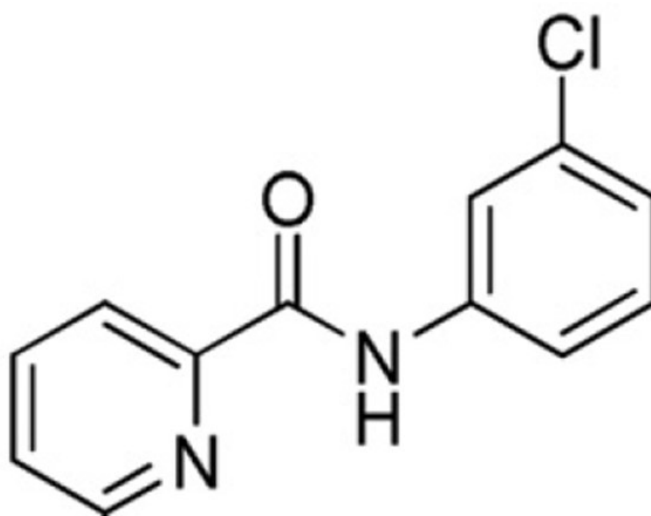


Figure 5.1: Chemical structure of N-(3-chlorophenyl)picolinamide (VU0364770 or ML292).

VU0364770 was dissolved in 5% DMSO and further diluted in a solution of 0.9% sterile saline and 10% Tween 80, before being sonicated to distribute the compound throughout the solution and injected as a suspension i.p. Image taken from Jones et al., 2012.

5.2 Experimental design

5.2.1 Chronic systemic VU0364770 treatment in 7.5µg lactacystin-lesioned rats: behavioural and neuroprotective effects

To investigate the neuroprotective and behavioural effects of the mGlu4 receptor PAM VU0364770 *in vivo*, male Sprague-Dawley rats (240-290g) were unilaterally lesioned in the SNc with 7.5µg lactacystin at day 0 (see Methods section 2.1.3 and experimental design, Figure 5.2). Animals were randomly assigned to a treatment group. Dosing began at day 4 post-lesioning, when lactacystin-induced cell death has begun but no overt behavioural deficits are observable, allowing the drug to be tested in a more clinically relevant manner. Treatment groups were as follows; 10mg/kg VU0364770 (n=7), 30mg/kg VU0364770 (n=7) and drug vehicle (n=8). Drug or drug vehicle treatment was carried out daily before midday from day 4 post-lesioning for 14 days (Figure 5.2). A battery of behavioural tests was used to regularly assess changes in motor symptoms in response to lesioning and drug treatment (see Methods section 2.1.5). Performance in the vertical cylinder test, vibrissae-evoked forelimb placement test, adjusted stepping test and spontaneous circling were all assessed prior to lesioning surgery to ascertain a baseline score. Animals were then re-tested behaviourally at intervals on days 7, 14 and 18 post-surgery to monitor deficit development and detect possible neuroprotective effects of VU0364770. VU0364770 was administered i.p. immediately after behavioural testing so that the drug did not directly impact on behaviour, and any neuroprotection-driven behavioural changes could be detected. On day 18, at the conclusion of the experiment and following behavioural testing, amphetamine-induced rotations (see Method section 2.1.5.2) were carried out to assess rotational asymmetry and determine the extent of the lesion and potential neuroprotection. On day 19, brains were removed and fixed for histological processing and immunohistochemical analysis. Correct needle placement was verified after Nissl counterstaining using the rat atlas to guide assessment (see Method section 2.2.2.6). Where lesioning was not in the correct place, animals were discounted from analysis (see Table 2.1 for lesion success rate). Staining of dopaminergic neurons was carried out on serial sections throughout the SNc utilising TH immunohistochemistry with Nissl counterstaining (see Method section 2.2.2) followed by stereological quantification. Additionally, activated microglia were immunolabelled with anti-OX-6 and macrophages were immunolabelled with anti-CD68 antibodies on serial sections throughout the SNc, followed by stereological quantification.

5.2.2 Chronic systemic VU0364770 treatment in 2.5µg lactacystin-lesioned rats: behavioural and neuroprotective effects

Due to the high degree of dopaminergic neuronal loss detected following lesioning with 7.5µg lactacystin after 19 days post-lesioning, a lower concentration of 2.5µg lactacystin was used for unilateral lesioning to create more of a partial lesion model which may unmask any neuroprotective effects of VU0364770. Only the higher 30mg/kg dose of VU0364770 was used in this study due to the limited amount of drug available and the lack of neuroprotection by VU0364770 administration in rats lesioned with 7.5µg of lactacystin. The same experimental design as that of the chronic study in 5.2.1 was employed (Figure 5.2). Male Sprague-Dawley rats (240-270g) were unilaterally lesioned in the SNc with 2.5µg lactacystin at day 0 and treated with either 30mg/kg VU0364770 (n=7) or drug vehicle (n=6) daily from day 4 post lesioning for a further 14 days. The same battery of behavioural tests was used to regularly assess changes in motor symptoms in response to lesioning and drug treatment, in order to detect subtler changes in motor deficits. Performance in the vertical cylinder test, vibrissae-evoked forelimb placement test, adjusted stepping test and spontaneous circling (see Method in 2.1.5) were all assessed prior to lesioning surgery to ascertain a baseline score. Animals were then re-tested behaviourally on days 7, 14 and 18 post-lesioning to monitor motor deficit development and detect possible neuroprotective effects of VU0364770. VU0364770 was administered i.p. immediately after behavioural testing so that the drug did not directly impact on behaviour, and any neuroprotection-driven behavioural changes could be detected. On day 18 at the conclusion of the experiment, amphetamine-induced rotations were carried out, in addition to the above battery of behavioural tests to assess rotational asymmetry and determine the extent of the lesion and any potential neuroprotection (see Method section 2.1.5.2). Brains were removed and fixed for histological processing and immunohistological analysis on day 19, and correct needle placement was verified after Nissl counterstaining using the rat atlas to guide assessment (see Method in 2.2.2.6). Where lesioning was not in the correct place, animals were discounted from analysis (see Table 2.1 for lesion success rate). Again, immunostaining of TH+ dopaminergic neurons was carried out alongside staining for OX-6+ and CD-68+ microglia on serial sections throughout the SNc, followed by stereological quantification.

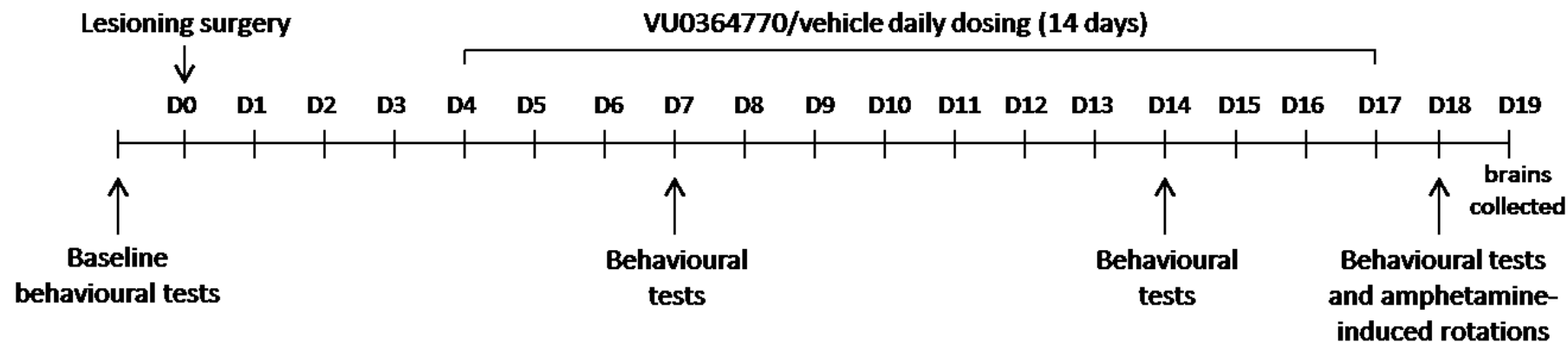


Figure 5.2: Schematic of experimental design for chronic VU0364770 treatment in lactacystin-lesioned animals

Rats were unilaterally lesioned with either 7.5ug or 2.5ug lactacystin at day 0. Animals were peripherally administered either vehicle or VU0364770 (10mg/kg or 30mg/kg) i.p. once daily from day 4 post-lesioning for a duration of 14 days. Baseline behaviour was assessed with a battery of behavioural tests prior to lesioning and again at days 7, 14 and 18 post-surgery to monitor deficit development and detect any neuroprotective effects of VU0364770. Amphetamine-induced rotations were also incorporated at day 18 to assess rotational asymmetry and therefore determine the extent of the lesion and detect any potential neuroprotection by VU0364770. VU0364770 was administered following scheduled behavioural tests so that the drug did not directly influence behavioural assessment and potential neuroprotection-driven changes could be detected.

5.2.3 Acute systemic VU0364770 treatment in lactacystin-lesioned rats: symptomatic changes

The effect of a single systemic dose of VU0364770 on motor deficits was investigated in lactacystin-lesioned rats to determine the acute effects of activating mGlu4 receptors. In the previous chronic drug treatment experiments detailed above, motor behaviour was assessed prior to the daily administration of VU0364770, thus facilitating the assessment of any neuroprotective effects of chronic VU0364770 administration. However, through its ability to modulate glutamatergic activity, VU0364770 may induce direct alleviation of motor deficits shortly after acute administration. Hence, any potential symptomatic alleviation of motor deficits by VU0364770 was subsequently assessed in fully lactacystin lesioned rats.

To investigate the acute effects of VU0364770 on motor deficits, male Sprague-Dawley rats (250-290g) were unilaterally lesioned in the SNc with 7.5µg lactacystin at day 0 (see experimental design, Figure 5.3). In order to ensure that the lactacystin had induced an extensive lesion of the nigrostriatal tract, amphetamine-induced rotations were carried out at day 14 for 30 minutes (see Methods section 2.1.5.2) and animals that rotated on average <1 net ipsiversive rotation per minute were discounted as poorly lesioned (n=1). Animals that would not rear in the vertical cylinder test (n=3) were also discounted from analysis. From day 21 post-lesioning onwards, animals (n=7 per group) were acutely treated with a single i.p. administration of 10mg/kg, 30mg/kg or 100mg/kg of VU0364770 or drug vehicle and behaviourally assessed. A minimum of a 4-day washout period between each test treatment was utilised to ensure no drug remained in the animal's system. As behavioural assessments were carried out at different time points after lesioning to allow wash out of test drugs (i.e. day 22, day 28, day 34), behavioural deficits gradually increased over the testing period, necessitating separate baseline measurements prior to each test, and data was normalised to a percentage of mean baseline scores. Baseline behaviour was assessed following drug vehicle administration 1 day prior to drug treatment to ascertain a baseline score and minimise habituation to tests. The following morning, animals were administered the test treatment (10mg/kg, 30mg/kg or 100mg/kg VU0364770 or drug vehicle i.p.) and behaviourally assessed 30 minutes later (based on pharmacokinetic data from Jones et al. 2012). Behavioural testing with a battery of tests took approximately 15 minutes per animal, so drug administration was staggered between animals on the testing day. Performance in the vertical cylinder test, adjusted stepping test and vibrissae-evoked forelimb placement test were all assessed during behavioural testing in the same order for each animal to control for changes in the concentration of VU0364770 in the brain over time. Spontaneous circling was not assessed in this study as well-lesioned animals tended not to rotate spontaneously or explore in the circling bowl. Drug vehicle testing was carried out to verify that repeat testing (over two days) did not alter behaviour.

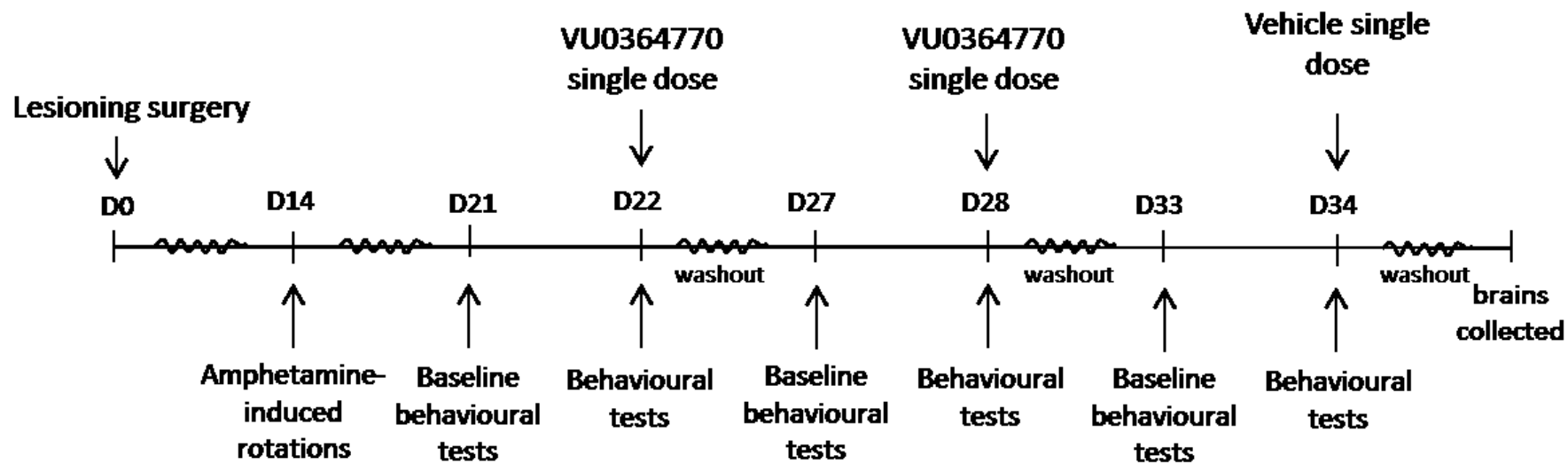


Figure 5.3: Schematic of experimental design for acute VU0364770 treatment in lactacystin-lesioned animals

Rats were unilaterally lesioned with 7.5µg lactacystin administered into the SNc at day 0 (D0). Amphetamine-induced rotations were used to confirm accurate lesioning 14 days after lesioning surgery (D14). At 21 days (D21) post-lesioning, behavioural deficits were clearly observable and testing began. Baseline behaviour was assessed with a battery of behavioural tests, and testing with VU0364770 or drug vehicle took place the following day on the 'test' day. Animals were peripherally administered either drug vehicle or VU0364770 (10mg/kg, 30mg/kg or 100mg/kg) as a single i.p. injection. 30 minutes later, the same battery of behavioural tests was again performed to detect any drug-induced changes to motor deficits.

5.2.4 Preparation of drug solution for peripheral administration

VU0364770 solution was made daily in saline from aliquots of stock in DMSO (66.6mg/ml) which was kept at -20°C, to give a final concentration of 5% DMSO and 10% Tween in saline as described in 2.1.4. Where a small amount of drug solution remained after dosing, it was kept at 4°C overnight and mixed with fresh compound for the following day's dosing. This was to minimise wastage of the compound and was not believed to affect the concentration of the drug in solution, as it has been shown to remain extremely stable, with 97% of its initial concentration remaining after 48 hours in PBS at 23°C (Engers et al. 2013).

5.2.5 Data analysis and statistics

All raw data was analysed in Microsoft Excel and statistical analysis was performed in Graphpad Prism Software Version 5 (San Diego, CA, USA). Behavioural tests were analysed according to the methods outlined in 2.1.5. Forelimb-use asymmetry is shown as percentage forelimb use in the vertical cylinder test, spontaneous circling is displayed as net rotations over 2 minutes, forelimb akinesia is shown as percentage contralateral steps relative to ipsilateral steps, and contralateral forelimb placements are shown relative to ipsilateral placements in the vibrissae-evoked forelimb placing test. In some cases, animals were excluded from vertical cylinder test analysis due to a lack of sufficient rearing activity, but were included in all other analysis. The numbers of animals included in each test are shown in figure legends below graphs.

To evaluate the effect of VU0364770 on behaviour in chronic treatment studies, comparisons between treatment groups were carried out with two-way repeated measures ANOVA followed by Bonferroni posthoc tests. Additionally, the area under the curve was calculated for each treatment group over time in each test: for all tests except the spontaneous rotations (for which regular area under the curve was carried out due to positive peaks), negative area under the curve was calculated from baseline to detect the effect of VU0364770, and comparisons between groups were carried out with unpaired t-tests (2 groups) or one-way ANOVA (>2 groups). Amphetamine-induced rotational asymmetry was analysed as net mean ipsiversive rotations per 5 minutes (over a 30 minute time period) and comparisons between treatment groups were carried out with unpaired t-tests or one-way ANOVA. Cell counts were analysed using two-way ANOVAs to compare contralateral and ipsilateral total cell estimates between hemispheres and between treatment groups. Data regarding cell loss is represented as percentage cell loss $\pm$ SEM relative to the unlesioned hemisphere.

For analysis of the effect of VU0364770 on motor behaviour in the acute treatment study, baseline data was normalised to 100% and 'test' data following vehicle or drug treatment was

calculated as a percentage of the mean baseline score. Two-way ANOVAs were used to compare baseline and 'test' values across treatments. All data in this chapter are presented as mean $\pm$ SEM.

5.3 Results

5.3.1 Chronic systemic VU0364770 treatment in 7.5µg lactacystin-lesioned rats

5.3.1.1 Effects of chronic systemic VU0364770 on behavioural deficits

Prior to lesioning, all animals demonstrated an equal use of both forelimbs at baseline in the vertical cylinder test for forelimb use asymmetry ($49.6 \pm 1.3\%$ contralateral forelimb use). Two-way ANOVA analysis of treatment groups showed a significant effect of lesioning over time (Figure 5.4 A: $F_{(3,68)}=5.38$ $p=0.002$) but no effect of VU0364770 treatment on forelimb-use asymmetry ($F_{(2,68)}=1.10$ $p=0.33$). Bonferroni post-hoc tests revealed a significant effect of lesion at day 18 compared to baseline in the vehicle-treated group ($p<0.05$), but not in VU0364770-treated groups. Negative area under the curve (AUC) analysis similarly showed no significant effect of VU0364770 treatment following lactacystin lesioning (Figure 5.4 B, one-way ANOVA $F_{(2,19)}=0.35$, $p=0.70$).

It was again observed that animals demonstrated some compensatory behaviour, such as weight shifting, when pushing off and landing in the vertical cylinder test which could mask their motor deficits during analysis. Therefore, wall exploration of the cylinder was assessed independently in the same tests as a more sensitive marker of forelimb use asymmetry. At baseline, all animals again displayed an equal use of both forelimbs for exploration of the cylinder wall (Figure 5.5 A: $47.2 \pm 4.26\%$ contralateral forelimb use). Two-way ANOVA analysis of treatment groups again showed an extremely significant effect of lesioning over time (Figure 5.5 A, $F_{(3,68)}=12.35$ $p<0.0001$) but no effect of VU0364770 treatment on forelimb-use asymmetry during wall exploration ($F_{(2,68)}=0.47$ $p=0.62$). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7, 14 and 18 compared to baseline in the 10mg/kg VU0364770-treated group (day 0 vs. days 7 and 18 $p<0.01$, day 0 vs. day 14 $p<0.001$), but not in vehicle or 30mg/kg-treated groups. Negative AUC analysis similarly showed no significant effect of VU0364770 treatment on forelimb-use asymmetry during wall exploration following lactacystin lesioning (Figure 5.5 B, one-way ANOVA $F_{(2,19)}=0.68$, $p=0.51$ ns).

