RGS PROTEINS IN EXPERIMENTAL PARKINSONISM AND L-DOPA-INDUCED DYSKINESIA

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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Abstract

Parkinson’s disease (PD) is a progressive neurodegenerative disorder producing a clinical syndrome of bradykinesia, rigidity and resting tremor. These motor symptoms appear due to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNC) and loss of dopamine in the striatum, which subsequently leads to an imbalance of the basal ganglia motor circuit. The most effective pharmacological treatment for PD is L-3,4-dihydroxyphenylalanine (L-DOPA), the immediate metabolic precursor of dopamine, which effectively restores motor function. L-DOPA is catabolised into dopamine and replaces neurotransmitter loss in PD. However, long-term L-DOPA treatment leads to abnormal involuntary movements (AIMs), such as L-DOPA-induced dyskinesia (LID), which reduces the quality of life in PD patients. Currently, there are no reliable pharmacological treatments for these motor complications. Clinical and preclinical studies have shown that development and expression of LID is linked to unregulated dopamine release and plasticity-induced changes of striatal dopaminergic and non-dopaminergic signalling pathways. The activities of these pathways can be modulated by neurotransmitter receptors of a specific classification, the G-protein-coupled receptor (GPCR) family. In turn, GPCRs are regulated by certain endogenous proteins, the regulators of G-protein signalling (RGS) proteins. Numerous RGS protein subtypes are expressed in the striatum but their roles in PD and LID remain poorly understood. Given the modulatory function of RGS proteins in the striatum, these endogenous factors may have pathophysiological roles in the expression of motor symptoms in PD and LID. The studies presented in this thesis investigated the roles of RGS proteins in the unilateral 6-hydroxydopamine (6-OHDA)-lesioned rat model of PD and LID.

Rats received unilateral 6-OHDA lesions of the right medial forebrain bundle to induce severe dopamine denervation. L-DOPA/benserazide (6/15 mg/kg) was then administered once daily for at least 21 days to induce stable abnormal involuntary movements (AIMs).

In Chapter 2 of this thesis, increased levels of RGS2 and RGS4 mRNA were found in the rostral striatum of the unilateral 6-OHDA-lesioned rat model of LID. Moreover, elevated levels of RGS4 mRNA were specific to sensorimotor regions and positively correlated with AIMs severity. These molecular and behavioural data suggest that RGS4 proteins are involved in the expression of LID.
In Chapters 3 and 4, behavioural studies conducted in the unilateral 6-OHDA-lesioned rat model of LID showed that acute inhibition of striatal RGS4 proteins reduced the expression of AIMs and improved overall motor function. Moreover, repeated de novo treatment with RGS4 protein inhibitors, in combination with L-DOPA, attenuated the development of AIMs and reduced the overexpression of preproenkephalin-B, a molecular marker of LID. These behavioural and molecular data suggest that blockade of RGS4 proteins can reduce the induction of LID.

In Chapter 5, in vivo microdialysis conducted in the unilateral 6-OHDA-lesioned rat model of LID showed that systemic administration of RGS4 protein inhibitors, in combination with L-DOPA, attenuated unregulated striatal dopamine efflux. These data suggest that RGS4 proteins may regulate specific G-protein coupled receptors, such as 5-HT\textsubscript{1A} receptors, that modulate striatal dopamine release.

In conclusion, the work presented in this thesis shows that RGS4 proteins play a pathophysiological role in the expression and development of LID. These proteins could mediate regulation of key neurotransmitter receptors involved in LID, making them a potential therapeutic target for the development of future treatments.
Declaration

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I dedicate this thesis to my Mum and Dad, for their unconditional love and support.
Abbreviations

°C  Degrees celsius
β-ME  β-mercaptoethanol
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine  MPTP
2-AG  2-arachidonoyl-glycerol
2-DG  2-deoxyglucose
3H  Hydrogen-3
5-HIAA  5-hydroxyindoleacetic acid
5-HT  5-hydroxytryptamine/ Serotonin
6-OHDA  6-hydroxydopamine
8-OH-DPAT  (±)-8-hydroxy-2-(dipropylamino)tetralin
14C  Carbon-14
35S  Sulphur-35
125I  Iodine-125
AADC  Amino acid aromatic decarboxylase
AC  Adenylate cyclase
Ach  Acetylcholine
AEA  N-arachidonoylethanolamine/ anandamide
AIMs  Abnormal involuntary movements
Akt  Protein kinase B
ALO  Axial, limb and orolingual
am  ante meridiem
AMPA  2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid
ANOVA  Analysis of variance
AP-1  Activating protein 1
ATP  Adenosine 5'-triphosphate
Ax  Axial
BAC  Bacterial artificial chromosome
C-terminal  Carboxyl-terminal
CaMK  Calmodulin-dependent protein kinase
cAMP  Cyclic adenosine monophosphate
CB  Cannabinoid
cdk  Cyclin dependent kinase
CDS  Continuous dopaminergic stimulation
ChAt  Choline acetyltransferase
Ci  Curie
CM  Centromedian nucleus
COMT  Catechol-O-methyltransferase
cpm  Counts per minute
CREB  Cyclic AMP response element-binding protein
CREs  cAMP-response elements
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>D&lt;sub&gt;1&lt;/sub&gt;R</td>
<td>Dopamine D&lt;sub&gt;1&lt;/sub&gt; receptors</td>
</tr>
<tr>
<td>D&lt;sub&gt;2&lt;/sub&gt;R</td>
<td>Dopamine D&lt;sub&gt;2&lt;/sub&gt; receptors</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine active transporter</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>Dopamine and cyclic AMP-regulated phosphoprotein 32kDa</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep brain stimulation</td>
</tr>
<tr>
<td>DEP</td>
<td>Disheveled/ EGL-10/ Pleckstrin</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DL</td>
<td>Dorsolateral</td>
</tr>
<tr>
<td>DM</td>
<td>Dorsomedial</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DRN</td>
<td>Dorsal raphe nuclei</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECB</td>
<td>Endocannabinoid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EP</td>
<td>Entopeduncular nucleus</td>
</tr>
<tr>
<td>EPSCs</td>
<td>Excitatory post-synaptic currents</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>fmol</td>
<td>Femtomoles</td>
</tr>
<tr>
<td>Fr</td>
<td>Friedman's statistic</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>G</td>
<td>Gauge</td>
</tr>
<tr>
<td>Gβ5</td>
<td>G-protein β subunit 5</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine-nucleotide binding protein</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-amino-butyric acid</td>
</tr>
<tr>
<td>GAIP</td>
<td>G-protein α subunit-interacting protein</td>
</tr>
<tr>
<td>GAPs</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>g&lt;sub&gt;AV&lt;/sub&gt;</td>
<td>Gravitational constant</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GGL</td>
<td>Gγ protein-like</td>
</tr>
<tr>
<td>Goloco</td>
<td>G&lt;sub&gt;αi/o&lt;/sub&gt;-Loco</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>GPI</td>
<td>Globus pallidus lateral/ external segment</td>
</tr>
<tr>
<td>GPm</td>
<td>Globus pallidus medial/ internal segment</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5′-triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Guanosine-5′-O-[gamma-thio]triphosphate</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein receptor kinase</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>h</td>
<td>Hour (s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IEGs</td>
<td>Immediate early genes</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>3,4-dihydroxyl-L-phenylalanine</td>
</tr>
<tr>
<td>Li</td>
<td>Limb</td>
</tr>
<tr>
<td>LID</td>
<td>L-DOPA-induced dyskinesia</td>
</tr>
<tr>
<td>Lo</td>
<td>Locomotor</td>
</tr>
<tr>
<td>LRKK2</td>
<td>Leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mAchR</td>
<td>Muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>MAGUK</td>
<td>Membrane-associated guanylate kinase</td>
</tr>
<tr>
<td>MAO</td>
<td>Monamine oxidase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
</tr>
<tr>
<td>mg/kg</td>
<td>Milligram per kilogram</td>
</tr>
<tr>
<td>mg/ml</td>
<td>Milligram per millilitre</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>ml/kg</td>
<td>Millilitre per kilogram</td>
</tr>
<tr>
<td>MPP</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MSK-1</td>
<td>Mitogen- and stress-activated kinase-1</td>
</tr>
<tr>
<td>MSNs</td>
<td>Medium spiny neurons</td>
</tr>
<tr>
<td>MTEP</td>
<td>3-((2-methyl-4-thiazolyl)ethynyl)pyridine</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino-terminal</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NAcc</td>
<td>Nucleus accumbens core</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAsh</td>
<td>Nucleus accumbens shell</td>
</tr>
<tr>
<td>NC</td>
<td>No change</td>
</tr>
<tr>
<td>nCi/g</td>
<td>Nanocurie per gram</td>
</tr>
<tr>
<td>ng/µl</td>
<td>Nanogram per microlitre</td>
</tr>
<tr>
<td>NHPs</td>
<td>Non-human primates</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>nmol</td>
<td>Nanomoles</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxidase synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>Ol</td>
<td>Orolingual</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OPA</td>
<td>o-phthaldialdehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>Pf</td>
<td>Parafascicular</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>pm</td>
<td>post meridiem</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomoles</td>
</tr>
<tr>
<td>PP-1</td>
<td>Protein phosphatase-1</td>
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<tr>
<td>PPE-A</td>
<td>Preproenkephalin-A</td>
</tr>
<tr>
<td>PPE-B</td>
<td>Preproenkephalin-B</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PPN</td>
<td>Pedunculopontine nucleus</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>PV⁺</td>
<td>Parvalbumin</td>
</tr>
<tr>
<td>R7BP</td>
<td>R7-binding protein</td>
</tr>
<tr>
<td>r.p.m</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RGS proteins</td>
<td>Regulators of G-protein signalling proteins</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>Substantia nigra pars reticulata</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic nucleus</td>
</tr>
<tr>
<td>STR</td>
<td>Striatum</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified Parkinson’s disease ratings scale</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>VA</td>
<td>Ventral anterior lateral nucleus</td>
</tr>
<tr>
<td>VAmc</td>
<td>Ventoanterior nucleus, magnocellular segment</td>
</tr>
<tr>
<td>VL</td>
<td>Ventrolateral</td>
</tr>
<tr>
<td>VLo</td>
<td>Ventral lateral nucleus, oral segment</td>
</tr>
<tr>
<td>VM</td>
<td>Ventromedial</td>
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<tr>
<td>VMAT2</td>
<td>Vesicular monoamine transporter 2</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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<td>w/v</td>
<td>Weight per volume</td>
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<tr>
<td>WAY-100635</td>
<td>[2-(4-(2-methoxyphenyl)-1-piperaziny-l)ethyl]-N-2-pyridinylcyclohexanecarboxamide</td>
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Chapter 1
General Introduction
1 Introduction