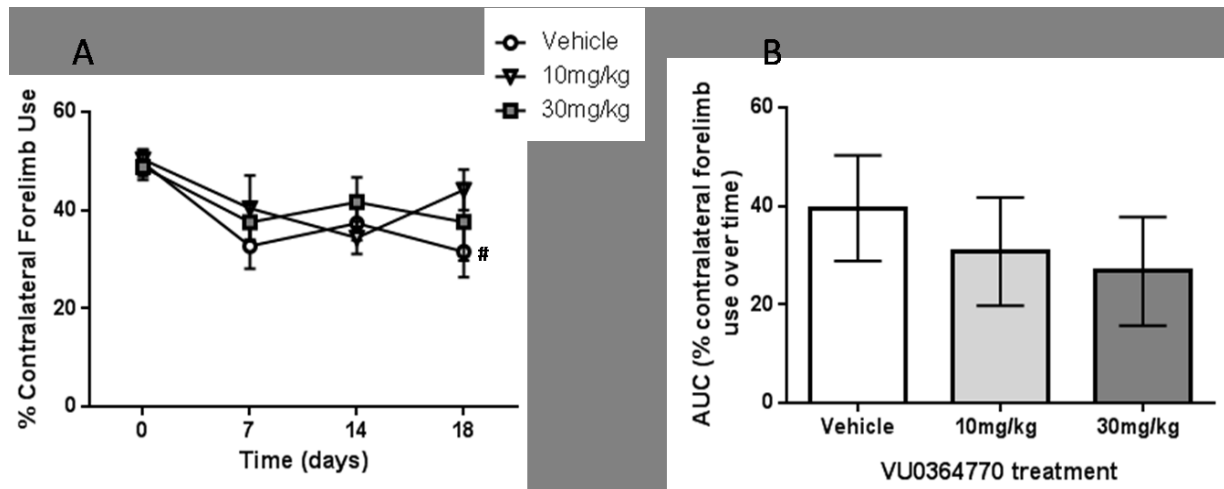


Figure 5.4: Effect of chronic VU0364770 treatment on forelimb use asymmetry in the vertical cylinder test. Rats were unilaterally lesioned in the SNc with 7.5 μ g lactacystin and treated with drug vehicle (n=7), 10mg/kg(n=7) or 30mg/kg (n=6) VU0364770i.p. from day 4 post-surgery for 14 days. Rats were tested in a vertical cylinder to determine forelimb use asymmetry prior to surgery at baseline (day 0), 7, 14 and 18 days after lesioning surgery. Data are shown as mean % contralateral forelimb use (push-off, cylinder wall exploration and landing combined) $\pm$ SEM.

A: There was a significant effect of lesion ($F_{(3,68)}=5.38$ $p=0.002$) but not VU0364770 treatment ($F_{(2,68)}=1.10$ $p=0.33$) on contralateral forelimb use when comparing the three treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at day 18 compared to baseline in the vehicle-treated group ($^{\#}p<0.05$), but not in VU0364770-treated groups.

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using one-way ANOVA analysis ($F_{(2,19)}=0.35$, $p=0.70$). AUC units are expressed as time x % contralateral forelimb use.

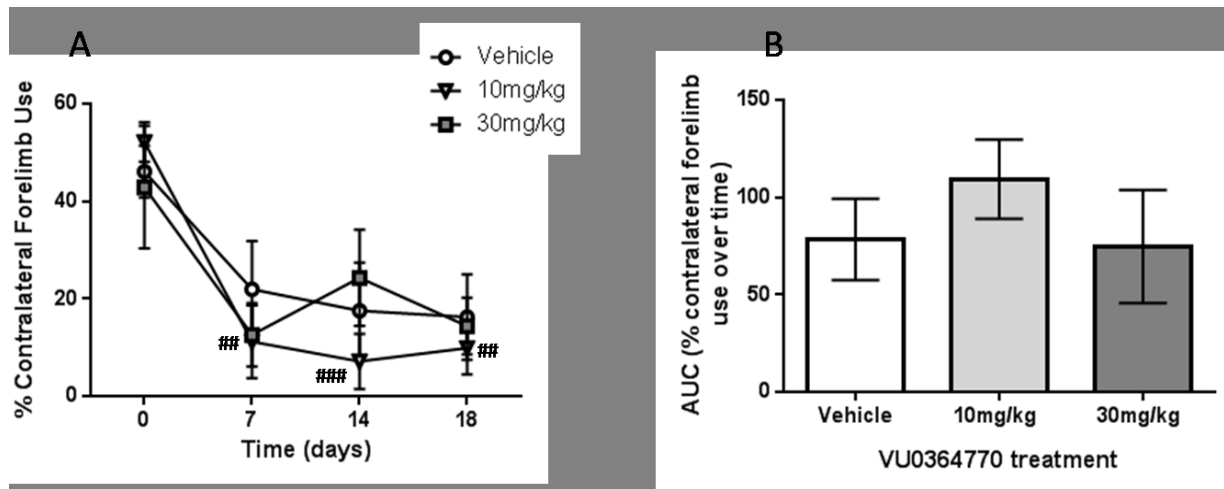


Figure 5.5: Effect of chronic VU0364770 treatment on forelimb-use asymmetry during wall exploration of the vertical cylinder. Rats were unilaterally lesioned in the SNc with 7.5 μ g lactacystin and treated with drug vehicle(n=7), 10mg/kg(n=7) or 30mg/kg(n=6) VU0364770 i.p. from day 4 post-surgery for 14 days. Rats were tested in a vertical cylinder to determine forelimb use asymmetry prior to surgery at baseline (day 0), 7, 14 and 18 days after lesioning surgery. Data are shown as mean % contralateral forelimb use (wall exploration alone) $\pm$ SEM.

A: There was a significant effect of lesion ($F_{(3,68)}=12.35$ $p<0.0001$) but not VU0364770 treatment ($F_{(2,68)}=0.47$ $p=0.62$) on contralateral forelimb use when comparing the three treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7, 14 and 18 compared to baseline in the 10mg/kg VU0364770-treated group ($^{##}p<0.01$, $^{###}p<0.001$), but not in vehicle or 30mg/kg-treated groups.

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using one-way ANOVA analysis ($F_{(2,19)}=0.68$, $p=0.51$). AUC units are expressed as time x % contralateral forelimb use.

It was noted that following unilateral lactacystin lesioning, rats tended to rotate spontaneously towards the contralateral side for up to 10 days post-lesioning, likely due to excess dopamine release from damaged dopaminergic cells in the lesioned SNc. At baseline prior to lesioning, animals demonstrated no rotational asymmetry (Figure 5.6 A). Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 5.6 A ($F_{(3,76)}=7.95$ $p=0.0001$) but no effect of chronic VU0364770 treatment on spontaneous rotational asymmetry ($F_{(2,76)}=0.47$ $p=0.62$ ns). Bonferroni post-hoc tests revealed a significant effect of lesion at day 7 compared to baseline in the vehicle-treated group ($p<0.05$), but not in 10mg/kg or 30mg/kg-treated groups. Area under the curve (AUC) analysis similarly showed no significant effect of VU0364770 treatment following 7.5µg lactacystin lesioning on spontaneous rotational asymmetry (Figure 5.6 B, one-way ANOVA $F_{(2,21)}=p=0.78$ ns).

Prior to lesioning, animals successfully stepped $97.6 \pm 1.5\%$ of total ipsilateral forelimb steps in the adjusted stepping test for forelimb akinesia (Figure 5.7 A). Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 5.7 A, $F_{(3,76)}=37.07$ $p<0.0001$) but no effect of chronic VU0364770 treatment on contralateral forelimb akinesia in the stepping test ($F_{(2,76)}=1.24$ $p=0.29$). Bonferroni post-hoc tests revealed a significant effect of lesion at days 14 and 18 compared to baseline in the vehicle-treated group ($p<0.001$ for both) and at days 7, 14 and 18 compared to baseline in the 10mg/kg and 30mg/kg VU0364770-treated groups ($p<0.001$ for all comparisons) as illustrated in Figure 5.7 A. Negative area under the curve (AUC) analysis similarly showed no significant effect of VU0364770 treatment following 7.5µg lactacystin lesioning on contralateral forelimb akinesia in the stepping test using one-way ANOVA (Figure 5.7B, $F_{(2,21)}=0.71$ $p=0.50$ ns).

Prior to lesioning at baseline, animals successfully placed their contralateral forelimb $99.3 \pm 1.7\%$ relative to total ipsilateral placements in the vibrissae-evoked forelimb placing test for forelimb akinesia (Figure 5.8 A). Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 5.8 A, $F_{(3,76)}=49.48$ $p<0.0001$) but no effect of chronic VU0364770 treatment on vibrissae-evoked contralateral forelimb placements ($F_{(2,76)}=0.73$ $p=0.48$ ns). Bonferroni post-hoc tests revealed a significant effect of lesion at days 14 and 18 compared to baseline in the vehicle-treated group ($p<0.001$) and at days 7, 14 and 18 compared to baseline in the 10mg/kg and 30mg/kg VU0364770-treated groups ($p<0.001$). Negative area under the curve (AUC) analysis similarly showed no significant effect of VU0364770 treatment following 7.5µg lactacystin lesioning on contralateral forelimb placements (Figure 5.8B, one-way ANOVA, $F_{(2,21)}=0.77$ $p=0.47$ ns).

Rotational asymmetry was investigated at the conclusion of the experiment (day 18 post lesioning) utilising amphetamine induced rotational behaviour as an indirect measure of lesion

magnitude. Drug vehicle treatment daily for 14 days starting 4 days post unilateral SNc lesioning with 7.5µg lactacystin was associated with a marked induction of circling behaviour following amphetamine administration on day 18 of the study, as illustrated in Figure 5.9. Similar to the effects of VU0364770 in the behavioural tests discussed above, administration of either 10 or 30mg/kg VU0364770 i.p. daily for 14 days starting on day 4 post lesioning failed to attenuate the circling behaviour induced after amphetamine administration as shown by one-way ANOVA analysis (Figure 5.9: net mean ipsiversive rotations/5 minutes for drug vehicle-treated animals: 25.8 ± 12.9 , 10mg/kg VU0364770: 38.6 ± 8.4 , 30mg/kg VU0364770: 9.4 ± 2.8 , $F_{(2,21)}=2.25$, $p=0.13$).

VU0364770 appeared to be well tolerated at the 10mg/kg and 30mg/kg doses used, and animals maintained normal weight gain (data not shown). Additionally, no gross behavioural abnormalities were observed following peripheral administration of VU0364770.

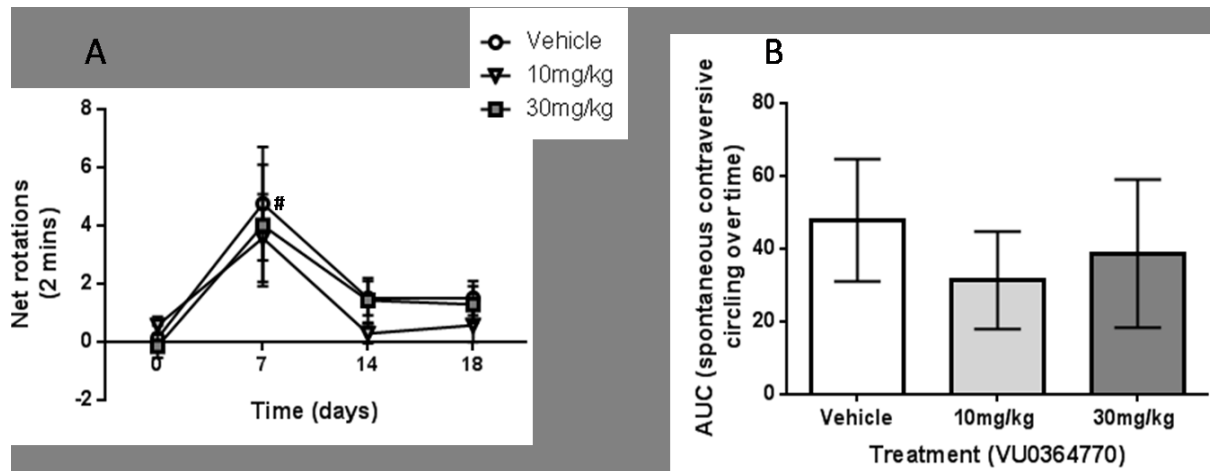


Figure 5.6: Effect of chronic VU0364770 treatment on spontaneous circling behaviour. Rats were unilaterally lesioned in the SNc with 7.5 μ g lactacystin and treated with drug vehicle (n=8), 10mg/kg (n=7) or 30mg/kg (n=7) VU0364770 i.p. from day 4 post-surgery for 14 days and tested for spontaneous circling in a circling bowl over a 2-minute period. Testing was carried out prior to surgery at baseline (day 0), 7, 14 and 18 days after lesioning surgery. Data are shown as net 360° rotations $\pm$ SEM, with positive values signifying net number of contraversive rotations, and negative values representing net number of ipsiversive rotations.

A: There was a significant effect of lesion ($F_{(3,76)}=7.95$ $p=0.0001$) but not VU0364770 treatment ($F_{(2,76)}=0.47$ $p=0.62$) on contralateral forelimb use when comparing the three treatment groups using a two-way ANOVA. Bonferroni post-hoc tests revealed a significant effect of lesion at day 7 compared to baseline in the vehicle-treated group ($^{\#}p<0.05$), but not in 10mg/kg or 30mg/kg-treated groups.

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using one-way ANOVA analysis ($F_{(2,21)}=p=0.78$). AUC units are expressed as time x % net contraversive rotations.

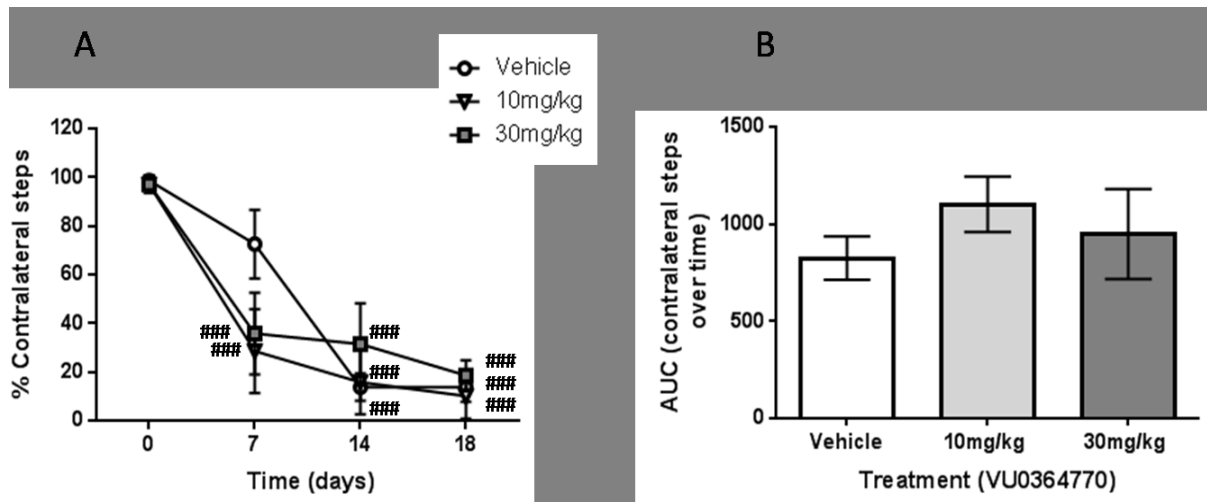


Figure 5.7: Effect of chronic VU0364770 treatment on forelimb akinesia in the stepping test. Rats were unilaterally lesioned in the SNc with 7.5µg lactacystin and treated with drug vehicle (n=8), 10mg/kg (n=7) or 30mg/kg (n=7) VU0364770 i.p. from day 4 post-surgery for 14 days and tested for contralateral forelimb akinesia in the adjusted stepping test. Testing was carried out prior to surgery at baseline (day 0), 7, 14 and 18 days after lesioning surgery. Data are shown as mean % contralateral steps relative to number of ipsilateral steps taken $\pm$ SEM.

A: There was a significant effect of lesion ($F_{(3,76)}=37.07$ $p<0.0001$) but not VU0364770 treatment ($F_{(2,76)}=1.24$ $p=0.29$) on contralateral forelimb use when comparing the three treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 14 and 18 compared to baseline in the vehicle-treated group (### $p<0.001$) and at days 7, 14 and 18 compared to baseline in the 10mg/kg and 30mg/kg VU0364770-treated groups(### $p<0.001$).

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using one-way ANOVA analysis ($F_{(2,21)}=0.71$ $p=0.50$). AUC units are expressed as time x % contralateral steps.

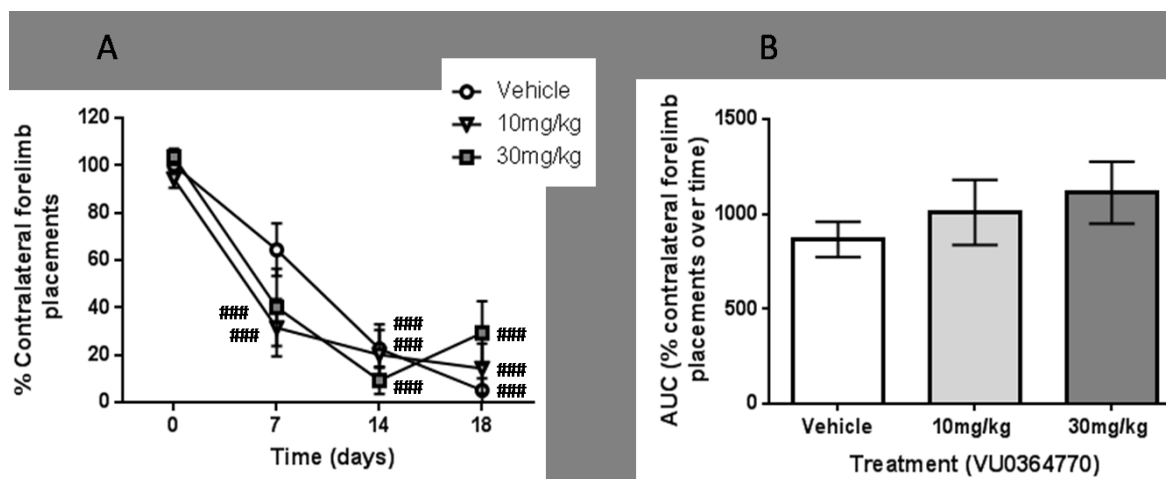


Figure 5.8: Effect of chronic VU0364770 treatment on vibrissae-evoked forelimb placement. Rats were unilaterally lesioned in the SNc with 7.5µg lactacystin and treated with drug vehicle (n=8), 10mg/kg (n=7) or 30mg/kg (n=7) VU0364770 i.p. from day 4 post-surgery for 14 days and tested for contralateral forelimb akinesia in the vibrissae-evoked forelimb placing test. Testing was carried out prior to surgery at baseline (day 0), 7, 14 and 18 days after lesioning surgery. Data are shown as mean % contralateral forelimb placements relative to number of ipsilateral placements (total 5 per side) ± SEM.

A: There was a significant effect of lesion ($F_{(3,76)}=49.48$ $p<0.0001$) but not VU0364770 treatment ($F_{(2,76)}=0.73$ $p=0.48$) on vibrissae-evoked contralateral forelimb placements when comparing the three treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 14 and 18 compared to baseline in the vehicle-treated group (### $p<0.001$) and at days 7, 14 and 18 compared to baseline in the 10mg/kg and 30mg/kg VU0364770-treated groups (#### $p<0.001$).

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb placements over time using one-way ANOVA analysis ($F_{(2,21)}=0.77$ $p=0.47$). AUC units are expressed as time x % contralateral forelimb placements.

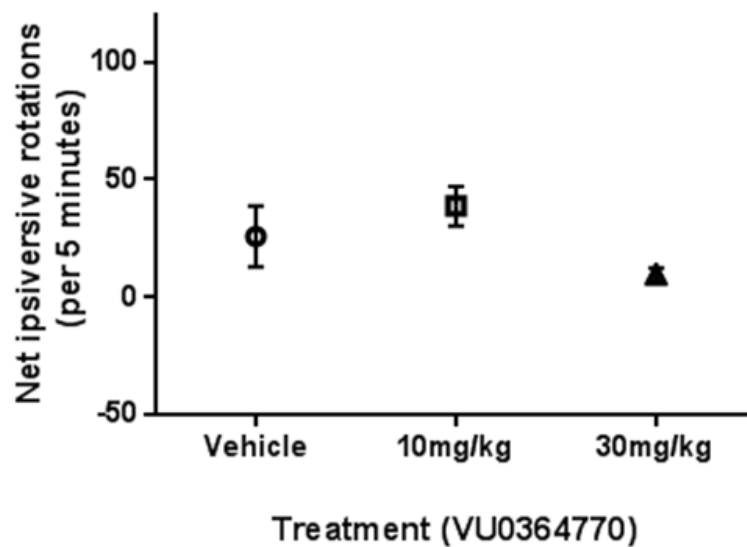


Figure 5.9: Effect of chronic VU0364770 treatment on rotational asymmetry at day 18 post lactacystin lesioning. Rats unilaterally lesioned in the SNc with 7.5µg lactacystin were treated with 5mg/kg amphetamine (i.p.) at the conclusion of the experiment, 18 days after lactacystin lesioning, to assess rotational asymmetry as an indication of lesion magnitude. Net ipsiversive rotations were measured 30 minutes after amphetamine administration for 30 minutes in 5-minute time-bins. Animals were treated with drug vehicle (n=8), 10mg/kg VU0364770 (n=7) or 30mg/kg VU0364770 (n=7) i.p. from day 4 post-lesioning for 14 days. One-way ANOVA analysis indicates that drug treatment did not attenuate rotational asymmetry ($F_{(2,21)}=2.25$ $p=0.13$). Data are shown as net mean ipsiversive rotations per 5 minutes $\pm$ SEM.

5.3.1.2 Effects of chronic peripheral VU0364770 administration on dopaminergic neuronal survival in the lactacystin lesioned SNc

In 7.5µg lactacystin lesioned animals, the SNc of the unlesioned hemisphere contained approximately 10,000 TH+ dopaminergic neurons, which is similar to the previous chapters and other studies (Baquet et al. 2009, Jackson-Lewis et al. 2000, Nair-Roberts et al. 2008) (Figure 5.10&5.11 A). Administration of 10 or 30mg/kg VU0364770 i.p. for 14 days starting 4 days post lesioning did not affect the numbers of TH+ dopaminergic neurons in the unlesioned SNc (Figure 5.11 A). Numbers of Nissl+ neurons detected in the unlesioned SNc was slightly higher at ~12,000, reflecting the fact that not all neurons in the SNc are dopaminergic. VU0364770 administration at 10 or 30mg/kg daily for 14 days did not affect the numbers of Nissl+ neurons in the unlesioned SNc (Figure 5.11 B).

Two-way ANOVA analysis revealed an extremely significant effect of lesioning with 7.5µg lactacystin on TH+ neurons in the ipsilateral SNc by day 19 for all treatment groups (Figure 5.11 A: $F_{(1,38)}=260.7$ $p<0.0001$), but no effect of VU0364770 treatment on TH+ neuronal estimates in the ipsilateral SNc ($F_{(2,38)}=0.62$ $p=0.53$). Similarly, two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning with 7.5µg lactacystin on Nissl+ neurons in the ipsilateral SNc by day 19 for all treatment groups (Figure 5.11 B: $F_{(1,38)}=233.9$ $p<0.0001$), but no effect of VU0364770 treatment on Nissl+ neuronal estimates in the ipsilateral SNc ($F_{(2,38)}=0.92$ $p=0.40$). In the animals receiving drug vehicle treatment for 14 days starting 4 days post-surgery, Bonferroni multiple comparisons tests showed lesioning of the left SNc with 7.5µg lactacystin resulted in a highly significant ($p<0.0001$) and severe neuronal loss, as assessed by both TH+ and Nissl+ estimates, in the ipsilateral, lesioned SNc compared to the contralateral, unlesioned SNc at day 19 post-lesioning (Figure 5.10 & 5.11). Bonferroni multiple comparisons tests similarly showed lesioning of the left SNc with 7.5µg lactacystin resulted in a highly significant ($p<0.0001$) and severe TH+ and Nissl+ neuronal loss in animals administered VU0364770 at 10 or 30mg/kg daily for 14 days starting 4 days post-surgery (Figure 5.10 & 5.11). No significant neuroprotection by VU0364770 of TH+ dopaminergic neurons against lactacystin toxicity was detected in the ipsilateral lesioned SNc (Figure 5.11 A: vehicle: 1179 ± 170 vs 10mg/kg: 1308 ± 217 and 30mg/kg: 1041 ± 157 TH+ cells remaining in the lesioned SNc). Similarly, no significant neuroprotection by VU0364770 of Nissl+ neurons against lactacystin toxicity was detected in the ipsilateral lesioned SNc (Figure 5.11 B: vehicle: 1763 ± 172 vs 10mg/kg: 2166 ± 263 and 30mg/kg: 1439 ± 168 Nissl+ cells remaining in the lesioned SNc).

Comparing percentage cell loss in the lesioned relative to the unlesioned SNc revealed no significant neuroprotective effect of VU0364770 treatment, as illustrated in Figure 5.11 for both TH+ (C) and Nissl+ (D) cell count estimates. Percentage loss of TH+ neurons after drug vehicle treatment was $88.7 \pm 1.3\%$ compared to $86.34 \pm 1.81\%$ for 10mg/kg and $86.84 \pm 1.81\%$ for 30mg/kg VU0364770-

treated animals. Similarly, the loss of Nissl+ neurons paralleled the loss of TH+ neurons in the lesioned SNc. The percentage loss of Nissl+ neurons following drug vehicle treatment was $86.3 \pm 1.2\%$ compared to $81.94 \pm 1.49\%$ for 10mg/kg and $85.8 \pm 2.63\%$ for 30mg/kg VU0364770-treated rats. Inflammatory cells were not investigated in this Chapter due to the lack of neuroprotection observed following chronic VU0364770 treatment, and therefore the likelihood that VU0364770 had no effect on microglial activation.

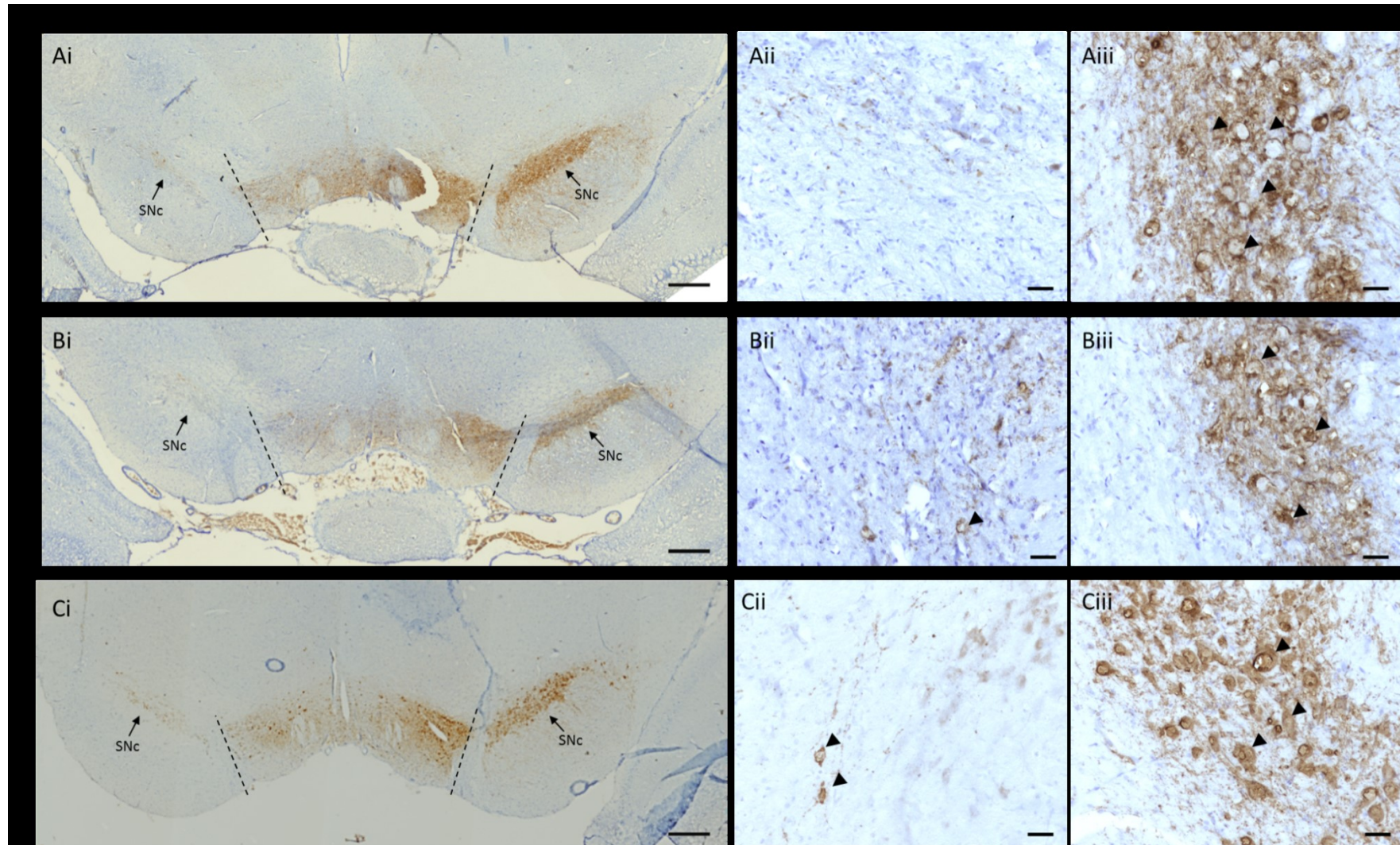


Figure 5.10: Effect of chronic VU0364770 treatment on TH+ dopaminergic neurons in the SNc of 7.5 μ g lactacystin-lesioned rats at day 19 post-lesioning. Representative photomicrographs of TH+ immunostaining. Rats unilaterally lesioned in the SNc with 7.5 μ g lactacystin were treated with either drug vehicle (Ai), 10mg/kg (Bi) or 30mg/kg (Ci) VU0364770 i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining. An extensive loss of TH+ dopaminergic neurons (brown neuronal staining) was seen in the ipsilateral SNc (Aii) compared to the contralateral SNc (Aiii) of drug vehicle treated animals. Similarly, an extensive loss of TH+ dopaminergic neurons was seen in the ipsilateral SNc (B and Cii) compared to the contralateral SNc (B and Ciii) of lactacystin lesioned animals treated daily with both doses of VU0364770. Arrows denote the SNc in the ipsilateral lesioned hemisphere and the contralateral unlesioned hemisphere (A, B and Ci). Arrowheads in magnified images denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100 μ m. High magnification images taken at 20x magnification, scale bar: 30 μ m.

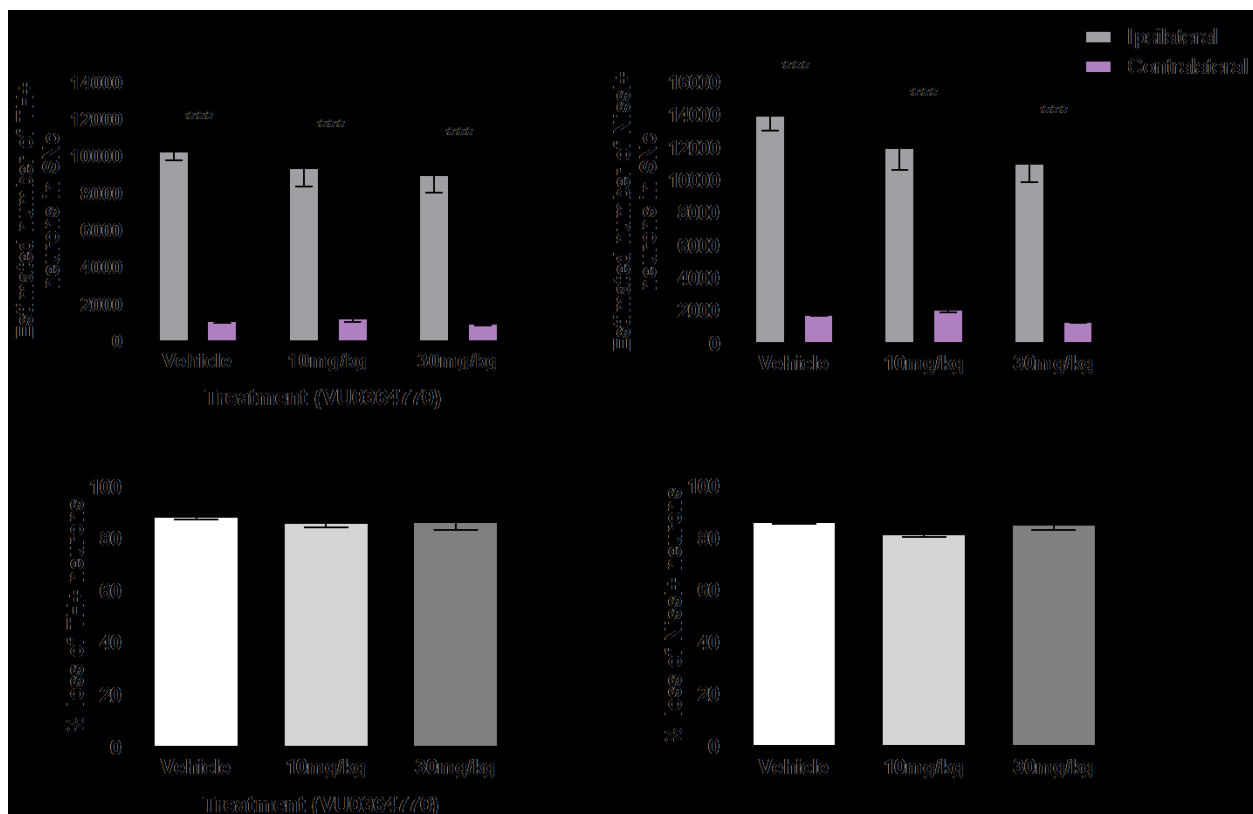


Figure 5.11: Effect of chronic VU0364770 treatment on the total number of TH+ dopaminergic and Nissl+ neurons in the SNc of 7.5µg lactacystin-lesioned rats at day 19 post-lesioning. Rats unilaterally lesioned in the SNc with 7.5µg lactacystin were treated with either drug vehicle, 10mg/kg or 30mg/kg VU0364770 i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining and cell quantification. Total neuronal cell numbers in the SNc were estimated in both the ipsilateral lesioned and contralateral unlesioned hemispheres of each 7.5µg lactacystin-lesioned rat and compared. For all comparisons, drug vehicle treated: n=8, 10mg/kg and 30mg/kg VU0364770: n=7 per group.

A: A significant reduction in the number of TH+ dopaminergic neurons was seen in the ipsilateral SNc compared to the contralateral SNc in all three treatment groups following lesioning ($F_{(1,38)}=260.7$ $p<0.0001$) when analysed by two-way ANOVA, but no effect of VU0364770 treatment was detected ($F_{(2,38)}=0.62$ $p=0.53$). *** $p<0.001$ ipsilateral vs. contralateral SNc. Data are shown as estimated mean total cell number $\pm$ SEM.

B: A significant reduction in the number of Nissl+ neurons was seen in the ipsilateral SNc compared to the contralateral SNc in all three treatment groups following lesioning ($F_{(1,38)}=233.9$ $p<0.0001$) when analysed by two-way ANOVA, but no effect of VU0364770 treatment was detected ($F_{(2,38)}=0.92$ $p=0.40$). *** $p<0.001$ ipsilateral vs. contralateral SNc. Data are shown as estimated mean total cell number $\pm$ SEM.

C: The percentage loss of TH+ dopaminergic neurons in the ipsilateral SNc relative to the contralateral SNc at day 19 following VU0364770 treatment did not differ from the drug vehicle treated group, indicating no neuroprotection occurred. Data are shown as mean % cell loss relative to the contralateral SNc $\pm$ SEM.

D: The percentage loss of Nissl+ neurons in the ipsilateral SNc relative to the contralateral SNc at day 19 following VU0364770 treatment did not differ from the drug vehicle treated group, reflecting the lack of neuroprotection of TH+ dopaminergic neurons. Data are shown as mean % cell loss relative to the contralateral SNc $\pm$ SEM.

5.3.2 Chronic systemic VU0364770 treatment in 2.5µg lactacystin-lesioned rats

5.3.2.1 Effects of chronic peripheral VU0364770 on behavioural deficits

Due to the high degree of dopaminergic neuronal loss detected following lesioning with 7.5µg lactacystin by 19 days post-lesioning, a lower concentration of 2.5µg lactacystin was used for unilateral lesioning to create more of a partial lesion model which may unmask any neuroprotective effects of VU0364770. Only the higher 30mg/kg dose of VU0364770 was used in this study due to the limited amount of drug available and the lack of neuroprotection by VU0364770 administration in rats lesioned with 7.5µg of lactacystin.

Prior to lesioning, all animals demonstrated an equal use of both forelimbs at baseline in the vertical cylinder test for forelimb-use asymmetry (Figure 5.12 A: $49.2 \pm 1.5\%$ contralateral forelimb use, $n=13$). Two-way ANOVA analysis of treatment groups showed a significant effect of lesioning over time (Figure 5.12 A, $F_{(3,33)}=3.06$ $p=0.041$) but no effect of 30mg/kg VU0364770 treatment on forelimb-use asymmetry ($F_{(1,11)}=0.03$ $p=0.86$ ns). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7 and 14 compared to baseline in the 30mg/kg VU0364770-treated group ($p<0.05$ for both) but not in the vehicle-treated group. Negative area under the curve (AUC) analysis similarly showed no significant effect of 30mg/kg VU0364770 treatment following 2.5µg lactacystin lesioning (Figure 5.12B, unpaired t-test $p=0.19$). As no forelimb use asymmetry could be detected in drug vehicle treated animals when assessing rearing behaviour in the vertical cylinder test, no effect of chronic VU0364770 treatment on forelimb use asymmetry could be ascertained with this test.