1.1 Overview

Parkinson’s disease (PD) is a neurodegenerative disease producing clinical symptoms which manifest as movement disorders such as bradykinesia, resting tremor and rigidity. These movement disabilities are caused by the progressive degeneration of nigrostriatal dopaminergic neurons, leading to an imbalance in basal ganglia circuitry.

The most effective pharmacological treatment for PD is 3,4-dihydroxyl-L-phenylalanine (L-DOPA), the immediate metabolic precursor of dopamine, which restores deficient dopamine levels in the striatum. However, long-term L-DOPA treatment in PD patients often leads to severe side-effects such as L-DOPA-induced dyskinesia (LID). The motor symptoms in LID may be caused by pulsatile dopamine receptor stimulation, which induces abnormal plasticity in dopaminergic and non-dopaminergic signalling pathways. The non-dopaminergic signalling pathways have major pathophysiological roles in motor symptoms of LID. Pharmacological agents that target the non-dopaminergic signalling pathways, such as the glutamatergic, serotonergic and opioid systems, can reduce dyskinesia in PD patients but cause adverse side-effects.

Many neurotransmitter receptors are G-protein coupled receptors (GPCRs) and are regulated by certain endogenous proteins (RGS proteins). These proteins negatively modulate both dopaminergic and non-dopaminergic signalling pathways and may be novel therapeutic targets for treatment of LID. Furthermore, due to their selective subunit regulation in GPCR signalling, RGS proteins may be suitable targets to dampen the adverse effects associated in combined treatment regimens. Understanding the roles of RGS protein subtypes in the pathophysiology of PD and LID may uncover potential therapeutic targets for future treatments.

In the work described in this thesis, the pathophysiological roles of RGS proteins in the unilateral 6-hydroxydopamine-lesioned rat model of Parkinson’s disease and L-DOPA-induced dyskinesia were investigated.
1.2 **Functional anatomy of the basal ganglia**

The basal ganglia are a group of subcortical nuclei functionally conveying information for both motor and non-motor processes. In primates, the subcortical nuclei include; the striatum (caudate nucleus and putamen), subthalamic nucleus, substantia nigra (pars reticulata, SNr and pars compacta, SNC), ventral tectal area and globus pallidus (medial/ internal and lateral/ external segments) (Crossman and Neary, 2000). In rodents, the nomenclature of subcortical nuclei is identical with the exception of the medial and lateral segments of the globus pallidus, which are referred to as the entopeduncular nucleus and globus pallidus, respectively (Paxinos and Watson, 1986).

Information is processed through parallel re-entrant loops connecting the cortex, basal ganglia and thalamus, in so called ‘cortico-basal ganglia-thalamocortical’ circuits, which remain segregated based on structure and function (Alexander and Culcher, 1990). These include the motor, oculomotor, associative, limbic and orbitofrontal circuits that project from separate regions of the basal ganglia and thalamus, to cortical target areas of the cerebral hemisphere (Alexander et al., 1986). The motor circuit remains the most well understood and dysfunction leads to severe movement disorders such as Huntington’s disease and PD (Crossman, 1990; DeLong, 1990).

The first study into the neocortical projections to the striatum used silver impregnation methods (Webster, 1961), which showed general anteroposterior and mediolateral topography. Later, McGeorge and Faull (1989), using a more sensitive axoplasmic transport technique, extended these observations and mapped longitudinal target regions from cortical projections in the rat striatum. In the monkey, studies have shown cortical motor areas (Area 4, Area 6 and the supplementary motor area) project in a topographical fashion to the striatum (Kunzle, 1975; Kunzle, 1977). Subsequent striatofugal efferent projections to the basal ganglia output nuclei (medial segment of globus pallidus and SNr) (Johnson and Rosvold, 1971; Parent et al., 1984), and, in turn, projections to the thalamic target nuclei (the oral part of the ventral lateral nucleus (VLo), lateral ventral anterior nucleus (VA), magnocellular part of the ventroanterior nucleus (VAmc) and the centromedian nucleus (CM)) were also found organised in a topographical manner (Kuo and Carpenter, 1973; Carpenter et al., 1976).
1.2.1 **Striatum**

The striatum, or neostriatum, is the major input structure to the basal ganglia. The dorsal and ventral (nucleus accumbens) regions show different efferent and afferent neuronal projections (McGeorge and Faull, 1989). In primates, in more caudal regions, the striatum is segregated into the caudate nucleus and putamen (Stephan et al., 1980). However, this remains a single structure in rodents (Paxinos and Watson, 1986).

The striatum is mainly composed of medium-sized neurons (12-18 µM diameter), which can be separated into at least two classes based on nuclear morphology (Wilson and Groves, 1980; Bolam et al., 1983; Graveland and DiFiglia, 1985) and axonal arborisation (Wilson and Groves, 1980; Izzo et al., 1987). The first cell type is commonly known as medium spiny neurons (MSNs), or Golgi type I cells, and represent ~95% of the neurons in the rat striatum. This is significantly greater than the population of MSNs (~75%) found in the monkey striatum (Graveland and DiFiglia, 1985).

The MSNs are the principal efferent projection cells of the striatum (Somogyi and Smith, 1979; Somogyi et al., 1981). Each cell has a smooth nuclear envelope (Bolam et al., 1983) and long axonal projections that form local collateral arborisations (Wilson and Groves, 1980). Light and electron microscopic studies in different species such as the monkey (Fox et al., 1971; DiFiglia et al., 1976), rat (Wilson and Groves, 1980), cat and ferret (Kemp and Powell, 1971), showed the dendrites of MSNs are occupied by a high number of spines. Approximately 15 spines per 10 µm of dendrite are found on MSNs in the cat and ferret caudate nucleus (Izzo et al., 1987).

The MSNs are morphologically different in dorsal and ventral regions (nucleus accumbens) of the striatum (Meredith et al., 2008). Notably the cell bodies are larger in the dorsal region compared to the ventral region, while the density of dendritic spines remains consistent (Meredith et al., 1992). MSNs efferent projections form the output of the striatum and mainly release γ-amino-butyric acid (GABA) as their neurotransmitter (Kita and Kitai, 1988). These neurons also express numerous neuropeptides such as enkephalin, prodynorphin, substance P and neurotensin, associated with GABA (McGinty, 2007).