Vertical wall exploration of the cylinder was assessed independently in the same tests as a more sensitive marker of forelimb use asymmetry. At baseline, animals displayed an equal use of both forelimbs for exploration of the cylinder wall ($51.7 \pm 5.9\%$ contralateral forelimb use). Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 5.13 A, $F_{(3,33)}=7.91$ $p=0.0004$) but no effect of chronic treatment with 30mg/kg VU0364770 on forelimb-use asymmetry for exploration of the cylinder wall ($F_{(1,11)}=0.28$ $p=0.60$). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7 and 14 compared to baseline in the vehicle-treated group ($p<0.05$ at both time points) and at days 7, 14 and 18 compared to baseline in the 30mg/kg VU0364770-treated group (day 0 vs. days 7 and 14 $p<0.05$, day 0 vs. day 18 $p<0.01$). Negative area under the curve (AUC) analysis similarly showed no significant effect of 30mg/kg VU0364770 treatment following 2.5µg lactacystin lesioning (Figure 5.13B, unpaired t-test $p=0.74$ ns).

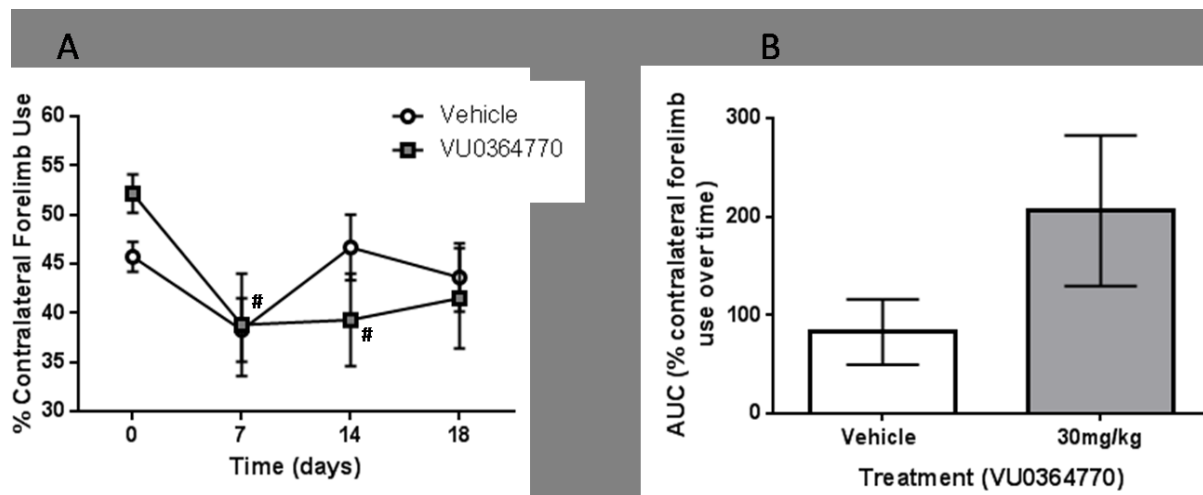


Figure 5.12: Effect of chronic drug vehicle or VU0364770 treatment on forelimb-use asymmetry in the vertical cylinder test. Rats were unilaterally lesioned in the SNc with 2.5 μ g lactacystin and treated with drug vehicle (white bars, n=6) or 30mg/kg VU0364770 i.p. (grey bars, n=7) daily from day 4 post-surgery for 14 days. Rats were tested in a vertical cylinder to determine forelimb-use asymmetry prior to surgery at baseline (day 0), 7 days, 14 days and 18 days after lesioning surgery. Data are shown as mean % contralateral forelimb use (push-off, cylinder wall exploration and landing combined) $\pm$ SEM

A: There was a significant effect of lesion ($F_{(3,33)}=3.06$ $p=0.041$) but not VU0364770 treatment ($F_{(1,11)}=0.03$ $p=0.86$) on contralateral forelimb use when comparing the two treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7 and 14 compared to baseline in the 30mg/kg VU0364770-treated group ($^{\#}p<0.05$) but not in the vehicle-treated group.

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using a paired t-test ($p=0.19$). AUC units are expressed as time x % contralateral forelimb use.

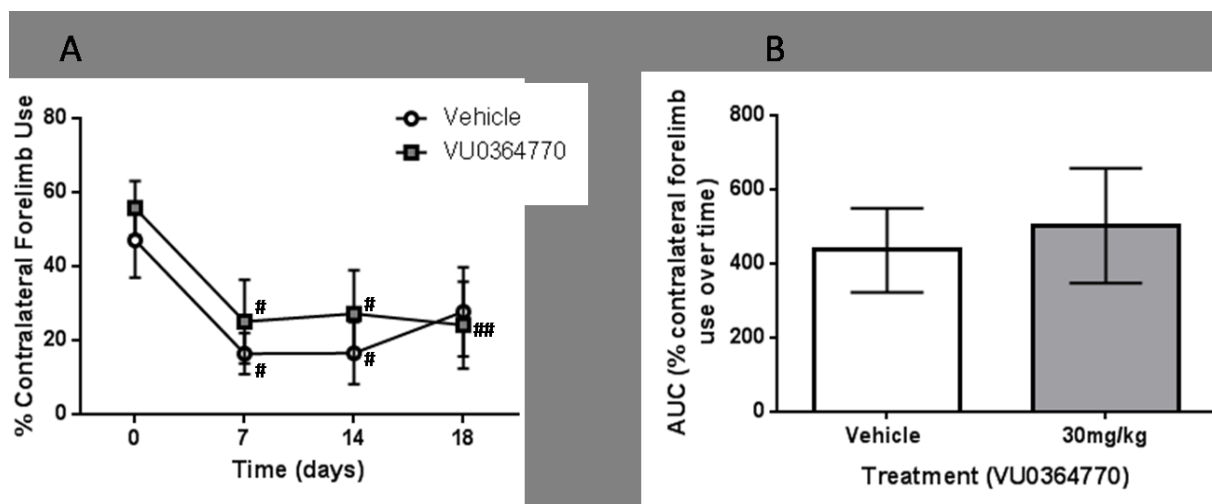


Figure 5.13: Effect of chronic vehicle or VU0364770 treatment on forelimb-use asymmetry for wall exploration in the vertical cylinder test. Rats were unilaterally lesioned in the SNc with 2.5 μ g lactacystin and treated with drug vehicle (white bars, n=6) or 30mg/kg VU0364770 i.p. (grey bars, n=7) daily from day 4 post-surgery for 14 days. Rats were tested in a vertical cylinder to determine forelimb-use asymmetry prior to surgery at baseline (day 0), 7 days, 14 days and 18 days after lesioning surgery. Data are shown as mean % contralateral forelimb use (cylinder wall exploration alone) $\pm$ SEM.

A: There was a significant effect of lesion ($F_{(3,33)}=7.91$ $p=0.0004$) but not VU0364770 treatment ($F_{(1,11)}=0.28$ $p=0.60$) on contralateral forelimb use when comparing the three treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7 and 14 compared to baseline in the vehicle-treated group ($^{\#}p<0.05$), and at days 7, 14 and 18 compared to baseline in the 30mg/kg VU0364770-treated group ($^{\#}p<0.05$, $^{##}p<0.01$).

B: No difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb use over time using a paired t-test($p=0.74$). AUC units are expressed as time x % contralateral forelimb use.

Following unilateral lactacystin lesioning, it was observed that 7.5µg lactacystin lesioned animals demonstrated a contraversive bias in spontaneous circling for up to 10 days post-surgery. Two-way ANOVA analysis of treatment groups showed a significant effect of lesioning over time (Figure 5.14 A, $F_{(3,33)}=4.43p=0.01$) but no effect of chronic 30mg/kg VU0364770 treatment on spontaneous rotational asymmetry ($F_{(1,11)}=1.57p=0.23$ ns). Bonferroni post-hoc tests revealed a significant effect of lesion at day 7 compared to baseline in the vehicle-treated group ($p<0.01$) but not in the 30mg/kg VU0364770-treated group. Area under the curve (AUC) analysis similarly showed no significant effect of 30mg/kg VU0364770 treatment following 2.5µg lactacystin lesioning on spontaneous rotational asymmetry (Figure 5.14B, unpaired t-test $p=0.13$ ns).

Prior to lesioning, animals successfully stepped $103.3 \pm 2.7\%$ of total ipsilateral forelimb steps in the adjusted stepping test for forelimb akinesia. Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 5.15 A, $F_{(3,33)}=7.56p=0.0006$) but no effect of chronic 30mg/kg VU0364770 treatment on contralateral forelimb akinesia in the stepping test ($F_{(1,11)}=0.50p=0.49$). Bonferroni post-hoc tests demonstrated a significant effect of lesion at days 14 and 18 compared to baseline in the vehicle-treated group ($p<0.05$ for both comparisons) and at days 7 and 18 compared to baseline in the VU0364770-treated group (day 0 vs. day 7 $p<0.001$, day 0 vs. day 18 $p<0.05$). Negative area under the curve (AUC) analysis similarly showed no significant effect of 30mg/kg VU0364770 treatment following 2.5µg lactacystin lesioning on contralateral forelimb akinesia in the stepping test (Figure 5.15B, unpaired t-test $p=0.27$).

Prior to lesioning, animals successfully placed their contralateral forelimb $96.9 \pm 3\%$ of the time relative to total ipsilateral placements in the vibrissae-evoked forelimb placing test. Following unilateral lesioning of the SNc with 2.5µg lactacystin, drug vehicle treated animals demonstrated a significant decline in vibrissae-evoked contralateral forelimb placements over time when analysed by one-way ANOVA (Figure 5.16 A, $F_{(3,15)}=4.30$, $p=0.022$). Bonferroni post-correction tests indicated a significant reduction in contralateral forelimb placement from baseline at day 14 (day 14: $48.6 \pm 18.4\%$, $p<0.05$). Administration of 30mg/kg VU0364770 daily for 14 days from day 4 post-lesioning did not attenuate contralateral forelimb akinesia, and animals demonstrated a significant decline in vibrissae-evoked contralateral forelimb placements when analysed by one-way ANOVA (Figure 5.16 B: $F_{(3,18)}=3.44$, $p=0.039$), with a significant reduction in contralateral forelimb placement from baseline detected by day 14 ($79.4 \pm 8.9\%$, $p<0.05$) (Figure 5.16 B). Two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning over time (Figure 5.16 A, $F_{(3,33)}=7.82p=0.0004$) but no effect of chronic 30mg/kg VU0364770 treatment on vibrissae-evoked contralateral forelimb placements ($F_{(1,11)}=0.007p=0.93$ ns). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7 and 14 compared to baseline in the vehicle-treated group (day 0 vs. day 7 $p<0.05$, day 0 vs. day 14 $p<0.01$) and at day 14 compared to baseline in the VU0364770-treated

group ($p < 0.05$). Negative area under the curve (AUC) analysis similarly showed no significant effect of 30mg/kg VU0364770 treatment following 2.5µg lactacystin lesioning on contralateral forelimb placements (Figure 5.16B, unpaired t-test $p = 0.79$).

Rotational asymmetry was investigated at the conclusion of the experiment (day 18 post lesioning) utilising amphetamine induced rotational behaviour as an indirect measure of lesion magnitude. Drug vehicle treatment daily for 14 days starting 4 days post unilateral SNc lesioning with 2.5µg lactacystin was associated with a marked induction of circling behaviour following amphetamine administration on day 18 of the study. Administration of 30mg/kg VU0364770 failed to attenuate the circling behaviour induced by amphetamine administration when analysed by an unpaired t-test (Figure 5.17: net mean ipsiversive rotations/5 minutes for drug vehicle treated animals: 32.3 ± 12.8 , 30mg/kg VU0364770: 33.6 ± 14.2 , $p = 0.87$ ns).

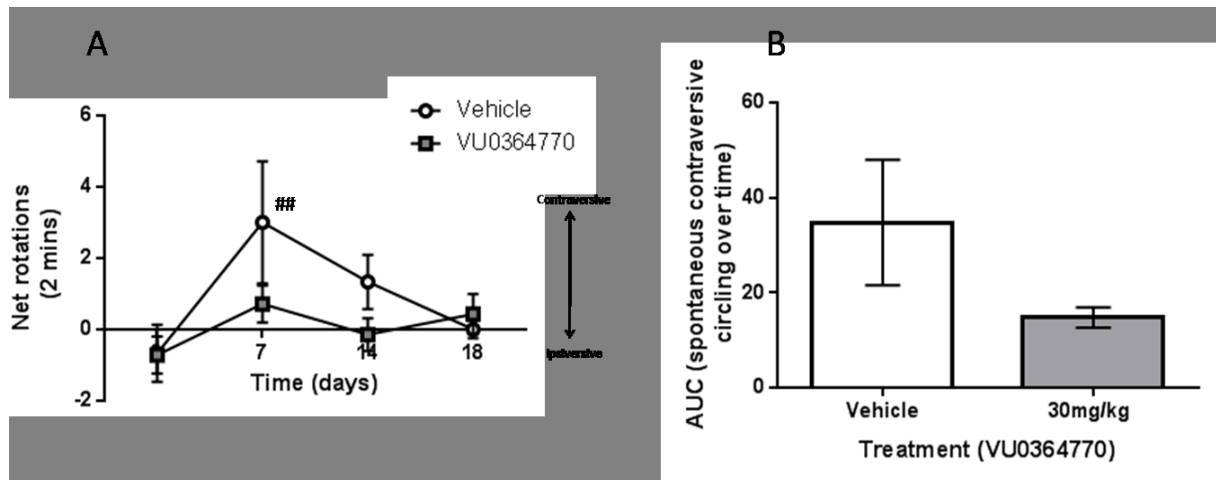


Figure 5.14: Effect of chronic vehicle or VU0364770 treatment on spontaneous circling behaviour. Rats were unilaterally lesioned in the SNc with 2.5µg lactacystin and treated with drug vehicle (white bars, n=6) or 30mg/kg VU0364770 i.p. (grey bars, n=7) daily from day 4 post-surgery for 14 days. Rats were tested for spontaneous circling in a Perspex circling bowl over a 2-minute period prior to surgery at baseline (day 0), 7 days, 14 days and 18 days after lesioning surgery. Data are shown as net 360° rotations ± SEM, with positive values signifying a contraversive bias in rotations, and negative values representing an ipsiversive bias.

A: There was a significant effect of lesion ($F_{(3,33)}=4.43$ $p=0.01$) but not VU0364770 treatment ($F_{(1,11)}=1.57$ $p=0.23$) on contralateral forelimb use when comparing the three treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at day 7 compared to baseline in the vehicle-treated group ($^{##}p<0.01$).

B: No difference between treatment groups was revealed when comparing area under curve of contralateral forelimb use over time using a paired t-test ($p=0.13$). AUC units are expressed as time x % contralateral forelimb use.

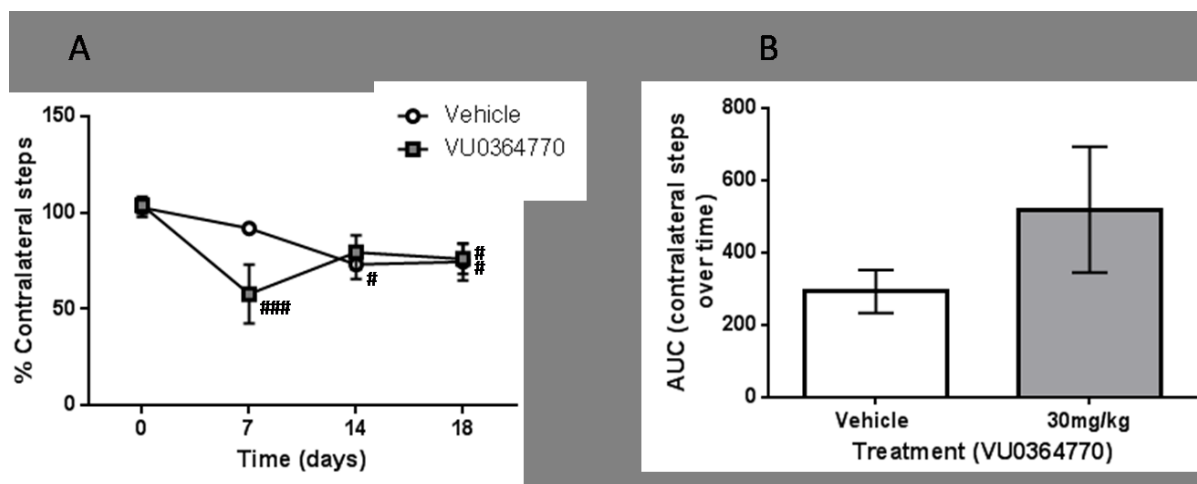


Figure 5.15: Effect of chronic vehicle or VU0364770 treatment on forelimb akinesia in the stepping test. Rats were unilaterally lesioned in the SNc with 2.5 μ g lactacystin and treated with drug vehicle (white bars, n=6) or 30mg/kg VU0364770 i.p. (grey bars, n=7) daily from day 4 post-surgery for 14 days. Rats were tested for forelimb akinesia in the adjusted stepping test prior to surgery at baseline (day 0), 7 days, 14 days and 18 days after lesioning surgery. Data are shown as mean % contralateral steps relative to number of ipsilateral steps taken $\pm$ SEM.

A: There was a significant effect of lesion ($F_{(3,33)}=7.56$ $p=0.0006$) but not VU0364770 treatment ($F_{(1,11)}=0.50$ $p=0.49$) on contralateral forelimb use when comparing the two treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 14 and 18 compared to baseline in the vehicle-treated group ($^{\#} p<0.05$) and at days 7 and 18 compared to baseline in the VU0364770-treated group ($^{\#} p<0.05$, $^{###} p<0.001$).

B: No significant difference between treatment groups was revealed when comparing negative area under curve of contralateral stepping over time when analysed by unpaired t-test ($p=0.27$). AUC units are expressed as time x % contralateral steps.