The other class of medium-sized neurons are aspiny interneurons, or Golgi type II cells, characterised by an indented nuclear envelope and intrinsic axonal arborisations located...
within the dendritic field (DiFiglia et al., 1980; Bishop et al., 1982; Bolam et al., 1983; Graveland and DiFiglia, 1985). In the primate, medium-sized aspiny neurons were found more prominent in the caudate nucleus of the dorsal striatum, while no regional differences of these interneurons were found in the rat striatum (Graveland and DiFiglia, 1985). These interneurons can be further divided based on neuronal activity and neurochemical analysis (see ‘striatal interneuron’ section below).

The nucleus accumbens (NAc) is a heterogeneous structure that can be sub-divided into the ‘shell’ and ‘core’ regions (Meredith et al., 1999; Meredith et al., 2008). This is based on differences in immunoreactivity for molecular markers such as calcium-binding protein, acetylcholinesterase and substance P (Jongen-Relo et al., 1994). In the rat nucleus accumbens, dopamine receptor levels are expressed in both a homogeneous and inhomogeneous pattern. For example, dopamine D<sub>1</sub> receptor binding is similar in core and shell regions, while dopamine D<sub>2</sub> receptor binding is significantly higher in the NAc core (Bardo and Hammer, 1991). Furthermore, the NAc shell and core regions have differences in synaptic organisation with lower spine density in NAc shell regions (Meredith et al., 1992).

The efferent projections from the NAc core and shell are segregated to different target regions (Heimer et al., 1991). Such segregated projections mediate different functional roles in motivation and reward associated behaviours. In rat studies, site-specific intrastralatal infusion of dopamine agonists into the NAc shell region increased certain unconditioned responses, such as locomotor and feeding behaviours (Swanson et al., 1997), while lesion of the NAc core region, but not NAc shell, specifically impaired goal-directed conditioned responses (Parkinson et al., 1999).

**Striatal interneurons**

A small percentage of interneurons exist in the striatum, with up to ~5% in rodents (Graveland and DiFiglia, 1985). Striatal interneurons are aspiny and differentiated by neurochemical analysis, the neurotransmitter utilised and kinetics of neuronal activity (Bolam et al., 1984; Kawaguchi, 1993; Tepper and Bolam, 2004).

Cholinergic interneurons are estimated to make up ~1% of the striatal neuron population and each cell has a large soma (35 µm) with extensive dendritic fields (Bolam et al., 1984).
Such organisation mediates a high degree of signal integration from extrinsic inputs. These inputs include; excitatory glutamatergic afferents from the centromedian/ parafascicular (cm/ pf) thalamic nuclei and, to a lesser extent, the frontal cortex (Meredith and Wouterlood, 1990; Lapper and Bolam, 1992), inhibitory GABAergic input from striatal neurons (Bolam et al., 1986), neuromodulatory dopaminergic (Kubota et al., 1987; Chang, 1988) and serotonergic inputs from SNC and raphe nucleus, respectively (Vizi et al., 1981; Lavoie and Parent, 1990; Blomeley and Bracci, 2005).

The cholinergic interneurons show spontaneous tonic activation (Wilson et al., 1990) and typically fire action potentials with long lasting after-hyperpolarisations (Wilson et al., 1990; Kawaguchi, 1993; Tepper and Bolam, 2004). These interneurons exert neuromodulatory effects on striatal neurons (Kawaguchi et al., 1995; Pakhotin and Bracci, 2007). Electrophysiological studies in the rat striatum have identified the muscarinic M1-like cholinergic receptors as crucial for corticostriatal synaptic plasticity (Calabresi et al., 1999).

Fast-spiking GABA interneurons are medium-sized and form intricate striatal microcircuits for feed-forward inhibition of MSNs (Kita et al., 1990). These interneurons are categorised into three main groups by neurochemical staining (Chesselet and Robbins, 1989; Kawaguchi, 1993; Tepper and Bolam, 2004). The first type express parvalbumin (PV⁺, a calcium binding protein) and are found predominantly in the lateral striatal regions from day nine of postnatal development in the rat (Schlosser et al., 1999). Electrophysiological recordings of PV⁺ neurons show rapid neuronal kinetics (Kawaguchi, 1993; Tepper and Bolam, 2004), which are mediated by co-expression of Shaw family potassium channel Kv3.1 in the dorsolateral striatum for fast-firing (Lenz et al., 1994) and, the presence of dendro-dendritic gap junctions for rapid signal transduction (Kita et al., 1990). The second type of interneuron is immunoreactive for neuropeptide Y, somatostatin and nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase, and these cells characteristically display persistent low-threshold spikes (Kawaguchi, 1993; Tepper and Bolam, 2004). The final interneuron type contains calretinin and display a rostral-caudal expression gradient in rodents (Wu and Parent, 2000). The functions of calretinin neurons remain to be fully elucidated but, interestingly, they are the only interneuronal type to show neurogenesis following neonatal hypoxia (Yang et al., 2008).
A small number of striatal interneurons immunoreactive for tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis (Molinoff and Axelrod, 1971), have been found present in the human, monkey and rat striata (Dubach et al., 1987; Tashiro et al., 1989; Prensa et al., 2000). However, the ability of these neurons to synthesise and release catecholamines, such as dopamine, remains to be clarified.

**Striatal compartments**

The striatum is organised in compartments shown by neurochemical analysis, displaying inhomogenous expression of receptors and enzymes, often referred to as the ‘patch-matrix’ organisation (Gerfen, 1984; Gerfen, 1992a). Areas with low staining levels of choline acetyltransferase (ChAt) correlate with μ-opioid receptor binding regions, called ‘striosomes’ or ‘patches’, respectively (Pert et al., 1976; Graybiel et al., 1986). Conversely, extrastriosomal striatal regions show specific calbindin immunoreactivity staining (Gerfen, 1985; Gerfen et al., 1985). The patch-matrix regions have excitatory glutamatergic inputs from distinct cortical layers. Projections to patch compartments are from deep layer V and layer VI limbic associated areas of the cortex (Gerfen, 1984; McGeorge and Faull, 1989; Gerfen, 1992b). In comparison, projections to matrix compartments are from supragranular layer V of sensorimotor cortical associated areas (Gerfen, 1989; McGeorge and Faull, 1989; Gerfen, 1992b).

Two types of pyramidal neurons that project from the cortex to the striatum are distinguished based on their projection paths (Reiner et al., 2010). The so called ‘PT-type’ neurons, project extra-telencephalically and form connections only to the ipsilateral striatum (Wilson, 1987; Levesque and Parent, 1998; Reiner et al., 2003). While the ‘IT-type’ neurons project intra-telencephalically and send afferents to both the ipsilateral and contralateral striatum (Wilson, 1987; Levesque and Parent, 1998; Reiner et al., 2003). These two types of neurons differ in neuronal kinetics; PT-type neurons relay information at a faster rate and have thicker axons (0.82 μm diameter) that fire during motor behaviours (Bauswein et al., 1989; Cowan and Wilson, 1994; Turner and DeLong, 2000; Reiner et al., 2003; Lei et al., 2004). In comparison, IT-type neurons have slower conduction rates, with thinner axons (0.41 μm diameter) that fire in preparation for movement (Bauswein et al., 1989; Turner and DeLong, 2000; Reiner et al., 2003; Lei et al., 2004). The PT-type and IT-type neurons preferentially innervate the ‘indirect’ and ‘direct’ projection pathways of the
basal ganglia, respectively. These neurons are part of the re-entrant loop circuit that facilitate and inhibit motor programmes (Reiner et al., 2010).

**Striatal afferent projections**

The striatum receives a range of afferent projections such as: excitatory glutamatergic fibres from the cortex, thalamus and subthalamic nucleus (STN); dopaminergic fibres from the midbrain; serotonergic fibres from dorsal and medial raphe nucleus; and noradrenergic fibres from the locus coerules (Lavoie et al., 1989; Lavoie and Parent 1990; Delfs et al., 1998; Groenewegen et al., 1999; Castle et al., 2005).