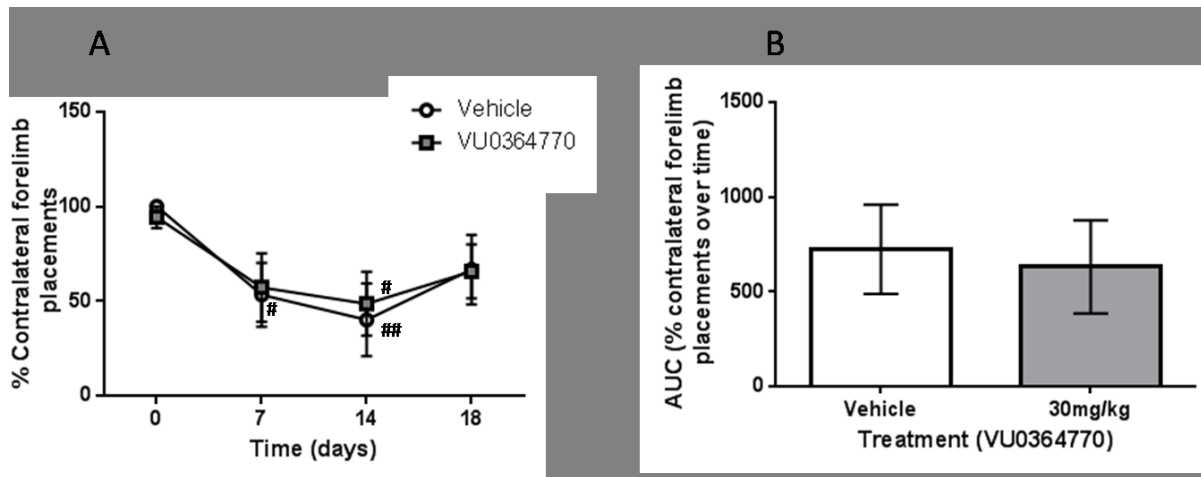


Figure 5.16: Effect of chronic vehicle or VU0364770 treatment on vibrissae-evoked forelimb placement. Rats were unilaterally lesioned in the SNc with 2.5µg lactacystin and treated with drug vehicle (white bars, n=6) or 30mg/kg VU0364770 i.p. (grey bars, n=7) daily from day 4 post-surgery for 14 days. Rats were tested for contralateral forelimb akinesia in the vibrissae-evoked forelimb placing test prior to surgery at baseline (day 0), 7 days, 14 days and 18 days after lesioning surgery. Data are shown as mean % contralateral forelimb placements relative to number of ipsilateral placements (total 5 per side) ± SEM.

A: There was a significant effect of lesion ($F_{(3,33)}=7.82$ $p=0.0004$) but not VU0364770 treatment ($F_{(1,11)}=0.007$ $p=0.93$) on contralateral forelimb use when comparing the two treatment groups (two-way ANOVA). Bonferroni post-hoc tests revealed a significant effect of lesion at days 7 and 14 compared to baseline in the vehicle-treated group ($^{\#}p<0.05$, $^{##}p<0.01$) and at day 14 compared to baseline in the VU0364770-treated group ($^{\#}p<0.05$).

B: No significant difference between treatment groups was revealed when comparing negative area under curve of contralateral forelimb placement over time when analysed by unpaired t-test ($p=0.79$). AUC units are expressed as time x % contralateral forelimb placements.

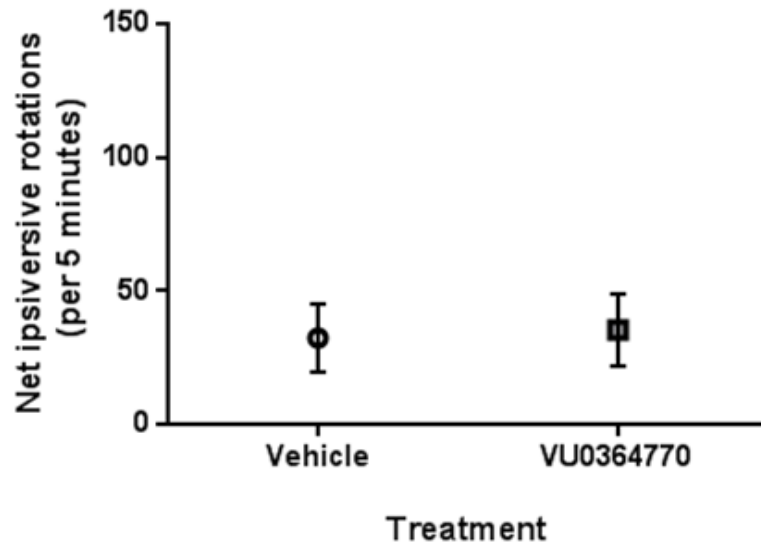


Figure 5.17: Effect of chronic VU0364770 treatment on rotational asymmetry at day 18 post lactacystin lesioning. Rats unilaterally lesioned in the SNc with 2.5µg lactacystin were treated with 5mg/kg amphetamine (i.p.) at the conclusion of the experiment, 18 days after lactacystin lesioning, to assess rotational asymmetry as an indication of lesion magnitude. Net ipsiversive rotations were measured 30 minutes after amphetamine administration for 30 minutes in 5-minute time-bins. Animals were treated with drug vehicle (n=6) or 30mg/kg VU0364770 i.p. (n=7) from day 4 post-lesioning for 14 days. Chronic drug treatment did not attenuate rotational asymmetry as analysed by an unpaired t-test. Data are shown as net mean ipsiversive rotations per 5 minutes $\pm$ SEM.

5.3.2.2 Effects of chronic peripheral VU0364770 on dopaminergic neuronal survival in the 2.5µg lactacystin lesioned SNc

In 2.5µg lactacystin lesioned animals, the SNc of the unlesioned hemisphere contained approximately 8,500 TH+ dopaminergic neurons, which is comparable to other studies (Baquet et al. 2009, Jackson-Lewis et al. 2000, Nair-Roberts et al. 2008) (Figure 5.18 & 5.19). Administration of 30mg/kg VU0364770 i.p. for 14 days starting 4 days post lesioning did not affect the numbers of TH+ dopaminergic neurons in the unlesioned SNc (Figure 5.19 A). Numbers of Nissl+ neurons detected in the unlesioned SNc was slightly higher at ~10,000, reflecting the additional non-dopaminergic neuronal populations in the SNc. Similarly, VU0364770 administration at 30mg/kg daily for 14 days did not affect the numbers of Nissl+ neurons in the unlesioned SNc (Figure 5.19 B).

Two-way ANOVA analysis revealed an extremely significant effect of lesioning with 2.5µg lactacystin on TH+ neurons in the ipsilateral SNc by day 19 for both treatment groups (Figure 5.18 and 5.19 A, $F_{(1,11)}=229.2, p<0.0001$), but no effect of 30mg/kg VU0364770 treatment on TH+ neuronal estimates in the ipsilateral SNc ($F_{(1,11)}=0.26, p<0.62$). Similarly, two-way ANOVA analysis of treatment groups showed an extremely significant effect of lesioning with 2.5µg lactacystin on Nissl+ neurons in the ipsilateral SNc by day 19 for both treatment groups (Figure 5.19 B, $F_{(1,11)}=319.5, p<0.0001$), but no effect of 30mg/kg VU0364770 treatment on Nissl+ neuronal estimates in the ipsilateral SNc ($F_{(1,11)}=0.19, p=0.67$). In the animals receiving drug vehicle treatment for 14 days starting 4 days post-surgery, Bonferroni multiple comparisons tests showed lesioning of the left SNc with 2.5µg lactacystin resulted in a highly significant ($p<0.0001$) and severe neuronal loss, as assessed by both TH+ and Nissl+ estimates, in the ipsilateral, lesioned SNc compared to the contralateral, unlesioned SNc at day 19 post-lesioning (Figure 5.18 & 5.19). Bonferroni multiple comparisons tests similarly showed lesioning of the left SNc with 2.5µg lactacystin resulted in a highly significant ($p<0.0001$) and severe TH+ and Nissl+ neuronal loss in animals administered 30mg/kg VU0364770 daily for 14 days starting 4 days post-surgery (Figure 5.18 & 5.19). No significant neuroprotection of TH+ dopaminergic neurons by VU0364770 against lactacystin toxicity was detected in the ipsilateral lesioned SNc (Figure 5.19 A, vehicle: 1187 ± 197 vs 30mg/kg VU0364770: 1117 ± 342 TH+ cells remaining in the lesioned SNc). Similarly, no significant neuroprotection of Nissl+ neurons by VU0364770 against lactacystin toxicity was detected in the ipsilateral lesioned SNc (Figure 5.19 B, vehicle: 1525 ± 276 vs 30mg/kg VU0364770: 1552 ± 370 Nissl+ cells remaining in the lesioned SNc).

Comparing percentage cell loss in the lesioned relative to the unlesioned SNc revealed no difference between drug vehicle and 30mg/kg DCPG-treated groups as illustrated in Figure 5.19 for both TH+ (C) and Nissl+ (D) cell count estimates. Percentage loss of TH+ neurons after drug vehicle treatment was $86 \pm 1.9\%$ compared to $86.8 \pm 3\%$ for 30mg/kg VU0364770-treated animals, indicating

no neuroprotective effect of 30mg/kg VU0364770 on neuronal survival. Similarly, the loss of Nissl+ neurons paralleled the loss of TH+ neurons in the lesioned SNc; following drug vehicle treatment, the percentage loss of Nissl+ neurons was $85.56 \pm 2.02\%$ compared to $84.81 \pm 2.71\%$ following 30mg/kg VU0364770 treatment.

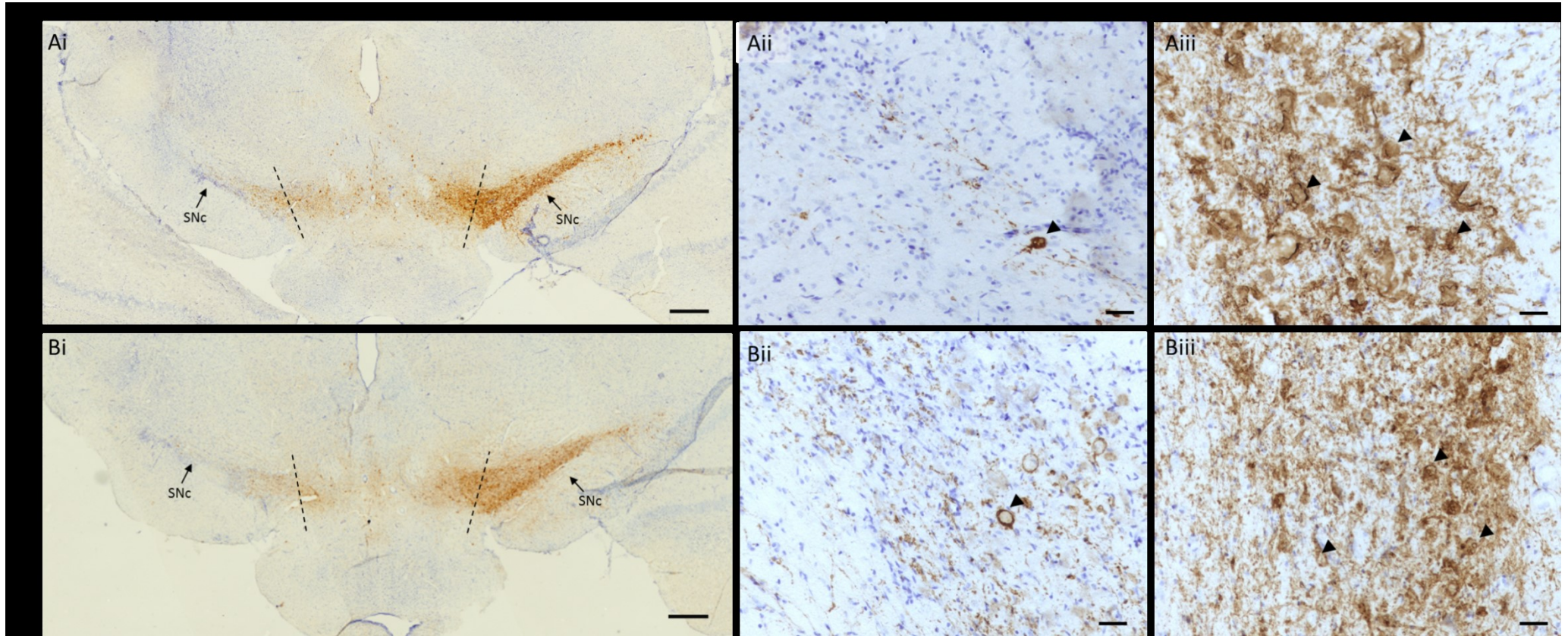


Figure 5.18: Effect of chronic VU0364770 treatment on TH+ dopaminergic neurons in the SNc of 2.5µg lactacystin-lesioned rats at day 19 post-lesioning. Representative photomicrographs of TH+ immunostaining. Rats unilaterally lesioned in the SNc with 2.5µg lactacystin were treated with either drug vehicle (Ai) or 30mg/kg VU0364770 (Bi) i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining. An extensive loss of TH+ dopaminergic neurons (brown neuronal staining) was seen in the ipsilateral SNc (Aii) compared to the contralateral SNc (Aiii) of drug vehicle treated animals. Similarly, an extensive loss of TH+ dopaminergic neurons was seen in the ipsilateral SNc (Bii) compared to the contralateral SNc (Biii) of lactacystin lesioned animals treated daily with VU0364770. Arrows denote the SNc in the ipsilateral lesioned hemisphere and the contralateral unlesioned hemisphere (A and Bi). Arrowheads in magnified images denote example immunopositive cells. Low magnification images taken at 4x magnification, scale bar: 100µm. High magnification images taken at 20x magnification, scale bar: 30µm.

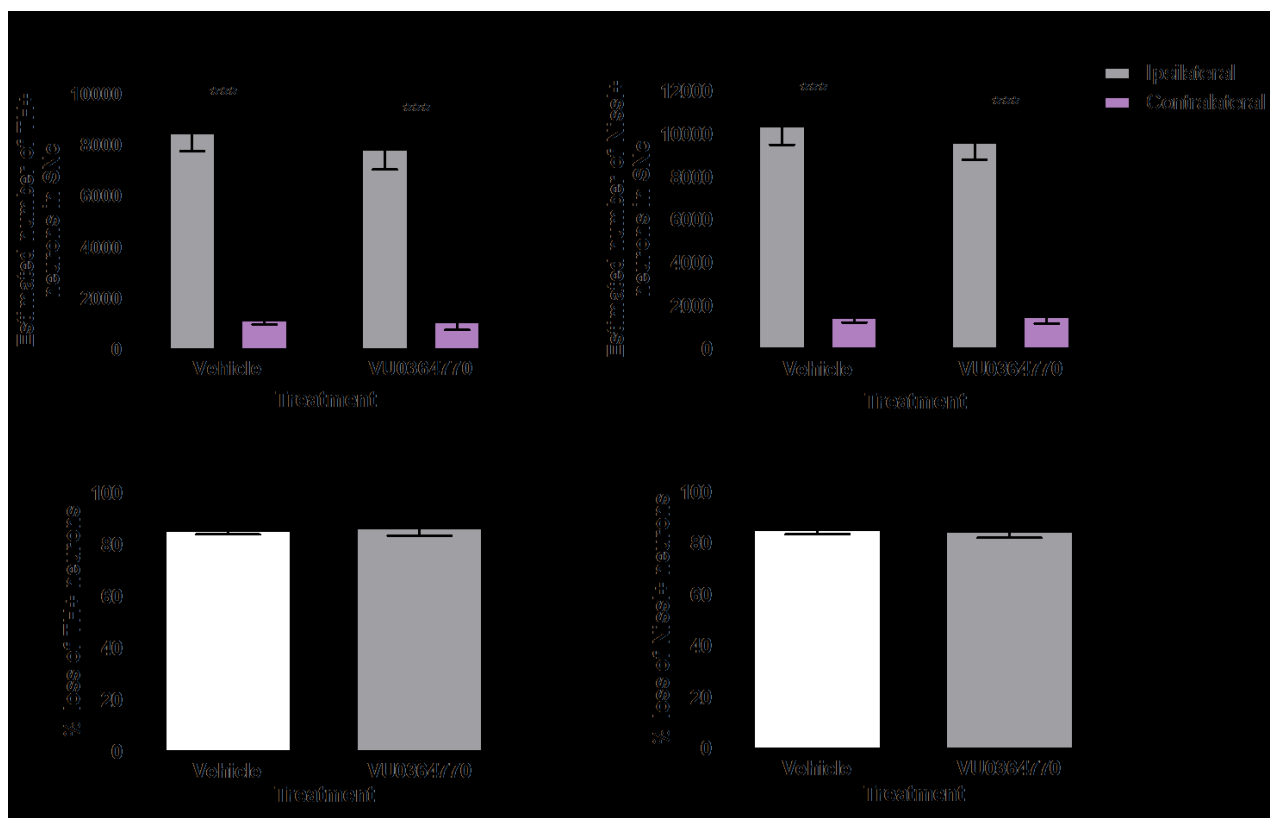


Figure 5.19: Effect of chronic VU0364770 treatment on the total number of TH+ dopaminergic and Nissl+ neurons in the SNc of 2.5µg lactacystin-lesioned rats at day 19 post-lesioning. Rats unilaterally lesioned in the SNc with 2.5µg lactacystin were treated with either drug vehicle or 30mg/kg VU0364770 i.p. daily from day 4 post-surgery for 14 days. At day 19 post-lesioning, brains were removed and processed for immunohistochemical staining and cell quantification. Total neuronal cell numbers in the SNc were estimated in both the ipsilateral lesioned and contralateral unlesioned hemispheres of each 2.5µg lactacystin-lesioned rat and compared. For all comparisons, drug vehicle treated group: n=6 and 30mg/kg VU0364770 treated group: n=7.

A: A significant reduction in the number of TH+ dopaminergic neurons was seen in the ipsilateral SNc compared to the contralateral SNc in both treatment groups following lesioning ($F_{(1,11)}=229.2$ $p<0.0001$) when analysed by two-way ANOVA, but no effect of VU0364770 treatment was detected ($F_{(1,11)}=0.26$ $p<0.62$). *** $p<0.0001$ ipsilateral vs. contralateral SNc. Data are shown as estimated mean total cell number $\pm$ SEM.

B: A significant reduction in the number of Nissl+ neurons was seen in the ipsilateral SNc compared to the contralateral SNc in both treatment groups following lesioning ($F_{(1,11)}=319.5$ $p<0.0001$) when analysed by two-way ANOVA, but no effect of VU0364770 treatment was detected ($F_{(1,11)}=0.19$ $p=0.67$). *** $p<0.0001$ ipsilateral vs. contralateral SNc. Data are shown as estimated mean total cell number $\pm$ SEM.

C: The percentage loss of TH+ dopaminergic neurons in the ipsilateral SNc relative to the contralateral SNc at day 19 following VU0364770 treatment did not differ from the drug vehicle treated group, indicating no neuroprotection occurred. Data are shown as mean % cell loss relative to the contralateral SNc $\pm$ SEM.

D: The percentage loss of Nissl+ neurons in the ipsilateral SNc relative to the contralateral SNc at day 19 following VU0364770 treatment did not differ from the drug vehicle treated group, reflecting the lack of neuroprotection. Data are shown as mean % cell loss relative to the contralateral SNc $\pm$ SEM.

5.3.3 Acute systemic VU0364770 treatment in 7.5µg lactacystin-lesioned rats

5.3.3.1 Effects of single peripheral administration of VU0364770 on behavioural deficits

Acute testing of 10mg/kg, 30mg/kg and 100mg/kg of VU0364770 was carried out using a battery of behavioural tests in 7.5µg lactacystin lesioned rats. Following unilateral lesioning of the SNc with 7.5µg lactacystin, rats were left for 21 days to allow measurable behavioural deficits to develop. At day 14 post-lesioning, animals were assessed for rotational asymmetry following amphetamine administration, and those that rotated on average <1 net ipsiversive rotation per minute were discounted from the study as poorly lesioned (n=2). Animals that would not rear in the vertical cylinder test (n=3) were also discounted from analysis. From day 21 post-lesioning onwards, animals (n=7 per group) were acutely treated with a single i.p. administration of 10mg/kg, 30mg/kg or 100mg/kg of VU0364770 or drug vehicle and behaviourally assessed. Animals demonstrated a strong forelimb use asymmetry and contralateral forelimb akinesia >21 days after unilateral lactacystin lesioning during baseline assessments. As behavioural assessments were carried out at different time points after lesioning to allow wash out of test drugs (i.e. day 22, day 28, day 34), behavioural deficits gradually increased over the testing period, necessitating separate baseline measurements prior to each test, and data was normalised to a percentage of mean baseline scores and analysed with two-way ANOVA.

In the vertical cylinder test, drug vehicle treated animals demonstrated no change in forelimb use asymmetry from baseline for either method of analysis (Figure 5.20 A and B), indicating that repeated testing (over 2 days) in the cylinder had no effect on contralateral forelimb use. No significant attenuation of forelimb use asymmetry from baseline was observed following acute administration of 10, 30 or 100mg/kg of VU0364770 where all parameters of the test were assessed (push-off, wall exploration and landing) (Figure 5.20 A: 10mg/kg VU0364770: $99.1 \pm 3.4\%$ of baseline, 30mg/kg VU0364770: $105.1 \pm 3\%$ of baseline, 100mg/kg VU0364770: $102.7 \pm 5.7\%$ of baseline, $F_{(3,48)}=0.47p=0.69$ ns). Similarly, no significant attenuation of forelimb use asymmetry from baseline was observed following acute administration of 10, 30 or 100mg/kg of VU0364770 where exploration of the cylinder wall alone was assessed (Figure 5.20 B: 10mg/kg VU0364770: $97.1 \pm 4.2\%$ of baseline, 30mg/kg VU0364770: $98.6 \pm 5.7\%$ of baseline, 100mg/kg VU0364770: $97.1 \pm 7.7\%$ of baseline, $F_{(3,48)}=0.15p=0.92$ ns). In the adjusted stepping test, drug vehicle treated animals demonstrated no change in forelimb akinesia from baseline, indicating no effect of repeat testing on contralateral stepping in this test. No significant reduction in forelimb akinesia from baseline was seen following 10, 30 or 100mg/kg of VU0364770 administration (Figure 5.20 C: 10mg/kg VU0364770: $99.1 \pm 3.4\%$ of baseline, 30mg/kg VU0364770: $105.1 \pm 3\%$ of baseline, 100mg/kg VU0364770: $102.7 \pm 5.7\%$ of baseline, $F_{(3,48)}=1.86p=0.14$ ns). Finally, in the vibrissae-evoked forelimb placement test, no effect of repeat testing on contralateral forelimb placement was seen in drug vehicle treated animals (Figure

5.20 D). Again, no significant improvement in contralateral forelimb placement was observed from baseline following acute drug vehicle or 10, 30 or 100mg/kg VU0364770 treatment (Figure 5.20 D: 10mg/kg VU0364770: $97.8 \pm 7.2\%$ of baseline, 30mg/kg VU0364770: $92.1 \pm 9.6\%$ of baseline, 100mg/kg VU0364770: $91.4 \pm 12.2\%$ of baseline, $F_{(3,48)}=0.10$ $p=0.95$ ns).

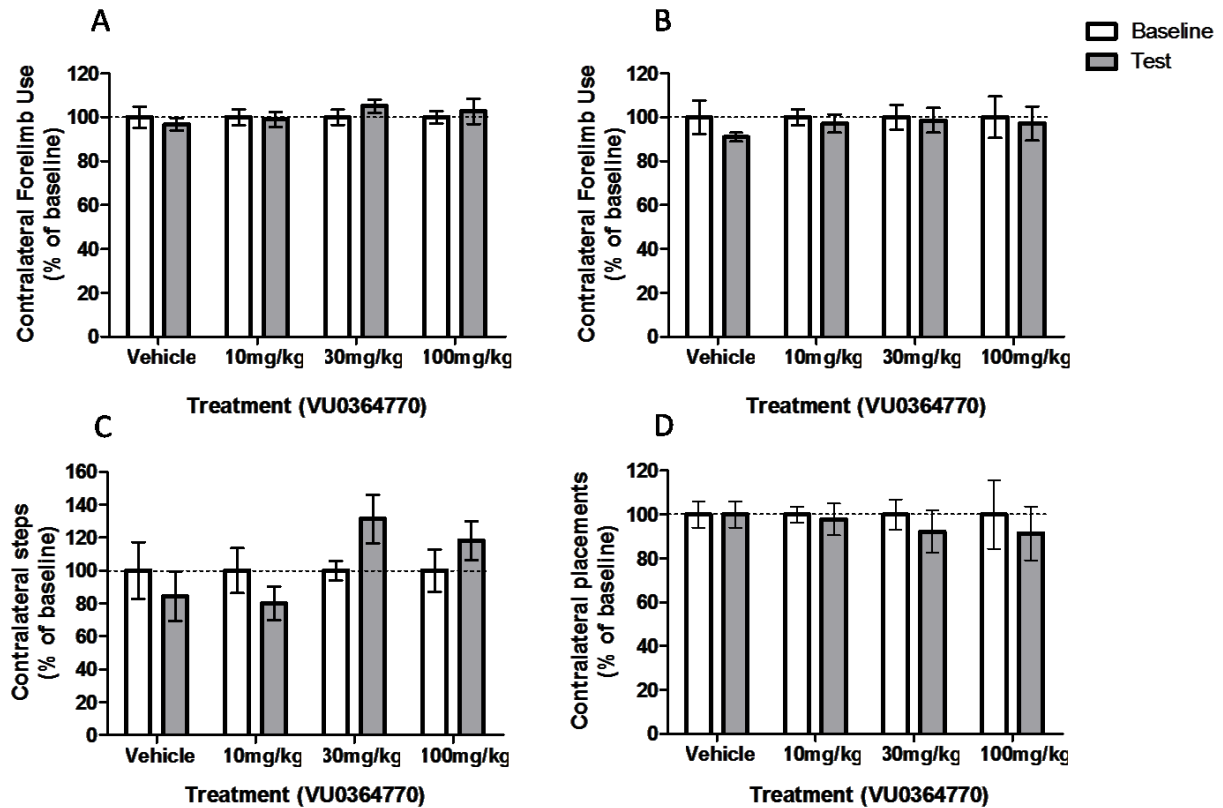


Figure 5.20: Effect of single VU0364770 administration on forelimb-use asymmetry and forelimb akinesia. Unilaterally lactacystin-lesioned rats were assessed in the vertical cylinder test to determine forelimb-use asymmetry, the adjusted stepping test to assess forelimb akinesia and in the vibrissae-evoked forelimb placement test to assess forelimb akinesia at both baseline (1 day prior to treatment, represented by white bars), and on the following ‘test’ day (represented by grey bars), after vehicle or VU0364770 (10mg/kg, 30mg/kg or 100mg/kg) i.p. treatment. All data was analysed by two-way ANOVA and is displayed as mean percentage of baseline score $\pm$ SEM, $n=7$ per treatment group.

A: Rats demonstrated no significant change from baseline following administration of 10, 30 or 100mg/kg VU0364770 when assessing contralateral forelimb use in the vertical cylinder for push-off, cylinder exploration and landing ($F_{(3,48)}=0.47$ $p=0.69$).

B: Rats showed no significant change in contralateral forelimb use for wall exploration of the vertical cylinder from baseline following administration of 10, 30 or 100mg/kg VU0364770 ($F_{(3,48)}=0.15$ $p=0.92$).

C: Rats showed no significant change in the number of contralateral steps from baseline following administration of 10, 30 or 100mg/kg VU0364770 ($F_{(3,48)}=1.86$ $p=0.14$).

D: Rats demonstrated no significant change in number of successful vibrissae-evoked contralateral forelimb placements from baseline following administration of 10, 30 or 100mg/kg VU0364770 ($F_{(3,48)}=0.10$ $p=0.95$).

5.4 Discussion

The aim of these studies was to elucidate the value of modulating the mGlu4 receptor in PD using the selective and brain permeable mGlu4 receptor PAM VU0364770 in the lactacystin model. Acute systemic dosing with VU0364770 was assessed to see whether it is able to alleviate the motor deficits in the lactacystin PD model, whilst chronic systemic dosing with VU0364770 was assessed to see whether VU0364770 is neuroprotective against the toxic effects of lactacystin.

5.4.1 Chronic peripheral VU0364770 administration provides no neuroprotection in lactacystin-lesioned animals

Similar to the previous chronic treatment study with DCPG in Chapter 4, motor deficits in drug vehicle treated 7.5µg lactacystin-lesioned animals were more severe than in drug vehicle treated 2.5µg lactacystin lesioned rats, limiting assessment of motor deficit improvement in the 2.5µg lactacystin lesioned animals treated chronically with VU0364770, although some mild deficits were detectable. Collectively, behavioural tests employed in this Chapter to assess motor deficits demonstrated no robust effect of chronic peripheral VU0364770 treatment from day 4 post-lesioning at either 10mg/kg or 30mg/kg doses in either high or low concentration (7.5µg or 2.5µg) lactacystin-lesioned rats, indicating no neuroprotection-driven symptomatic improvement by delayed-start chronic VU0364770 treatment. Supporting the lack of neuroprotection-driven behavioural improvement, dopaminergic cell counts indicate that delayed-start chronic systemic treatment with 10 or 30mg/kg VU0364770 provides no neuroprotection of dopaminergic neurons in the SNc of rats unilaterally lesioned with 7.5µg or 2.5µg lactacystin.

To date, there have been no published studies investigating the neuroprotective effects of VU0364770; however, *in vivo* neuroprotection evidence exists for other mGlu4 receptor PAMs. The mGlu4 receptor PAM PHCCC provides neuroprotection in animal models of PD when infused into the GPe and when given once systemically prior to toxin administration, despite its low efficacy at the mGlu4 receptor and poor CNS exposure (Battaglia et al. 2006). Similarly, the mixed allosteric agonist/PAM VU0155041 has been shown to provide functional neuroprotection in the unilateral 6-OHDA model when given supranigrally, an effect linked in part to a reduction in the inflammatory response (Betts et al. 2012). However, comparison of the efficacy of these compounds with VU0364770 is somewhat complicated by PHCCC's antagonistic properties at the mGlu1 receptor and VU0155041's partial allosteric agonist properties at the mGlu4 receptor.

As there have been no neuroprotective studies published on this compound to date, VU0364770 doses were selected based on the antiparkinsonian findings of Jones et al. (2012) in the

haloperidol model (0.75mg/kg i.p.) of PD and its good brain exposure at 10mg/kg. However, as no antiparkinsonian effects were seen in the lactacystin model following acute dosing with VU0364770 up to 100mg/kg (as discussed below in 5.4.3), it is possible that the doses of 10 and 30mg/kg given in the chronic treatment studies were not high enough to adequately attenuate glutamatergic signalling at the STN:SNc synapse to prevent further damage by excitotoxicity. Similarly, the selected doses may not have been high enough to reduce inflammatory damage via attenuation of microglial activation, and perhaps there might have been detectable neuroprotection at a dose of 100mg/kg if given chronically. Additionally, pharmacokinetic data reveals a reduction in brain exposure at around 2 hours after systemic administration of VU0364770, and although it is still present in the system 6 hours after dosing (Engers et al. 2013, Jones et al. 2012), it is likely that between daily doses of VU0364770, the compound was not maintained at effective concentrations to be active in the brain. However, the financial and practical constraints of administering the compound more than once daily at high doses limited investigation into this. Also, it may be that VU0364770 is unable to provide robust neuroprotective effects in the lactacystin model due to the delay in administering the treatment, and questions the translational value of preclinical studies demonstrating neuroprotective effects of compounds when treatment prior to the induction of neurodegeneration is used.

5.4.2 Motor deficits are not reversed by acute peripheral DCPG administration in 7.5µg lactacystin-lesioned animals

Although no neuroprotection-driven improvements in motor deficits were demonstrated following chronic treatment with 10 or 30mg/kg VU0364770 in the previous study, it was important to determine if acute treatment with VU0364770 could provide symptomatic relief. However, no improvement in forelimb-use asymmetry was apparent in the vertical cylinder test following VU0364770 treatment at any dose, and analysing forelimb-use for wall exploration alone also showed no change from baseline. Similarly, no change in contralateral forelimb akinesia was detected following VU0364770 administration in the adjusted stepping test or the vibrissae-evoked forelimb placement test. Collectively, these tests demonstrate no effect of acute administration of ≤ 100 mg/kg VU0364770 on motor deficits, suggesting that VU0364770 is not effective as an antiparkinsonian agent at these doses in the lactacystin model.

VU0364770 has previously been shown to reverse drug-induced catalepsy and akinesia in rats at 10, 30 and 56.6mg/kg (Jones et al. 2012); however the lactacystin model more closely resembles the unilaterally lesioned 6-OHDA model than these transient drug-induced models. VU0364770's apparent lack of antiparkinsonian efficacy in the lactacystin model contradicts the findings of Jones et al. (2012), where 100mg/kg was found to be modestly effective (32%) at improving forelimb

asymmetry in the unilateral 6-OHDA model. Interestingly, Jones et al. (2012) reported that VU0364770's activity as a MAO inhibitor was not responsible for the modest improvement in forelimb asymmetry they detected as no change in striatal dopamine levels was found, suggesting its main action is via the mGlu4 receptor. The hypothesis that targeting the mGlu4 receptor in the BG can normalise overactive signalling within the indirect pathway in PD is well established (see section 1.6). Given its high level of presynaptic localisation at strategic points in the affected BG pathways, it stands to reason that potentiation of mGlu4 receptor activation with selective PAMs could modulate transmission and result in antiparkinsonian effects. Certainly, mGlu4 receptor selective agonists have been shown to reduce signalling at corticostriatal, striatopallidal and subthalamonigral synapses, confirming the receptor's key role here (Beurrier et al. 2009, Cuomo *et al.* 2009, Valenti et al. 2003). Additionally, the mGlu4 receptor PAM PHCCC reverses reserpine-induced akinesia in rats when administered centrally and systemically (Battaglia et al. 2006, Broadstock et al. 2012, Marino et al. 2003). Similarly, the mixed allosteric agonist/PAM VU0155041 demonstrates antiparkinsonian activity in models of PD when injected intracranially (Betts et al. 2012, Niswender et al. 2008a). However, comparison of the efficacy of these compounds with VU0364770 is somewhat complicated by PHCCC's mGlu1 receptor antagonistic properties and VU0155041's partial allosteric agonist properties at the mGlu4 receptor.

The disparity between the present study's findings and the findings of Jones and colleagues (2012) may be the result of using different types of toxin-based models of PD; 6-OHDA selectively kills dopaminergic neurons, whereas lactacystin is a non-selective proteasome inhibitor, and can cause dysfunction of other cell populations (see section 1.3.2), perhaps worsening the pathology. Additionally, Jones and colleagues used 6-OHDA to lesion the MFB, which induces a less severe loss of SNc dopaminergic neurons than when injected directly into the SNc (see section 1.3.2). However, Jones et al. did not quantify the loss of nigral dopaminergic neurons in their study, only the loss of striatal dopamine (>99%), so the models cannot be directly compared here, as striatal dopamine levels are found to decrease prior to dopaminergic neuronal cell death (Deumens et al. 2002). Thus, the severity of the lesion following lactacystin administration into the SNc in the present study could potentially limit the detection of a moderately effective antiparkinsonian treatment. Furthermore, the assessment and analysis of forelimb asymmetry by Jones et al. in the 6-OHDA model differed slightly from that used in this study; exposure to a conical-shaped vertical cylinder was assessed for 10 minutes by Jones and colleagues, almost twice as long as in the present study, and a composite measure of forelimb usage for wall contacts and landing (but not pushing-off) was calculated as in Lundblad et al. (2004). This method of analysis resulted in more surface contacts to assess, perhaps allowing smaller changes in behaviour to be distinguished. However, it is likely that the battery of

behavioural tests used in the present study would have detected any changes in motor deficits following VU0364770 administration. It is possible that the route of administration of VU0364770 altered its efficacy; in the present study, difficulties with solubilisation of the compound in saline alone resulted in a larger volume of drug mixed with possible irritants to the skin, necessitating i.p. administration. In comparison, Jones and colleagues administered the compound s.c., perhaps causing some minor differences in body distribution and rate of excretion. However, it is likely that i.p. administration would actually reach circulation faster than s.c. administration, and the compound would have more of an effect, suggesting the administration route used in this Chapter likely played no role in VU0364770's lack of efficacy.

Importantly, systemic administration of selective mGlu4 receptor PAMs alone has not provided robust antiparkinsonian activity comparable to that seen with the novel mGlu4 receptor selective agonist LSP1-2111 or classic L-DOPA in more disease-relevant dopamine depletion models such as the 6-OHDA model (Bennouar et al. 2013, Beurrier et al. 2009, Jones et al. 2012, Le Poul et al. 2012). This raises doubts about mGlu4 receptor PAM efficacy at key BG synapses. Indeed, PHCCC, VU0155041 and Lu AF21934 all fail to significantly reduce IPSCs in the GP (Bogenpohl *et al.* 2012, Gubellini *et al.* 2014, Marino et al. 2003), suggesting mGlu4 receptor PAMs are not efficacious at the striatopallidal synapse when administered alone. These findings question the hypothesis that mGlu4 receptor PAMs alone can improve motor symptoms, in particular by inhibiting GABAergic signalling at the striatopallidal synapse. Recent findings by Gubellini et al. (2014) suggest that the endogenous concentrations of glutamate within the GP in both physiological and disease states may not be sufficiently high to provide the levels of mGlu4 receptor stimulation required for PAM activity. Indeed, the mGlu4 receptor PAM Lu AF21934 only provides an inhibitory effect in the GP following a significant increase in synaptic glutamate levels caused by glutamate transporter blockade (Gubellini et al. 2014). It may be that PAM activity at the corticostriatal or subthalamonigral synapse is more effective due to higher endogenous glutamate levels; however, this may not be enough to translate directly into symptomatic improvement. It is possible therefore that in the lactacystin model the basal levels of synaptic glutamate are not high enough to permit VU0364770 to modulate activity at key BG synapses in order to provide symptomatic improvement.

Although Jones and colleagues demonstrated a fairly small efficacy of VU0364770 in PD models alone, they showed a far greater effect on motor deficits when the compound was given alongside an ineffective dose of L-DOPA (Jones et al. 2012). Targeting the mGlu4 receptor with other selective PAMs such as ADX88178 and Lu AF21934 has been shown to provide antiparkinsonian effects in models of PD, but only when given in conjunction with L-DOPA or an mGlu4 receptor agonist (Bennouar et al. 2013, Gubellini et al. 2014, Le Poul et al. 2012). Recent reports have demonstrated

synergistic effects resulting from a combination of orthosteric agonists with mGlu4 receptor PAMs (Bennouar et al. 2013, Kłak et al. 2007, Valenti et al. 2005), as well as potentiating other treatments such as L-DOPA, adenosine A2A antagonists and dopamine receptor agonists (Jones et al. 2012, Le Poul et al. 2012). These findings strongly suggest that mGlu4 receptor PAMs may be more effective as a combination therapy with low doses of L-DOPA or orthosteric agonists, with the added therapeutic potential to reduce L-DOPA induced dyskinesia experienced by patients after years of increasing L-DOPA treatment (Bennouar et al. 2013).