**Corticostriatal projection**

Across different animal species the cortex projects massively to the striatum (Kunzle, 1975; Kunzle, 1977; McGeorge and Faull, 1989). In primates and rats, the striatum can be divided into associative, sensorimotor and limbic territories (McGeorge and Faull, 1989; Parent and Hazrati, 1995) that form separate parallel circuits with different cortical areas (Alexander and Crutcher, 1990; Obeso et al., 2008).

The most rostral region of the striatum forms part of the associative circuit and receives inputs from associative cortical areas such as frontal, temporal and parietal lobes (Goldman and Nauta, 1977; Kunzle, 1977; Ragsdale and Graybiel, 1981). In this circuit, the basal ganglia output relays to the thalamic VA nucleus and neurons from this structure feed-back to the dorsolateral prefrontal cortex and orbitofrontal cortices (Haber et al., 1995a; Obeso et al., 2008).

In the primate brain, the putamen forms part of the motor circuit, where sensorimotor afferents from the primary motor and somatosensory cortices project in a somatotopic manner to the putamen (Kunzle, 1977; Romanelli et al., 2005). The representation of the leg is mapped dorsal, followed by the arm, while the facial representation lays most ventral in the putamen (Romanelli et al., 2005). The massive corticostriatal projection converges onto sensorimotor fields of striatal neurons, with an estimated 5000:1 synaptic ratio (Alexander et al., 1986). As part of the motor circuit, the basal ganglia output nuclei projects to the VLo thalamic nucleus and neurons from this structure feed-back to motor cortical areas (Carpenter et al., 1976; Alexander et al., 1986).
In the rat striatum, sensorimotor territories are more superior in the dorsolateral rostral striatum compared to the ventromedial side (McGeorge and Faull, 1989; Nakano, 2000; Voorn et al., 2004). Within the dorsolateral quadrant, the rostral sensorimotor cortex (representation of head areas) innervates the central region, while the caudal sensorimotor cortex (representation of limb areas) innervates the dorsal region of the dorsolateral striatum (McGeorge and Faull, 1989). On the medial side of the rostral striatum, afferent corticostriatal projections from the auditory cortex innervate the ventromedial quadrant (McGeorge and Faull, 1989; Nakano, 2000; Voorn et al., 2004), while corticostriatal projections from the visual cortex innervate the dorsomedial quadrant. However, there is some degree of functional overlap between the two medial regions (McGeorge and Faull, 1989).

The limbic circuit arises from limbic cortical areas, such as the amygdala, which projects to the nucleus accumbens (McGeorge and Faull, 1989; Haber et al., 1995). Dysfunction of the limbic circuit is thought to occur in complex behavioural disorders such as addiction and obsessive compulsive disorder (Modell et al., 1989; Modell et al., 1990).

**Thalamostriatal projections**

The centromedian/ parafascicular (CM/ Pf) thalamic complex sends excitatory glutamatergic projections to the striatum and the STN (Nakano, 2000; Castle et al., 2005). These projections form positive and negative feed-back loops, respectively, which are speculated to modulate basal ganglia activity in attention to behavioural stimuli (Obeso et al., 2008). However, further studies are needed to confirm this hypothesis.

**Pedunculopontine nucleus basal ganglia connection**

The pedunculopontine nucleus (PPN) projects to different nuclei of the basal ganglia (Hammond et al., 1983; Jackson and Crossman, 1983; Lavoie and Parent, 1994) and forms reciprocal connections to the STN, medial globus pallidus and SNC (Kim et al., 1976; DeVito et al., 1980; Carpenter and Jayaraman, 1990; Nakano et al., 1990; Mena-Segovia et al., 2004). The PPN also projects to the spinal cord and has been proposed as a potential neurosurgical target for improving gait and postural stability in movement disorders such as Parkinson’s disease (Jenkinson et al., 2006; Hamani et al., 2007).
Nigrostriatal neurons

The basal ganglia receive dopaminergic projections from ventral midbrain neurons located in the retrorubral area (A8), SNc (A9) and ventral tegmental area (VTA, A10) (Smith and Kieval, 2000).

The nigrostriatal system is primarily composed of dopaminergic neurons of the SNc which provides dense innervation to the striatum (Moore et al., 1971; Carpenter and Peter, 1972). These dopaminergic neurons segregate to the patch-matrix compartments (Gerfen et al., 1987; Prensa et al., 2000). In rats, thick and varicose fibres from the ventral tier of the SNc project to patch compartments, while thinner and less varicose projections from the dorsal tier of the SNc project to matrix compartments (Gerfen, 1985; Prensa and Parent, 2001; Smith and Villalba, 2008).

Ventral midbrain dopaminergic neurons project to different functional regions of the striatum in primates. Neurons from the VTA and dorsal SNc project to limbic ventral striatum, while the ventral SNc innervates the sensorimotor striatal region, and the densocellular part of the ventral SNc innervates the associative striatal region (Haber and Fuge, 1997; Smith and Kieval, 2000; Prensa and Parent, 2001; Smith and Villalba, 2008). Interestingly, projections to striatal target regions correlate to the expression of calbindin protein, which is expressed in neurons that target the limbic, but not sensorimotor, striatal regions (Gerfen et al., 1985; Smith and Kieval, 2000).

In rats, lesion of the dopaminergic SNc has shown to induce deficits in learning and working memory (Zis et al., 1974; Da Cunha et al., 2003; Braga et al., 2005). Moreover, studies in primates have shown tonically active dopaminergic neurons fire robustly to reward-related stimuli and in anticipation of positive behavioural reinforcement (Romo and Schultz, 1990; Schultz and Romo, 1990; Schultz et al., 1998). Such responses are suggested to mediate behavioural-reinforced learning (Kimura and Matsumoto, 1997).

Most dopaminergic afferent terminals form synapses at the dendritic spine ‘neck’ of MSNs (Pickel et al., 1981; Bouyer et al., 1984; Moss and Bolam, 2008) exerting postsynaptic modulation on corticostriatal projections (Smith and Bolam, 1990; Dani and Zhou, 2004). Moreover, dopamine D2 receptors present on presynaptic neurons of corticostriatal
afferents can directly modulate glutamate release into the striatum (Bamford et al., 2004a; Bamford et al., 2004b).

Dopamine can also elicit parasynaptic modulation on distant receptors following diffusion (Descarries et al., 1996), which is aided by morphologically distinct ‘open’ synaptic contacts for volume transmission (Moss and Bolam, 2008; Rice and Cragg, 2008). The temporal effects of dopamine in the striatum are somewhat dependent on the extent of extracellular clearance by dopamine active transporters (DAT) (Cenci and Lundblad, 2006; Rice and Cragg, 2008). Indeed, clearance of dopamine is slower in the ventral striatum where DATs are expressed at lower levels (Kuhr and Wightman, 1986; Stamford et al., 1988).

1.2.2 Basal ganglia output nuclei

Substantia nigra pars reticulata (SNr)

The SNr and medial globus pallidus form the basal ganglia output nuclei (Albin et al., 1989; Alexander and Crutcher, 1990). These nuclei receive glutamatergic afferents from the STN (Kita and Kitai, 1987; Parent and Smith, 1987; Parent and Parent, 2007) and GABAergic afferents from the striatum (Graybiel and Ragsdale, 1979; Parent and Hazrati, 1995). The SNr is primarily composed of GABAergic neurons (Oertel and Mugnaini, 1984), which are expressed along a lateromedial gradient across the layered compartmentalised structure (Smith et al., 1987; Deniau et al., 2007). These neurons project to the ventral medial and parafascicular thalamic nuclei. In turn, neurons from these structures project to the motor cortex (Clavier et al., 1976; Di Chiara et al., 1979; Herkenham, 1979; Deniau and Chevalier, 1992; Deniau et al., 2007). The SNr also projects to the superior colliculus and pontine tegmentum, and neuronal activity of these projections may have roles in orientation, posture, muscular tone and locomotion (Takakusaki et al., 2004; Deniau et al., 2007).

Early studies using electrophysiology in awake monkeys showed that SNr neurons are tonically active at high frequency (50-100 Hz) during rest (Hikosaka and Wurtz, 1983a; Chevalier and Deniau, 1990) but reduce in firing activity during saccadic eye movements (Hikosaka and Wurtz, 1983a; Hikosaka and Wurtz, 1983b; Hikosaka and Wurtz, 1983c). These findings suggest that disinhibition of thalamic motor nuclei occurs during motor initiation (Chevalier and Deniau, 1990; Gerfen, 1992b).
**Medial segment of globus pallidus (GPm)**

The GPm (or entopeduncular nucleus, EP, in rats) receives striatal GABAergic afferents in a topographical manner (Staines and Fibiger, 1984; Parent and Hazrati, 1995). Anterograde axonal tracing of biotinylated dextran amine was used to map the topographical regions of the GPm. These regions include; the motor territory located ventrolateral of the caudal GPm, the premotor territory located rostromedial and the supplementary motor area, located intermediate between the two aforementioned structures (Francois et al., 1994; Nakano, 2000).

Electrophysiological studies in animals have shown that activation of the GPm/SNr nuclei induces GABA-mediated inhibition of the thalamic and brainstem motor nuclei, which inhibits movement (Chevalier and Deniau, 1990; Turner and Anderson, 2005). Moreover, site-specific administration of muscimol, a potent GABA<sub>A</sub> receptor agonist, in the ventral medial thalamic nuclei induces catalepsy and rigidity in rats (Klockgether et al., 1985). These results suggest that the functional effect of GABAergic-mediated inhibition of thalamic motor nuclei is a reduction of movement.

### 1.2.3 Striatal efferent projections: ‘direct’ and ‘indirect’ pathways

In the 1980s, anatomical and neurochemical data revealed that the striatal MSNs form two main output pathways, the so called ‘direct’ and ‘indirect’ pathways, to the GPm/SNr (Figure 1; Alexander et al., 1986; Albin et al., 1989).

The ‘direct’ pathway is monosynaptic, directly projecting to the GPm/SNr. Activation of the direct pathway is thought to facilitate movement and initiate desired motor sequences (Alexander and Crutcher, 1990; Hauber, 1998). Neurons of this pathway primarily express dopamine D<sub>1</sub> receptors and pro-opioid peptide preproenkephalin-B (PPE-B). PPE-B is subsequently cleaved to substance P and prodynorphin (Gerfen, 1992b).

In contrast, the ‘indirect’ pathway is polysynaptic. Striatofugal neurons of this pathway first project to the lateral segment of the globus pallidus (GPI). GPI GABAergic efferents project to the STN and in turn, the STN projects via excitatory glutamatergic projections to the GPm/SNr. Activation of the indirect pathway is thought to inhibit unwanted motor programmes and ‘smooth’ cortical initiated motor patterns (Alexander and Crutcher, 1990;
Hauber, 1998). The striatopallidal neurons primarily express dopamine D_2 receptors and peptide preproenkephalin-A (PPE-A). PPE-A is subsequently cleaved to enkephalin (Gerfen, 1992b).

**Updated concepts of the ‘direct’ and ‘indirect’ pathways**

The classic model of the basal ganglia circuitry remains oversimplified and important neuronal connections are often overlooked (Figure 1). These include reciprocal projections that exist between the striatum and target nuclei, such as the GPI and the SNc (Haber et al., 2000; Sato et al., 2000). These connections demonstrate the multi-directional flow of information between nuclei of the basal ganglia.

As part of the indirect pathway, the STN is reciprocally connected to GPI (Kita et al., 2005; Parent and Parent, 2007) and also projects directly to the striatum in a topographical manner (Parent and Smith, 1987; Nakano et al., 1990). The STN is a major input station of the basal ganglia (Nambu et al., 2002) and receives cortical (Monakow et al., 1978; Kitai and Deniau, 1981; Inase et al., 1999), CM/ Pf thalamic and brainstem afferent projections (Lanciego et al., 2004; Castle et al., 2005). Corticosubthalamic projections form the so-called ‘hyperdirect pathway’ and convey faster excitatory input to the basal ganglia output nuclei (Nambu et al., 2002). Activation of the hyperdirect pathway is suggested to promote voluntary motor behaviour in a ‘center-surround’ fashion, which may be reinforced by activity of the direct pathway (Nambu et al., 2000; Nambu et al., 2002).
Classic model of the neuronal connections in the basal ganglia

Figure 1. A schematic diagram illustrating the neuronal connections in the classic model of the basal ganglia. Dopamine mediates opposing effects on dopamine receptor subtypes. D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; Enk, enkephalin; Dyn, prodynorphin; STR, striatum; GPI, lateral globus pallidus; GPM, medial globus pallidus; STN, subthalamic nucleus; SNr, substantia nigra pars reticulata; SNC, substantia nigra pars compacta; VL thalamus, ventrolateral thalamic motor nucleus.
1.3 Guanine-nucleotide binding protein coupled receptors (GPCRs)

GPCRs are the largest family of membrane proteins and mediate most cellular responses including neurotransmission, hormonal responses, olfaction and taste (Kandel et al., 2000; Pierce et al., 2002). Many neurotransmitter receptors including dopamine, noradrenergic, serotonin and opioid receptors are GPCRs (Markovic and Challiss, 2009). These are characteristically heptahelical, spanning the plasma membrane with seven α-helical structures, and are associated with a heterotrimeric guanine-nucleotide binding protein (G-protein, G\(_{\alpha/\beta\gamma}\)) (Unger et al., 1997; Teller et al., 2001). Ligand binding to the GPCR leads to conformation changes, specifically in orientation of α-helices 3 and 6, which activates the heterotrimeric (G\(_{\alpha/\beta\gamma}\)) complex by exposing G-protein binding sites (Farrens et al., 1996; Hamm, 1998). This subsequently leads to heterotrimer dissociation as the G\(_{\alpha}\) subunit exchanges guanosine diphosphate (GDP) for guanosine-5’-triphosphate (GTP). The separated G\(_{\alpha}\) and G\(_{\beta\gamma}\) subunits target downstream effector molecules that activate multiple signal cascades (Hamm, 1998; Bridges and Lindsley, 2008). For example, free G\(_{\alpha}\) and G\(_{\beta\gamma}\) subunits can modulate ion channel activity and activate second messenger cascades that promote gene transcription, cell proliferation and changes in organ physiology (Clapham and Neer, 1997; Hamm, 1998; Rosenbaum et al., 2009). The G-protein signalling mechanism is terminated following GTP hydrolysis by intrinsic GTPase activity mediated by the G\(_{\alpha}\) subunit. Subsequently, the heterotrimeric (G\(_{\alpha/\beta\gamma}\)) complex is reformed (Gilman, 1987).

GPCRs are abundantly expressed in tissues and organs, and show pathological changes in many human disease states (Dalrymple et al., 2008; Markovic and Challiss, 2009). Indeed, GPCRs are a major target for the pharmaceutical industry, with approximately 60% of all pharmacological agents targeting GPCRs. G-protein signalling mechanisms are reliant on scaffolding proteins (Pierce et al., 2002; Abramow-Newerly et al., 2006; Neitzel and Hepler, 2006), which include additional co-factors that bind to G-protein subunits to prevent sensitisation and regulate signal transduction (Andreeva et al., 2007). For example, phosphorylation of cytoplasmic GPCR segments by G-protein receptor kinases (GRKs) initiates the binding of β-arrestins (Lohse et al., 1990; Claing et al., 2002), which leads to receptor internalisation via clarithrin-coated pit machinery (Pierce et al., 2002). This process reduces GPCR signalling by recycling GPCRs via endocytosis or, alternatively, degradation by lysosomes (Ariano et al., 1997; Kim et al., 2004; Paspalas et al., 2006; Xiao et al., 2007).
1.3.1 Dopamine D₁ receptors

Dopamine receptors are grouped according to common downstream pathways mediated by the Gₐ subtype of the G-protein; D₁-like (D₁ and D₅) and D₂-like receptors (D₂, D₃ and D₄) (Neve et al., 2004). D₁-like receptors signal via Gₐs and Gₐolf subunits, which, in turn, bind to the C2 cytosolic domain of adenylate cyclase (Tesmer et al., 1997b). Subsequent adenylate cyclase activation leads to catalysis of adenosine 5'-triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The cAMP molecules disinhibit the catalytic subunits of protein kinase A (PKA) for phosphorylation of downstream targets such as; dopamine and cyclic AMP-regulated phosphoprotein 32kDa (DARPP-32), ion channels, glutamate receptors and cyclic AMP response element-binding protein (CREB) (Figure 2; Hemmings et al., 1984; Konradi et al., 1994; Cantrell et al., 1997; Flores-Hernandez et al., 2002).