5.5 Conclusions

The findings in the studies described in this chapter demonstrate that delayed-start chronic peripheral VU0364770 administration up to 30mg/kg dose provides no neuroprotection against lactacystin toxicity, with no measurable neuroprotection-driven attenuation of motor deficits or histological protection of dopaminergic neurons in the lesioned SNc. Additionally, acutely administered VU0364770 demonstrates no antiparkinsonian effects, indicating VU0364770 cannot modulate BG pathways sufficiently to attenuate motor deficits in the lactacystin model. Differences in experimental assessment and animal model may account for the disparity between the modest antiparkinsonian effects demonstrated by Jones et al (2012) and the lack of antiparkinsonian activity found here. However, this study and several others question the efficacy of mGlu4 receptor PAMs within the BG when administered alone, and it may be that they are more effective as antiparkinsonian and neuroprotective agents when given as a combination therapy.

Chapter 6: General Discussion

6.1 Overview

Although current treatments such as dopamine replacement therapy are able to ameliorate the symptoms in the early phases of PD, as the disease progresses and the remaining population of functional dopaminergic neurons that mediate their effects are lost, they become less effective. Patients often develop debilitating side effects such as LIDs and on-off motor fluctuations. There is currently a significant unmet need for disease modifying or neuroprotective drugs to slow the rate of disease progression and provide long-term symptomatic relief. Novel therapeutics that can provide symptomatic relief whilst attenuating the ongoing neurodegeneration are therefore required. The targeting of mGlu receptors has become a therapeutic focus in recent years, demonstrating modulatory effects on neurotransmission and yielding symptomatic improvements combined with significant neuroprotective properties (Hovelsø *et al.* 2012), as well as more favourable safety and tolerability profiles than drugs targeting iGlu receptor types. Group III mGlu receptors were the focus of this thesis as they currently hold the most therapeutic promise for PD, with evidence suggesting activation of these receptors not only modulates aberrant neurotransmission in the BG to provide symptom relief, but also provides neuroprotective effects in the nigrostriatal system (Hovelsø *et al.* 2012). However, the specific receptor subtypes responsible for these effects and their underlying mechanisms of efficacy are still being determined. Recent advances in the development of subtype-selective and potent ligands that are able to cross the BBB provides the ability to explore the variety of functions associated with targeting individual group III mGlu receptor subtypes in a clinically relevant manner. The overarching hypothesis of this thesis was that targeting specific group III mGlu receptors may hold therapeutic promise in PD, both by modulating aberrant neurotransmission in the basal ganglia to provide symptom relief and by providing neuroprotective effects in the nigrostriatal system through a variety of mechanisms including attenuation of inflammatory-induced damage. In order to test this hypothesis, the antiparkinsonian activity and neuroprotective effects of group III mGlu receptor subtypes were investigated using novel selective ligands in the lactacystin model of PD, and their putative underlying mechanisms of efficacy were explored. Many classical rodent models of PD have failed to display one of the key neuropathological features of PD: protein accumulation and aggregation. In this thesis, the lactacystin model was employed as it exhibits the cardinal features of PD including protein accumulation and progressive neuronal degeneration. Additionally, to ensure the treatment would be more clinically applicable, a delayed-start-to-treatment design following lesioning was utilised. Chronic dosing with selective ligands was assessed to determine their neuroprotective efficacy against the toxicity of lactacystin, whilst acute dosing with the same ligands was assessed to determine their ability to alleviate the motor deficits in the lactacystin model of PD.

6.2 Consolidation of findings

6.2.1 Lack of efficacy of the mGlu8 receptor agonist DCPG in models of Parkinson's disease

Selective activation of the mGlu8 receptor has previously been demonstrated to have antiparkinsonian activity in rodent models with prolonged dopamine depletion (Johnson et al. 2013), and is neuroprotective in animal models of epilepsy and anxiety (Moldrich et al. 2001, Schmid & Fendt 2006), but there has been no published evidence demonstrating the neuroprotective effects of targeting the mGlu8 receptor in any *in vivo* model of neurodegeneration. The hypotheses of these studies was that activation of the mGlu8 receptor with the mGlu8 selective agonist DCPG would provide neuroprotection and symptomatic relief in the lactacystin rat model of PD, and provide protection of SNc dopaminergic neurons against inflammatory-mediated damage via attenuation of microglial pro-inflammatory activation. In order to test this, DCPG was assessed for its antiparkinsonian and neuroprotective potential in the lactacystin model using a more clinically relevant delayed-start-to-treatment experimental design and peripheral administration. Additionally, the ability of DCPG to provide neuroprotection *in vivo* against inflammatory-induced neurodegeneration was examined, and its ability to attenuate microglial activation induced by LPS was investigated as a potential neuroprotective mechanism of action. Delayed-start chronic peripheral DCPG administration resulted no histological neuroprotection or attenuation of motor deficits in lactacystin-lesioned animals, providing no evidence of neuroprotection by DCPG in the lactacystin model of PD. Additionally, DCPG demonstrated no antiparkinsonian/symptomatic effects when administered acutely, indicating it cannot modulate the abnormal signalling in BG pathways sufficiently to attenuate motor deficits in the lactacystin model.

When investigated using the LPS model, chronic DCPG treatment again demonstrated no effect on motor behaviour or cell death, however a trend towards a small degree of neuroprotection was apparent (~24%), although no statistical significance could be detected, likely due to the underpowering of these experiments as discussed in Chapter 4. If further experiments confirm that the neuroprotective trend apparent in the LPS model is true neuroprotection, these results suggest that DCPG may protect against inflammatory-induced neurodegeneration in the LPS model, preventing inflammatory-mediated damage of neurons, but is not effective in the lactacystin model. However, DCPG treatment was not associated with attenuation in the number of activated microglia or macrophages observed in the lesioned SNc *in vivo* in either the lactacystin or LPS models, nor did it reduce LPS-induced activation of primary microglia in culture, indicating that DCPG does not reduce microglial activation as initially hypothesised, but could perhaps act through another unknown mGlu8 receptor-mediated mechanism protecting against inflammatory damage in the LPS model such as via

astrocytes or directly on neurons. The numbers of activated microglia within the SNc remained unchanged following DCPG treatment in lactacystin model, likely due to the lack of DCPG-induced neuroprotection detected, and therefore no reduction in the cellular damage and debris that microglia react to. In contrast, the increased neuronal survival and corresponding reduction in cellular damage and debris observed previously in the 6-OHDA model may account for the reduction in activated microglial numbers in this model. Furthermore, the lack of attenuated microglial activation observed in the LPS and lactacystin models in this thesis compared to the apparent reduction in microglial activation in the 6-OHDA model previously demonstrated in our lab could be due to differences in the mechanism of microglial activation by the different lesioning toxins. The indirect microglial activation that occurs in response to 6-OHDA lesioning may be easier to resolve than the direct, more aggressive activation that occurs in response to LPS. In the 6-OHDA model, microglia are activated via the disruption of the CD200R neuronal-microglial linkage and activators such as ATP and metalloproteinases (MMPs) being released by distressed neurons (Walker & Lue 2013, Kim *et al.* 2007), which DCPG may be able to attenuate more easily. In contrast, LPS directly activates pattern receptors on microglia, particularly TLR4, triggering a robust proinflammatory response that may be harder to dampen than activation via alternative pathways. This could explain the lack of attenuation in microglial activation in the LPS model in this thesis compared to the attenuation of microglial activation in the 6-OHDA model previously shown. Whether DCPG treatment could result in decreased microglial activation when activated via a different pathway is questionable, but this could be tested in microglial cultures activated via MMP9 or MMP3 rather than TLR activation by LPS.

Although there is no clear evidence for neuroprotection by DCPG in the studies described in this thesis, the trend towards neuroprotection detected in the LPS model combined with the neuroprotective evidence from other studies discussed previously suggests that, if DCPG is neuroprotective in the LPS model following further higher powered studies, it may be acting on astrocytic activation or via mGlu8 receptors on neurons directly to provide neuroprotection. As discussed in Chapter 4, activation of mGlu8 receptors may increase neuronal resilience to inflammatory-mediated damage by acting directly on anti-apoptotic signalling pathways or intracellular cell survival signalling pathways within neurons, or may act indirectly by increasing astrocyte release of neurotrophic factors such as GDNF, uptake of glutamate and/or reduction of ROS via increased production of antioxidants like GSH.

6.2.2 Lack of efficacy of the mGlu4 receptor PAM VU0364770 in the lactacystin model of Parkinson's disease

In contrast to the limited publications available on the efficacy of targeting the mGlu8 receptor in PD, the mGlu4 receptor has been demonstrated in numerous studies to be the most promising subtype of the group III mGlu receptors so far, displaying both antiparkinsonian and neuroprotective effects in various models of PD (MacInnes et al. 2004, Marino et al. 2003, Valenti et al. 2003). Additionally, with the development of subtype selective PAMs, investigating the effects of selectively targeting the mGlu4 receptor is now possible. PAMs are more selective for mGlu receptor subtypes due to their allosteric binding sites being more distinct than the orthosteric site. Additionally, due to their lipophilic nature, PAMs cross the BBB more easily than agonists so can be administered peripherally. However, they require binding of glutamate at the orthosteric site before an effect can be elicited. The novel, selective and brain permeable mGlu4 receptor PAM VU0364770 was used in this thesis to elucidate the value of allosteric modulation of the mGlu4 receptor in PD, and to determine if the compound could reproduce the antiparkinsonian effects demonstrated by Jones et al. (2012) as well as provide neuroprotection in the lactacystin model of PD. Other selective PAMs targeting the mGlu4 receptor have previously been shown to provide both symptomatic relief and neuroprotective effects in classical models of PD, and although VU0364770 is a recently characterised, novel compound with improved specificity and potency for the mGlu4 receptor, its neuroprotective potential has not been investigated until this point. The hypothesis of this study was that activation of the mGlu4 receptor with the mGlu4 selective PAM VU0364770 could provide neuroprotection and symptomatic relief in the lactacystin rat model of PD. To test this, chronic dosing with VU0364770 using a more clinically relevant delayed-start-to-treatment experimental design was assessed to see whether it was neuroprotective against the toxic effects of lactacystin. Additionally, acute dosing with VU0364770 was assessed to see whether it was able to alleviate the motor deficits in the lactacystin PD model, as previously demonstrated in the 6-OHDA model. However, no neuroprotective or antiparkinsonian effects were observed following treatment with VU0364770 in the lactacystin model.

Many published studies using the mGlu4 PAM PHCCC have demonstrated neuroprotection against classical toxins modelling PD, making the finding that VU0364770 had no effect against lactacystin in this thesis somewhat unexpected. Moreover, the finding that VU0364770 was recently shown to possess antiparkinsonian properties in other models of PD (Jones et al. 2012) but that this was unable to be replicated in the lactacystin model in this thesis raises important questions about the model itself. As previously discussed in Chapter 5, the disparity between the antiparkinsonian behavioural findings by Jones et al. (2012) and the lack of them in this study could also be attributed to the slight differences in experimental design, however given the additional finding of no

antiparkinsonian efficacy of DCPG in the lactacystin model in this project, it seems more likely that these differences are due to the model itself.

6.3 Different models of PD yield differing results

The findings in this thesis question the efficacy of acutely modulating signalling within the BG with mGlu4 receptor PAMs and mGlu8 receptor agonists. However, as both of these selective compounds have previously demonstrated antiparkinsonian efficacy in the 6-OHDA model at the same doses as those used in this project, these findings indicate a clear and fundamental difference between the models themselves. The non-specificity of lactacystin's toxicity and the large lesions observed in this thesis compared to 6-OHDA's selectivity for catecholaminergic neurons is likely to be a key factor in this disparity. The effects of the loss of both dopaminergic and non-dopaminergic signalling in response to lactacystin lesioning may have a very different impact on downstream BG activity than the loss of dopaminergic neurons alone. Lactacystin-induced damage has been shown to spread to extranigral brain regions; indeed, a clear loss of TH+ VTA neurons adjacent to the lesioned SNc was visible in this thesis, and research within our lab has shown a loss of approximately 20% of TH+ VTA neurons following smaller nigral lesions than those induced in this thesis (Harrison. I, unpublished observation, 2014). Lactacystin lesioning may also lead to a secondary neuropathology, as animals administered 10µg lactacystin into the L-MFB have demonstrated a thinning of the primary motor cortex alongside decreases in thalamic and striatal volume (Vernon et al. 2011). These structural changes, whether induced by a functional reorganisation of circuitry following dopamine depletion or by direct toxin damage, are likely to impact behaviour and may fundamentally differ between models of PD, depending on the toxin used and the location of administration. This may explain the antiparkinsonian effects of both DCPG and VU0364770 in both 6-OHDA and temporary drug-induced dopamine depletion models (Johnson et al. 2013, Jones et al. 2012), but its lack of detectable antiparkinsonian efficacy in the lactacystin model in this thesis. Indeed, there is some recent evidence suggesting that the BG pathways and behaviour are affected differently between the 6-OHDA and lactacystin models. Konieczny et al. (2014a) detected differences in rotational behaviour between 6-OHDA and lactacystin lesioned rats in response to L-DOPA, with acute L-DOPA treatment inducing rotational behaviour in the 6-OHDA model, but not in the lactacystin model. Additionally, although repeated L-DOPA treatment did induce contralateral rotations in both of the models, it was much less effective in the lactacystin model. The researchers ruled out any differences in dopamine depletion levels and found comparable increases in levels of L-DOPA in the SN and striatum between models, and suggested the difference in behavioural response to L-DOPA was due to the distinctly different mechanisms of neurodegeneration evoked by the toxins. They then extended their research further

to demonstrate by stereological analysis that whilst nigral dopaminergic neurons are particularly vulnerable to its toxicity, lactacystin also induces a partial lesion of non-dopaminergic nigral neurons, with a clear impact on motor behaviour (Konieczny *et al.* 2015). They established that alongside dopaminergic neuronal loss, nigral GABAergic neurons are also lost, with slightly reduced levels of GABA found in the SN of lactacystin lesioned rats compared to no change in 6-OHDA lesioned rats. The stereological cell counts performed in this thesis support these findings; a consistently greater loss of Nissl+ cells than TH+ cells following lactacystin lesioning was apparent when considering neuronal population size, with approximately 10-20% of non-dopaminergic Nissl+ neurons lost, confirming that a small population of non-dopaminergic neurons in the SNc are killed by lactacystin also. When treated intranigally with muscimol, a GABA_A agonist, lactacystin-lesioned rats rotated in a contralateral direction significantly less than 6-OHDA-lesioned rats (Konieczny *et al.* 2015), further confirming a clear difference in motor behaviour between models. It therefore seems likely that the disparity in antiparkinsonian activity by DCPG and VU0364770 between this project and other publications is due to the differences in the toxins used to model PD.

The neuroprotection that has been demonstrated by DCPG *in vitro* following MPP+ exposure (Jantas *et al.* 2014), *in vivo* in the 6-OHDA model of PD previously shown in our lab (Chan H., unpublished observation, 2010), and the possible hint towards it in the LPS model in this thesis compared to the findings of no neuroprotection by DCPG in the lactacystin model suggests a fundamental difference in the mechanisms of neurodegeneration between these more classical toxins and lactacystin. 6-OHDA mediates cell death primarily via OS, iron accumulation and mitochondrial dysfunction (Sachs & Jonsson 1975), and cell death is exacerbated by the localised inflammatory response. LPS toxicity is entirely mediated by inflammatory damage, and acute MPTP or MPP+ toxicity is mediated via mitochondrial dysfunction and OS. However, none of these models acutely induce proteasomal dysfunction or protein accumulation or aggregation, key factors in the pathogenesis of PD. In contrast, lactacystin treatment induces irreversible UPS inhibition leading to an accumulation of cytotoxic proteins and gradual cell impairment, alongside mitochondrial dysfunction and oxidative stress, resulting in increased apoptosis and necrosis of cells (Zhou *et al.* 2010, Fenteany & Schreiber 1998). The lack of neuroprotection by DCPG seen in the lactacystin model in this thesis may be due to DCPG's inability to protect neurons against this irreversible proteasome inhibition, the primary inducer of cell death in this model. Additionally, if the potential neuroprotective effects of DCPG are mediated via astrocytes as we have previously hypothesised, their vulnerability to lactacystin is also a key factor that may limit DCPG's neuroprotective capabilities in this model. Importantly, numbers of astrocytes have been shown not to be significantly altered by lactacystin in doses up to 20µg *in vivo* (McNaught *et al.* 2002b), suggesting lactacystin toxicity does not induce astrocytic death. Instead,

lactacystin could perhaps prevent them from providing effective neuroprotection, despite mGlu8 receptor stimulation. Indeed, lactacystin can alter astrocytic morphology, causing a reorganisation of the cytoskeleton and inducing a morphological change from a flattened polygonal shape to a stellate morphology, as well as decreasing proliferation (Ren *et al.* 2009). Astrocytic morphology is increasingly believed to correspond to their function (Lei *et al.* 2008, März *et al.* 2007), so it is possible that lactacystin could limit or inhibit astrocytic-derived neuroprotective functions induced by DCPG. DCPG's potential neuroprotective actions, if mediated via astrocytes, could be inhibited by lactacystin-induced astrocytic proteasomal dysfunction, but perhaps not by the indirect neurotoxin LPS or the selective neurotoxins 6-OHDA and MPP+. This would explain the lack of neuroprotection induced by DCPG observed in the lactacystin model compared to the potential trend towards protection seen in the LPS model in this thesis, and the more substantial protection in the 6-OHDA model observed in previous studies by our group.