In the striatum, Gₐolf is abundantly expressed and transduces dopamine D₁ receptor signals to specifically activate adenylate cyclase subtype 5 (Zhuang et al., 2000; Corvol et al., 2001; Herve et al., 2001). This so called ‘canonical’ signalling pathway has two well established outcomes: (1) phosphorylation of DARPP-32 at threonine residue 75 (Thr75) by cyclin dependent kinase (cdk5) leads to inhibition of PKA, and (2) PKA phosphorylation of DARPP-32 at Thr34 (phospho-Thr34-DARPP-32) leads to inhibition of protein phosphatase-1 (PP-1), which, subsequently, prevents the dephosphorylation of PKA targets (Figure 2; Hemmings et al., 1984; Svenningsson et al., 2004). The phospho-Thr34-DARPP-32 signal transduction pathway increases the expression of immediate early genes; c-fos, zif268 and arc (Svenningsson et al., 2000a; Gerfen et al., 2002), and activates extracellular regulated kinases (ERK) (Bertran-Gonzalez et al., 2008), which are crucial for synaptic plasticity of corticostriatal synapses (Calabresi et al., 2000b). Indeed, dopamine D₁ receptor-mediated activation of PKA leads to phosphorylation of N-methyl-D-aspartic acid (NMDA) receptor subunits and activation of CREB, which facilitates long-term synaptic plasticity in striatal neurons (Dudman et al., 2003; Nishi et al., 2005; Calabresi et al., 2008; Cenci and Konradi, 2010).

1.3.2 Dopamine D₂ receptors

Dopamine D₂ receptors can be alternatively spliced at the third cytoplasmic loop to produce two functional receptor isoforms; D₂s and D₂L. These receptors activate Gₐi/o subunits (Figure 2; Watts et al., 1998; Nickolls and Strange, 2003), which inhibit the adenylate
cyclase-mediated signal cascade by binding to the C1 cytosolic domain of the adenylate cyclase protein (Robinson and Caron, 1997). Thus, dopamine D2 receptor activation reduces cAMP-mediated PKA activation, decreasing phospho-Thr34-DARPP-32 (Nishi et al., 1997; Neve et al., 2004), while dopamine D2 receptor blockade elevates phospho-Thr34-DARPP-32 (Svenningsson et al., 2000b).

Dopamine D2 receptors have a strong crosstalk with other GPCRs, especially adenosine A2a receptors. These receptors are most abundantly expressed in the striatum and co-localise with dopamine D2 receptors, modulating dopamine signalling and motor control (Rosin et al., 2003, Schiffmann et al., 2003). However, in contrast to dopamine D2 receptors, the adenosine A2a receptors activate adenylate cyclase via Gɑsolf subunits (Kull et al., 2000). Thus, it appears a balance between adenosine A2a and dopamine D2 receptor activation determines the activation or inhibition of adenylate cyclase, and subsequent downstream signalling cascades (Ferre et al., 1991; Lerner and Kreitzer, 2012).

In addition to the canonical pathways, dopamine receptors can activate non-canonical signalling pathways, which are cascades not directly downstream of cAMP/ PKA (Figure 2). For example, dopamine D1 receptors can, in parallel, activate the Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1) and, subsequently, the mitogen-activated protein kinase kinase (MEK), as part of the MAPK pathway for synaptic plasticity (Sweatt, 2001; Fasano et al., 2009). While dopamine D2 receptor activation initiates protein scaffold formation with β arrestin, protein kinase B (Akt) and protein phosphatase 2A (PP2A), activating glycogen synthase kinase 3 (GSK-3) for cellular adaptation (Jope and Johnson, 2004; Beaulieu et al., 2005; Forde and Dale, 2007).

In the past decade, studies have revealed GPCRs are also expressed on the plasma membrane as dimers in homo- and hetero-oligomeric complexes (Angers et al., 2001; Carriba et al., 2007; Franco et al., 2008; Panetta and Greenwood, 2008). In vitro cell studies have shown heteroligomerised combinations of dopamine D1, D2, mGluR5 metabotropic glutamate and adenosine receptors (Gines et al., 2000; Canals et al., 2003; Fuxe et al., 2003). Such receptor formations exhibit different pharmacological and physiological responses compared to single receptors (Franco et al., 2008). Interestingly, heteroligomerisation of dopamine D1-D2 receptors in rat striatal neurons activates a different cell signalling pathway via Gɑq subunit, which initiates phospholipase C-mediated
calcium signalling and activation of calmodulin-dependent protein kinase IIa (CaMKII) (Lee et al., 2004; Rashid et al., 2007; Hasbi et al., 2010).

1.3.3 The effect of dopamine on corticostriatal synaptic plasticity
Dopamine receptor signalling can induce long-term potentiation (LTP) and long-term depression (LTD) for motor learning (Calabresi et al., 2000a; Calabresi et al., 2007). Activation of both dopamine D<sub>1</sub> receptors and NMDA receptors in striatal neurons induces synaptic plasticity, which may underly development of certain hyperkinetic movement disorders (Calabresi et al., 2008). In contrast, dopamine D<sub>2</sub> receptors mediate LTD. An electrophysiological study showed that LTD was abolished in corticostriatal neurons of dopamine D<sub>2</sub> receptor knockout mice (Calabresi et al., 1997). Moreover, co-incidental activation of multiple receptors is needed for induction of LTD. A recent study has shown that activation of dopamine D<sub>2</sub> and muscarinic M<sub>1</sub> cholinergic receptors and, in turn, Cav1.3 calcium channel signalling is needed to induce LTD in corticostriatal neurons (Wang et al., 2006).
Dopamine $D_1$ and $D_2$ receptor signalling mechanisms

Figure 2. A schematic diagram of the canonical and non-canonical dopamine receptor signalling pathways. Dopamine $D_1$ and $D_2$ receptors have opposite effects on activation of adenylate cyclase and, thus, activation of protein kinase A-mediated signalling. Activation of heteroligomeric dopamine receptors lead to increased intracellular calcium. MEK, mitogen-activated protein kinase kinase; ERK, extracellular-signal-regulated kinases; p-CREB, phosphorylated- cAMP response element binding; RasGRF, Ras-specific guanine nucleotide releasing factor; PKA, protein kinase A; DARPP-32$^{Thr34}$, dopamine- and cAMP-regulated phosphoprotein 32 phosphorylated at threonine residue 32; PP-1, protein phosphatase-1; p-GRK, phosphorylated G-protein receptor kinase; Akt, protein kinase B; pp2A, protein phosphatase 2A; GSK3β, glycogen synthase kinase 3; DAG, diacylglycerol; PKC, protein kinase C; IP3, inositol trisphosphate; IP3R, inositol trisphosphate receptor; ER, endoplasmic reticulum; CaMKII, calcium/ calmodulin-dependent protein kinases II.
1.4 Regulators of G-protein signalling (RGS) proteins

The first RGS protein, factor Sst2p, was shown to negatively modulate GPCR signalling in yeast *Saccharomyces cerevisiae* (Dohlman et al., 1995; Dohlman et al., 1996). RGS protein genes remain conserved in different plants and mammals, suggesting an important role in the regulation of eukaryotic GPCR signalling (Ross and Wilkie, 2000). RGS proteins reduce GPCR transmission by acting as GTPase activating proteins (GAPs), rapidly attenuating the G-protein signal (up to 1000 fold) and promoting reformation of the inactive G_αβγ heterotrimer (Figure 3; Hepler, 1999).

RGS proteins can be found located at the plasma or nuclear membranes, or in the cytosol, where they are recruited to the plasma membrane following GPCR-ligand binding (Druey et al., 1998; Roy et al., 2003). Using molecular crystallisation methods, Tesmer et al. (1997a) was first to show that RGS proteins bind with highest affinity to the G_α-GTP hydrolysis complex. These data demonstrated that RGS proteins stabilise the G_α subunit transition complex and enhance G_α-GTPase enzyme activity (Tesmer et al., 1997a).

RGS proteins can also form multi-protein complexes through additional sub-domains, which reflect their diversity in cellular signalling (Abramow-Newerly et al., 2006; Neitzel and Hepler, 2006). Mutations of G-protein subunits in *in vitro* cell preparations have shown that certain RGS proteins can still inhibit downstream signalling pathways (Hepler et al., 1997; Schesonoka et al., 2000). These data suggest that some RGS proteins can inhibit G-protein signalling, without necessarily binding directly to the G_α subunit (Roy et al., 2006).

In mammalian cells, over 30 RGS proteins have been identified (Abramow-Newerly et al., 2006; Bansal et al., 2007). All RGS proteins have a conserved ~125 amino acid ‘RGS domain’, which is a 9α helical structure that folds into two globular domains. Specifically, helices 4-7 of the RGS domain contact three ‘switch regions’ of the G_α subunit to stabilise the G_α-GTP hydrolysis complex (Tesmer et al., 1997a). In humans, a total of 16 G_α subunits are separated into four main G_α subfamilies (Table 1; McCudden et al., 2005; Milligan and Kostenis, 2006). Most of these G_α subunits are regulated by RGS proteins (Table 2; Jean-Baptiste et al., 2006; Bansal et al., 2007).
<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Subunit subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{\alpha s}$</td>
<td>$G_{\alpha s}$ and $G_{\alphaolf}$</td>
</tr>
<tr>
<td>$G_{\alpha i}$</td>
<td>$G_{\alpha i1-3}$, $G_{\alpha o}$, $G_{\alpha z}$, $G_{\alpha t-cone}$, $G_{\alpha t-rod}$ and $G_{\alpha gust}$</td>
</tr>
<tr>
<td>$G_{\alpha q}$</td>
<td>$G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$ and $G_{\alpha 16}$</td>
</tr>
<tr>
<td>$G_{\alpha 12}$</td>
<td>$G_{\alpha 12}$ and $G_{\alpha 13}$</td>
</tr>
</tbody>
</table>

**Table 1.** The four main $G_{\alpha}$ subunit subfamilies found in humans.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Distribution of mRNA</th>
<th>RGS proteins</th>
<th>Regulated $G_{\alpha}$ subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/ RZ</td>
<td>Brain, Heart, Lung, Liver, Retina</td>
<td>RGS17 (RGSZ2), RGS19 (GAIP), RGS20 (RGSZ1)</td>
<td>$G_{\alpha z}$, $G_{\alpha i/o}$, $G_{\alpha q}$</td>
</tr>
<tr>
<td>B/ R4</td>
<td>Brain, Heart, Lung, Liver, Retina, Muscle, Pituitary</td>
<td>RGS1, RGS2, RGS3, RGS4, RGS5, RGS8, RGS13, RGS16, RGS18, RGS21</td>
<td>$G_{\alpha q}$, $G_{\alpha i/o}$</td>
</tr>
<tr>
<td>C/ R7</td>
<td>Brain, Retina, Pancreas</td>
<td>RGS6, RGS7, RGS9, RGS11</td>
<td>$G_{\alpha i}$</td>
</tr>
<tr>
<td>D/ R12</td>
<td>Brain, Heart, Liver, Lung, Spleen</td>
<td>RGS10, RGS12, RGS14</td>
<td>$G_{\alpha i/o}$</td>
</tr>
</tbody>
</table>

**Table 2.** The four main subfamilies of regulators of G-protein signalling (RGS) protein classified based on amino acid sequence homology and presence of additional subdomains. RGS proteins show regional expression and regulate different $G_{\alpha}$ subunits in G-protein signalling. Adapted from Hepler (1999).
RGS proteins are separated into four main subfamilies based on amino acid sequence homology and presence of additional sub-domains (Table 2; Hepler, 1999). The expression levels of these RGS proteins can vary greatly, with some subtypes ubiquitously expressed in multiple organs (Nomoto et al., 1997). Other RGS protein subfamilies also exist (Jean-Baptiste et al., 2006; Bansal et al., 2007) but are not exclusively known for their RGS protein activity. Such proteins include GRKs and RhoGEFs, which have characterised roles in receptor desensitisation and structural protein-protein interactions, respectively (Zheng et al., 2001; Day et al., 2004; Vazquez-Prado et al., 2004).

1.4.1 Subfamily A/ RZ
The RZ family members include; RGS17 (RGSZ2), RGS19 (G-protein α subunit-interacting protein; GAIP) and RGS20 (RGSZ1). RZ RGS proteins regulate G\textsubscript{ai/o}, G\textsubscript{aq} and G\textsubscript{az} subunits (Mao et al., 2004; Garzon et al., 2005b; Garzon et al., 2005c). Specific RZ members, RGS17 and RGS20, have cysteine rich regions in the N-terminal domain that are modified by post-translational glycosylation, which initiates protein scaffolding in opioid receptor desensitisation (Garzon et al., 2004; Garzon et al., 2005b; Rodriguez-Munoz et al., 2007).

1.4.2 Subfamily B/ R4
The R4 family members are effective GAPs for G\textsubscript{aq} and G\textsubscript{ai/o} subunits, but not the G\textsubscript{as} subunit (Hepler et al., 1997; Ross and Wilkie, 2000). R4 proteins are generally small with an N-terminal amphipathic helix. Conserved cysteine residues within this structure are pamitoylated for plasma membrane recruitment (Dunphy and Linder, 1998; Srinivasa et al., 1998). The N-terminal of RGS2 can bind directly to \(\alpha\textsubscript{1A}\) adrenergic or M\textsubscript{1}-cholinergic receptors at intracellular loop 3 (Bernstein et al., 2004; Hague et al., 2005). The RGS domain for this subfamily of proteins is located at the C-terminal and interacts with the G-protein to promote the G\textsubscript{o}-GTP hydrolysis transition complex (Neitzel and Hepler, 2006).

1.4.3 Subfamily C/ R7
The R7 family members; RGS6, RGS7, RGS9 and RGS11 have additional sub-domains such as Disheveled/ EGL-10/ Pleckstrin (DEP) and G\textgamma protein-like (GGL) motifs located at the N-terminal (Martemyanov et al., 2003; Garzon et al., 2005a; Jean-Baptiste et al., 2006). The RGS domain located at the C-terminus negatively modulates G\textsubscript{ai} subunits.
The C-terminal length of the RGS9 protein determines localisation to different target regions. Two splice variants have been identified to date, the shorter RGS9-1 modulates photoreceptor signalling in the retina (Rahman et al., 1999), while RGS9-2 (with an additional 209 amino acids) is specifically expressed in the striatum and modulates dopaminergic signalling (Rahman et al., 1999; Zachariou et al., 2003; Gold et al., 2007).

RGS proteins of the R7 subfamily have additional sub-domains that play important roles in functional specificity to receptors. For example, removal of the DEP domain compromises co-localisation of RGS9-2 to dopamine D2 receptors, suggesting that additional motifs underlie specific receptor targeting (Burchett, 2000). Various R7 RGS proteins are found in vivo as heterodimers with G-protein β subunit 5 (Gβ5) (Witherow and Slepak, 2003), connected via the GGL domain (Cabrera et al., 1998; Snow et al., 1998). In Gβ5 knockout mice, RGS9-2 and RGS7 protein levels are abolished in the striatum, which suggests the heterodimeric complex stabilises these RGS proteins (Chen et al., 2003). Moreover, RGS-Gβ5 interactions have physiological roles in ion channel conductance, specifically accelerating the kinetics of G-protein-gated K+ channels (Kovoor et al., 2000).

Interestingly, RGS9-2 also binds R7-binding protein (R7BP) for proteolytic stability in striatal neurons (Anderson et al., 2007a; Anderson et al., 2007b). This interaction leads to localisation of RGS9-2 from the plasma membrane to the postsynaptic density (Anderson et al., 2007a). The R7BP is regulated by palmitoylation and mediates vesicular transport of RGS7 between the plasma membrane and nucleus (Drenan et al., 2005; Hepler, 2005).

### 1.4.4 Subfamily D/ R12

The R12 subfamily (RGS10, RGS12 and RGS14) have a diverse distribution across the brain, heart, lungs, liver and spleen (Hunt et al., 1996; Snow et al., 1997). These proteins primarily attenuate Gαi/o-mediated signalling. RGS10 is distinctly small (173 amino acids), while RGS12 and RGS14 are larger with additional sub-domains such as ras-binding (RBD), phosphotyrosine binding (PTB) and Gαi/o-Loco (Goloco) motifs (Snow et al., 1997; Jean-Baptiste et al., 2006). The Goloco domain provides an additional inhibitory mechanism by blocking GDP release (Kimple et al., 2001).
1.4.5 RGS proteins in neurotransmission

RGS proteins modulate a wide range of neurotransmitter receptor signalling at the G-protein level, which includes modulation of dopaminergic, serotonergic, opioid, adrenergic and cholinergic systems (Bansal et al., 2007; Hooks et al., 2008). Moreover, RGS proteins can also modulate second messenger signals such as adenylate cyclase, mitogen-activated protein kinase (MAPK) and potassium conductance in neurons (Burchett, 2000; Marinissen and Gutkind, 2001; Goldsmith and Dhanasekaran, 2007).