The original hypothesis of this thesis was that targeting the mGlu8 receptor with DCPG and the mGlu4 receptor with VU0364770 could reduce lactacystin-induced neuroinflammatory damage in order to provide neuroprotection, most likely by attenuating microglial activation and its associated detrimental effects. However, the studies in this thesis indicate that activation of the mGlu4 or mGlu8 receptors with these selective compounds is unable to provide any neuroprotection in the lactacystin model of PD. Given that the broad group III mGlu receptor activating compounds L-AP4 and RS-PPG can reduce LPS-induced microglial activation and the associated neurotoxicity *in vitro* (Taylor *et al.* 2003), and the findings in this thesis that DCPG does not attenuate microglial activation in either the lactacystin or LPS models, it can be reasonably surmised that the mGlu4 receptor is the likely candidate for mediating the effects of these compounds on microglia, especially as rat microglia do not express the mGlu7 receptor (Taylor *et al.* 2003). The fact that the mGlu4 PAM VU0364770 does not provide any neuroprotection in the lactacystin model may be due to the particular mechanisms driving neurodegeneration, and it remains to be seen if VU0364770 is neuroprotective in other more classical models of PD. As the inflammatory response induced in the lactacystin model is secondary to proteasome inhibition, it may be that the main purpose of the microglial activation observed in the SNc is simply to clear dead cells and debris, and hence does not have a central role in driving the degenerative process. Indeed, recent studies in our research group have shown that there is a resolution of inflammation, as measured by microglial activation, by 5 weeks post-lactacystin lesioning, once cell death and clearance of debris has occurred (Harrison. I, unpublished observation, 2014). Additionally, if neuroinflammatory-induced neuronal damage is limited in the lactacystin model, the role of glutamate in this model will also be small, as it is released by detrimental pro-inflammatory M1-like activated microglia (Piani *et al.* 1992). In contrast, in the 6-OHDA model, both

the innate inflammatory response and glutamate release may help to drive the neurodegenerative process and hence the model may be more responsive to DCPG or VU0364770. If correct, this theory would indicate that anti-inflammatory compounds that are effective in the classical toxin models of neurodegeneration would not provide any neuroprotection in the lactacystin model. In fact, a recent study by Konieczny *et al.* (2014b) using a lactacystin-lesioned rat model observed no neuroprotective effects of Celastrol, a compound with potent anti-inflammatory and anti-oxidant properties previously shown to be neuroprotective in MPTP and genetic models of PD (Cleren *et al.* 2005, Faust *et al.* 2009, Allison *et al.* 2001). Celastrol has a narrow therapeutic window, but is neuroprotective in the MPTP model at 3mg/kg (Cleren *et al.* 2005). However, when given at the same dose, this anti-inflammatory compound could not induce neuroprotection under conditions of UPS inhibition by lactacystin in the rat brain, and in fact worsened the nigrostriatal degeneration. The authors suggested that this was due to fundamental differences between the models of PD, and that Celastrol's additional actions as a mild proteasome inhibitor likely worsened the ongoing UPS inhibition. Importantly, this study demonstrates that the anti-oxidant and anti-inflammatory properties of Celastrol were unable to protect against proteasome impairment.

6.4 Implications of findings

Collectively, the findings in this thesis demonstrate that the mGlu8 receptor agonist DCPG is unable to protect against lactacystin-induced UPS inhibition, nor does it provide robust antiparkinsonian effects in this model. The mGlu4 receptor PAM VU0364770 similarly demonstrates no antiparkinsonian or neuroprotective activity in the lactacystin model, despite being moderately effective on motor deficits in the 6-OHDA model in other labs. These findings are of importance as they suggest that the toxin selected to model PD and its mechanisms of neurodegeneration has a large impact on the neuroprotective and antiparkinsonian efficacy of a drug. Intranasally administered lactacystin may more closely recapitulate the pathogenesis of human PD than other classical toxin models do, demonstrating both the characteristic protein accumulation and aggregation that other acute toxin models do not develop alongside a more progressive neurodegeneration. Additionally, the non-specificity of lactacystin toxicity for non-dopaminergic neurons may more accurately model the pathological changes observed in PD than selective toxins like 6-OHDA, as although dopaminergic neurons do seem to be preferentially lost in both the lactacystin model and in PD, non-dopaminergic systems are also affected. Furthermore, the astrocytic dysfunction that may occur following lactacystin exposure is a feature of many neurodegenerative diseases including PD, with post mortem studies demonstrating the presence of process-bearing stellate astrocytes in the SN of PD patients

(Ferrer & Blanco 2000). However, the effects of lactacystin on glial morphology and function should be further elucidated, as there has thus far been limited research into this.

Substantial neuroprotection and neurorestoration have been previously demonstrated *in vivo* in the lactacystin model, with compounds such as histone deacetylase inhibitors (HDACIs) (Harrison. I, 2014, manuscript in preparation), MAO-B inhibitors (Zhu *et al.* 2008) and iron chelators (Zhu *et al.* 2007) all showing efficacy in this model. Additionally, antiparkinsonian activity has been demonstrated in the lactacystin model following treatment with L-DOPA, albeit a weaker response compared to that seen in the 6-OHDA model (Konieczny *et al.* 2014a). All of this data indicates that other mechanisms can and have been targeted to result in antiparkinsonian or neuroprotective outcomes in the lactacystin model. However, there is obviously a clear lack of knowledge regarding the downstream effects of lactacystin lesioning and its impact on signalling in the BG. It is possible that the lactacystin model may more completely reproduce PD pathology than other models due to its accumulation of aberrant proteins to induce cell death, and the very limited or complete lack of neuroprotection and antiparkinsonian activity afforded by DCPG and VU0364770 treatment in the lactacystin model in this thesis brings sharply into question whether group III mGlu receptors are truly suitable targets for the treatment of PD. Furthermore, although a slight trend towards neuroprotection by DCPG was observed in the LPS model in this study, a much more robustly significant neuroprotection would be required if it is to translate into any real clinical benefit in humans. Indeed, no compounds targeting any of the group I and II mGlu receptor subtypes that have been shown to be neuroprotective in rodent or primate models of PD have yet been successfully translated into the clinic (Duty & Jenner 2011, Finlay & Duty 2014). A few mGlu5 receptor antagonists and NAMs have reached clinical trials focussed on treating LIDs, with some demonstrating anti-dyskinetic efficacy. However, all of the compounds had unacceptable side effects at the doses used, necessitating further research and development (Amalric 2015, Rascol *et al.* 2014, Picconi & Calabresi 2014). Clinical trials of group III mGlu receptor targeting compounds are yet to be carried out.

6.5 Limitations of these studies

There are general limitations to using toxins to model PD in rodents and how well they recapitulate the true pathogenesis of human PD. The LPS model is controversial as it only reproduces one aspect of PD pathology, and neuroinflammation may not be the primary mediator of neurodegeneration in PD. However, it was a valuable tool to investigate the anti-inflammatory mechanisms of DCPG in this thesis. Similarly, UPS inhibition by lactacystin may not represent the true initial trigger of neurodegeneration in PD. Direct injection of toxins into the brain at high concentrations means that toxin exposure is acute and leads to a rapid destruction of dopaminergic neurons rather than

progressive degeneration. Even the lactacystin model, which demonstrates a slower, more progressive cell loss compared to 6-OHDA and acute MPTP models, induces neurodegeneration far faster than the progressive decline seen in human PD over decades. Additionally, toxin administration is performed in young animals, which does not reflect the aged brain affected in PD. Furthermore, the physically disruptive nature of a stereotaxic injection likely contributes to the inflammation and cell death seen in the brain for at least several weeks post-surgery, so degeneration cannot be completely attributed to the toxin itself, and sham lesioned animals would have been a useful addition to investigate this. There are further limitations of using stereotaxic surgery, as individual differences between animals makes it difficult to control exactly where the toxin is injected despite using the same coordinates, and the accuracy and degree of toxin exposure to the target region of the brain is unknown until brain tissue is later histologically studied. In this project, this led to expensive drug treatments being administered to animals that were not accurately lesioned in the SNc, necessitating later exclusion from analysis.

The spreading of lactacystin cytotoxicity to areas far from the injection site (Vernon et al. 2011) and its non-specific toxicity are hard to limit when an effective lesion of the nigrostriatal pathway is required. A substantial cell loss in the VTA and the SNr was observed in lactacystin lesioned animals in this project, suggesting a spreading of lactacystin toxicity to adjacent neuronal populations. This finding is supported by previous research by our group and others (I. Harrison 2014 in press, Vernon et al. 2010). The neighbouring dopaminergic neurons of the VTA are relatively preserved in human PD compared to the loss seen in the SN (Sulzer 2007), although whether the degree of neuronal loss in the VTA following lactacystin lesioning mirrors that of human PD or is far greater needs to be further explored. Damage to extranigral regions is thought to impact behaviour (Vernon et al. 2011), and the loss of neurons in the SNr may have contributed to the variability in motor dysfunction following lactacystin lesioning in this thesis. Cell loss in the SNr caused by lactacystin may alter the signalling in the BG and paradoxically improve behavioural deficits by rebalancing aberrant signalling from the output nuclei. This would make testing for improvements in motor impairment difficult, and may explain why motor deficits in some animals appeared not to worsen significantly following 7.5µg lactacystin lesioning, yet an accurate lesion placement and large degree of neuronal loss in the SNc was later confirmed histologically. It is also interesting that 2.5µg lactacystin was unable to induce the degree of partial lesion we expected, or increase the level of neuroprotection induced by DCPG, suggesting cell death induced by lactacystin is non-linear. This is despite other groups claiming dose-dependent results with intranigral lactacystin (Mackey et al. 2013), and suggests the model may not be as reproducible between groups as other toxin-based models are.

Behavioural testing for studies was very time consuming as much handling was needed to ensure rats could be used in forced behavioural tests such as the adjusted stepping test without stress impacting on their behaviour. Additionally, following lesioning, rats often became akinetic, no longer exploring their surroundings and making observational tests such as the vertical cylinder test and spontaneous rotations test difficult to carry out, with some animals unable to be assessed in these tests due to their lack of behaviour. Akinetic motor symptoms have been demonstrated in other rodent models of PD, and have been associated with a substantial reduction in striatal dopamine levels (Sedelis *et al.* 2001). Limited motor deficit development was observed in 2.5µg lactacystin lesioned rats and LPS lesioned rats, likely due to the short time course of the experiment and the limited neuronal death, as this may not have been long enough for rats to develop measurable motor deficits. However, extended studies would have required prolonged drug treatment and therefore much higher experimental costs. A more robustly significant antiparkinsonian effect of DCPG may have been detected if a larger number of animals had been used in behavioural testing, as behavioural data is often highly variable and subtle effects of compounds can be missed if experiments are underpowered. As the initial experimental group size was chosen based on TH+ cell counts from previous studies within our lab in the lactacystin model (n=7 per group), it is likely that all experiments in this project were somewhat underpowered to detect more subtle behavioural improvements. Indeed, looking at the data retrospectively, for a 20% improvement in contralateral forelimb use in the vertical cylinder test in 7.5µg lactacystin lesioned animals, a sample size of 9 animals per treatment group would be required to detect an effect, providing >80% power and a significance level of 5%. Furthermore, to detect a 20% improvement in rotational asymmetry induced by amphetamine, a sample size of 25 animals per treatment group would have been required. Again, this was limited by high experimental costs and time. Importantly, whilst it is likely that the study was underpowered to statistically detect neuroprotection by DCPG in the LPS model, as discussed in Chapter 4, the lack of antiparkinsonian and neuroprotective effects by DCPG and VU0364770 in the lactacystin model suggests that these compounds were not effective in this model, and an increase in sample size would not have changed this outcome. The use of primary cell culture in this project was necessary to further examine the potential mechanisms of DCPG's efficacy in isolation. It is a useful technique as many variables can be controlled such as the concentration and length of exposure to toxins and test compounds. However, the environment for cells is artificial, so they may behave very differently compared to the *in vivo* environment. Indeed, neurons are thought to have a modulatory effect on glia, as microglial reactivity to LPS can be suppressed by contact with neurons (Chang *et al.* 2001). Additionally, cell culture medium might not reflect the true environment that cells are exposed to in disease states, such as increased levels of ROS. Although mGlu8 receptors are present on microglia,

expression of the receptor may be age dependent, and immature primary microglia derived from foetal rat brain tissue may not accurately represent the susceptibility to toxin exposure or sensitivity to drug compounds of mature human microglia in PD.

6.6 Directions for future research

In order to extend the research in this thesis, it would be interesting to further investigate DCPG's effects in the LPS model with a larger sample size, and if neuroprotection is confirmed statistically, to elucidate DCPG's mechanisms of neuroprotection in this model. Investigating DCPG's effects on astrocytes exposed to LPS *in vitro* by quantifying growth factor and neurotrophic factor release in the cell culture medium using ELISAs, as well as both released and cellular GSH levels, may provide further insight into DCPG's mechanisms of neuroprotection against inflammatory-mediated damage. Another aspect that could be explored is the co-administration of DCPG with L-DOPA or other mGlu receptor targeting compounds. This could potentially result in synergistic effects, enhancing potential neuroprotection or reversing motor deficits. In terms of extending the research into the mGlu4 receptor PAM, it would be interesting to establish whether systemically administered VU0364770 can provide any neuroprotection in the classical models of PD where it could not in the lactacystin model. However, it is important to be realistic about the value of pursuing further research into the potential neuroprotective and antiparkinsonian effects of targeting group III mGlu receptors; it would only be worthwhile pursuing targets that can provide dramatic neuroprotection in models of PD, so that they have any chance of translating into a clinically significant outcome. The combined research in this thesis suggests that DCPG and VU0364770 are unlikely to be effective disease modifying agents in the clinic, so pursuing research into their mechanisms of efficacy would perhaps waste resources that could be used to study other, more promising approaches for the treatment of PD. However, although the evidence presented in this thesis suggests that targeting the mGlu8 receptor with DCPG does not provide robust neuroprotection or relief from motor symptoms, it may be that it plays a more valuable role in treating some of the debilitating non-motor symptoms often present in PD. Indeed, mGlu8 receptor knockout mice demonstrate an anxiety-like phenotype (Duvoisin *et al.* 2005, Linden *et al.* 2002), and DCPG has been shown to be effective as an anxiolytic in mice (Duvoisin *et al.* 2010), as well as in a rat model of chronic pain (Palazzo *et al.* 2008). DCPG may therefore be more effective as an adjunct therapy for treatment of some of the non-motor symptoms of PD, and its effects on features of PD such as anxiety, depression and chronic pain could be explored behaviourally in models of PD, as many demonstrate both non-motor and motor features of PD (McDowell & Chesselet 2012).

The lack of antiparkinsonian activity by VU0364770 detected in this thesis and the recent evidence demonstrating limited efficacy of other mGlu4 receptor PAMs in models of PD when

administered alone indicates that PAMs may be less effective for treatment of PD than previously thought. Additionally, no disease-modifying effects have yet been demonstrated by these compounds. However, these findings do not entirely rule out the mGlu4 receptor as a promising target in PD, and it may be that the newly developed, systemically active and highly selective mGlu4 receptor orthosteric agonists LSP1-2111 and LSP4-2022 can provide more adequate neuroprotection and antiparkinsonian effects than PAMs (Beurrier et al. 2009, Goudet et al. 2012). These compounds have shown antiparkinsonian efficacy in the haloperidol model of PD, so it would be of interest to examine whether these effects can be replicated in the lactacystin model following acute administration. Additionally, recent studies suggest that mGlu4 receptor PAMs are mainly ineffective when administered alone, but demonstrate synergistic effects on symptom reversal when combined with adenosine 2A antagonists or sub-threshold doses of L-DOPA in rodent models of PD, indicating L-DOPA sparing abilities (Bennouar et al. 2013, Gubellini et al. 2014, Le Poul et al. 2012, Jones et al. 2011). This provides the possibility of reducing the need for escalating doses of L-DOPA and decreasing the side-effects common to prolonged L-DOPA usage such as LIDs and on-off motor fluctuations (Bennouar et al. 2013, Jones et al. 2012). The novel mGlu4 receptor PAM, LuAF21934, was recently shown to reduce LID development but not severity in rats (Bennouar et al. 2013), suggesting mGlu4 receptor PAMs may prolong the benefits of current PD therapies, likely by reducing glutamatergic transmission in the corticostriatal pathway (Amalric 2015, Bennouar et al. 2013). A similar synergy resulting from a combination of mGlu receptor orthosteric agonists with mGlu4 receptor PAMs has been shown in several studies (Bennouar et al. 2013, Kłak et al. 2007, Valenti et al. 2005). In certain situations, mGlu receptors can function as heterodimers and this is thought to affect their pharmacological profile (Kammermeier 2012). For example, mGlu2/4 receptor heterodimers need to be co-bound by their respective agonists to be fully activated. As co-administration of different mGlu receptor ligands has been shown to result in synergistic effects, this heterodimerization of receptors may be one of the contributing factors to their efficacy. This finding highlights that there is still much to learn about the complexity of mGlu receptor functioning, and further studies using combinations of subtype-selective ligands for acute dosing and behavioural testing are needed, perhaps in several different models of PD.

The use of lactacystin to model PD has previously been limited to a small number of research groups (McNaught 2004, McNaught et al. 2002b, Pan et al. 2008, Vernon et al. 2010, Zhu et al. 2007), and only very recently have groups started to investigate its differences compared to other toxin-based models (Konieczny et al. 2014a). Further research to characterise the mechanisms of toxicity induced by lactacystin *in vivo* is necessary, in particular its effects on non-dopaminergic neuronal populations and corresponding brain regions, and how this impacts on signalling within the BG. This

could be explored using immunohistochemical and stereological quantification techniques as used in this thesis, as well as electrophysiological techniques. Additionally, the effects of lactacystin on non-neuronal cells should be further characterised to expand our understanding of this model. This could be carried out *in vitro* with microglial and astroglial cell lines exposed to different concentrations of lactacystin under controlled conditions, and further investigated in primary astrocytes and microglia. A variety of viability assays and examination of UPS dysfunction using an assay measuring the rate of proteolytic cleavage of a fluorophore-linked polypeptide (Calbiochem Ltd) could be used to assess proteasomal function in cells.

The decision in this thesis not to investigate the effects of targeting the mGlu7 receptor was based on the limited compounds available to target it. The mGlu7 receptor agonist AMN082 is rapidly metabolised to Met-1, a metabolite that no longer acts on mGlu7 receptors, and so was not considered for systemic administration. However, recent work indicates it can protect against excitotoxic damage (Domin *et al.* 2015), and may be worth investigating. However, other studies suggest that mGlu7 receptor activation is anxiogenic (Palazzo *et al.* 2008), potentially worsening anxiety or depression, common non-motor features of PD. Research into the effects of mGlu7 receptor activation in PD is limited until more potent and stable compounds are developed, but if drugs with improved pharmacokinetics do become available, the neuroprotective and antiparkinsonian effects of the mGlu7 receptor as a target can be further examined in models of PD.

6.7 Final conclusions

Overall, the results in this thesis reject the overarching hypothesis that targeting specific group III mGlu receptors may hold therapeutic promise in PD, both by modulating aberrant neurotransmission in the basal ganglia to provide symptom relief and by providing neuroprotective effects in the nigrostriatal system through a variety of mechanisms including attenuation of inflammatory-induced damage. However, although targeting group III mGlu receptors may not hold as much neuroprotective or antiparkinsonian potential as initially hoped, it may be that combining several mGlu receptor selective compounds that target different aspects of neurodegeneration and BG signalling will provide more effective therapies in the future. The results in this thesis highlight the importance of testing candidate compounds in a variety of PD models with differing mechanisms of neurodegeneration, as different models represent different aspects of PD neuropathology, and can therefore yield very differing results. The disparity in findings between models may provide some explanation as to why many drugs that are effective when tested in classic models of PD such as the 6-OHDA and MPTP model then fail to translate into effective compounds in the clinic. It is possible that the lactacystin model is one step closer to recapitulating the neuropathological features of PD, and provides a

platform for testing old and new compounds. However, we are limited by our current understanding of the underlying causes of the disease. It is therefore difficult to know which is the best model to use when testing potentially disease-modifying compounds; several exhibit key characteristics of PD, yet none completely recapitulate the complexity of human PD. A model that better resembles true PD pathology, its time course and behavioural symptoms would present us with a greater possibility of predicting clinical efficacy of disease modifying agents to treat PD in the future.

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Appendices

Appendix 1

Parts of this thesis include sections from *Journal of neurochemistry*, 129, pages 4-20: **Williams and Dexter (2014)** "Neuroprotective and symptomatic effects of targeting group III mGlu receptors in neurodegenerative disease" with permission from John Wiley and Sons.

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