In situ hybridisation has been commonly used to show the distribution of RGS protein gene expression in normal brain tissue (Gold et al., 1997; Geurts et al., 2002; Stanwood et al., 2006) and in disease states (Mirnics et al., 2001; Geurts et al., 2003). Although the lack of suitable antibodies for detecting RGS proteins has limited the immunohistochemical characterisation of RGS protein spatial distribution, studies have been able to quantify protein levels by using western blot analysis (Larminie et al., 2004; Gold et al., 2007).

The RGS protein subtypes: RGS2, RGS4, RGS7, RGS8, RGS9-2, RGS10, RGS17 and RGS20 are found highly expressed in the brain (Gold et al., 1997; Larminie et al., 2004). Some of these subtypes, such as RGS4, are ubiquitously expressed and are found in regions such as the prefrontal cortex (Gibbons et al., 2008), basal ganglia and locus coeruleus (Gold et al., 1997). Other RGS proteins, such as RGS9-2, remain largely localised in discrete areas such as the striatum of the basal ganglia (Gold et al., 1997; Gold et al., 2007).

The roles of RGS proteins in the basal ganglia are yet to be fully understood. RGS4 and RGS9-2 modulate multiple neurotransmitter receptors in the striatum (Bansal et al., 2007; Hooks et al., 2008) and have functional roles in motor production (Grillet et al., 2005; Blundell et al., 2008) and addiction (Hooks et al., 2008; Traynor, 2010). However, the specific actions of these RGS proteins on the direct and indirect pathways of the basal ganglia remain to be elucidated (Herrera-Marschitz et al., 2010).

RGS protein regulation of dopamine receptors

One of the best characterised RGS proteins to date is RGS9-2. This protein is found expressed throughout the striatum in MSNs and cholinergic interneurons (Cabrera-Vera et al., 2004). Electrophysiological data in rat striatal slices has shown that RGS9-2 specifically attenuates dopamine D2 receptor-mediated calcium Cav2.2 signalling in
cholinergic interneurons (Cabrera-Vera et al., 2004). Moreover, behavioural studies have demonstrated the functional relevance of RGS9-2 proteins in MSNs, which has uncovered their potential as a therapeutic target in disease states (Traynor and Neubig, 2005). For example, in rats, unilateral viral-mediated overexpression of RGS9-2 in the striatum caused rotation bias towards the operated side following dopamine agonist treatment (Rahman et al., 2003). This suggests that overexpression of RGS9-2 in the treated hemisphere attenuates dopamine receptor signalling, leading to supersensitivity of dopamine receptors in the untreated hemisphere following dopamine agonist treatment (Ungerstedt and Arbuthnott, 1970). It was then suggested that RGS9-2 may be a therapeutic target for the attenuation of excessive dopamine receptor signalling. This idea was employed in the treatment of LID in parkinsonian non-human primates (NHPs), where viral-mediated overexpression of RGS9-2 specifically reduced dopamine D2, but not D1, receptor agonist-induced dykinesia in L-DOPA-primed animals (Gold et al., 2007).

Molecular data suggests other RGS proteins expressed in the striatum may also be potential therapeutic targets in disease states where abnormal dopamine neurotransmission is a causative factor. For example, striatal RGS2 and RGS4 mRNA show preferred localisation to dopamine D1 and D2 receptors, respectively (Taymans et al., 2004). Moreover, RGS2 and RGS4 mRNA expression levels are rapidly modulated following dopamine agonist challenge in dopamine-denervated rats (Taymans et al., 2004).

**RGS protein regulation of opioid receptors**

Opioid receptors (μ, δ, κ) are GPCRs that signal through G-proteins Gαz and Gi/o (Garzon et al., 2005a; Garzon et al., 2005b; Garzon et al., 2005c). In opioid addiction, opioid receptors mediate development of symptoms such as tolerance and dependence (Matthes et al., 1996; Zachariou et al., 2003). Following acute or chronic morphine treatment, striatal μ-opioid receptors expressed in dendritic spines of MSNs are redistributed (Svingos et al., 1996; Haberstock-Debic et al., 2003). RGS protein subtype 19 (GAIP) can facilitate opioid receptor internalisation by endocytosis via clarith-coat pit machinery (Elenko et al., 2003; Xie and Palmer, 2005). Moreover, RGS19 shares a common promoter with opioid receptor-like 1 gene (Ito et al., 2000; Sierra et al., 2002), which suggests an intimate regulatory response of this receptor.
It is worth noting that most molecular adaptations in addiction occur downstream of the GPCR (Zachariou et al., 2003; Garzon et al., 2005b; Psifogeorgou et al., 2007; Hooks et al., 2008). For example, RGS9-2 gene expression is down-regulated following chronic morphine treatment, which may underlie addiction (Zachariou et al., 2003). In support of this, RGS9-2 knockout mice show increased sensitivity to morphine-induced analgesia and reward (Zachariou et al., 2003). RGS9-2 may also play a role in the development of tolerance to the acute effects of morphine treatment. In transfected cell studies, RGS9-2 prevents µ-opioid receptor internalisation and inhibits ERK signal transduction pathways (Psifogeorgou et al., 2007), which may underlie µ-opioid receptor desensitisation.

Understanding the roles of RGS proteins in the basal ganglia may help uncover the pathophysiological mechanisms in neurological disorders such as PD and LID. Moreover, by elucidating the functions of RGS proteins in disease states it may uncover therapeutic targets for future treatments.
The role of RGS proteins in GPCR signalling

Figure 3. The heterotrimeric G-protein ($G_{\alpha/\beta\gamma}$) remains in close proximity with the guanine nucleotide coupled receptor (GPCR) in its inactive state. (1) Following ligand binding to the GPCR, (2) the G-protein is activated and (3) $G_{\alpha}$ binds guanosine-5'-triphosphate (GTP) causing dissociation of the $G_{\alpha/\beta\gamma}$ heterotrimeric complex. The subunits target their effectors and activate second messenger signal cascades. (4) RGS proteins stabilise an intermediate complex of GTP hydrolysis on the $G_{\alpha}$ subunit causing rapid attenuation of G-protein signal and (5) reassociation of the $G_{\alpha/\beta\gamma}$ heterotrimeric complex.
1.5 Parkinson’s disease (PD)
PD was first described by James Parkinson in 1817 in his famous monograph “An essay on the shaking palsy”. He described six patients with characteristic resting tremor and the progressive development of motor disabilities.

1.5.1 Clinical symptoms in PD
PD is a progressive neurodegenerative disorder producing a clinical syndrome of bradykinesia, rigidity and resting tremor (Marsden, 1994). These motor disabilities arise from degeneration of dopaminergic neurons in the SNc leaving pathological hallmarks called Lewy bodies (Bethlem and Den Hartog Jager, 1960). PD can occur in young patients termed ‘young-onset’ PD (Golbe, 1991) but prevalence is higher in the elderly, affecting up to 1% of the population over 50 years of age (Marsden, 1994).

In the 1960s, Ehringer and Hornykiewicz showed loss of dopamine in the caudate nucleus and putamen in PD patients (Ehringer and Hornykiewicz, 1960). Following approximately 80% loss of striatal dopamine, clinical cardinal features appear (Marsden, 1982) and are often asymmetric (Fearnley and Lees, 1991). Dopamine deficiency correlates to severity of bradykinesia, which is the ‘slowness of performed movement’ in simple daily tasks (Vingerhoets et al., 1997). Bradykinesia can also be caused by psychological illnesses such as depression, a phenomenon known as ‘kinesia paradoxica’ (Quinn, 1998; Jankovic, 2008). Meanwhile, rigidity is the raised difficulty in movement from increased resistance in muscles and joints, which is consistent during the course of limb movement.

One of the most common symptoms in PD is resting tremor and often appears unilateral, with a high probability of developing over the course of PD (Rajput et al., 1991; Hughes et al., 1993). Resting tremor typically worsens with anxiety and is absent during sleep or performing action. The characteristics of resting tremor are different from essential tremors (Jankovic, 2008). For example, resting tremor occurs at a frequency of 4-6 Hz, while essential tremors occur between 5-10 Hz. Other common motor features of PD include ‘freezing’, a form of akinesia, and postural/ gait disturbances.

A range of non-motor symptoms are present in PD patients but are often overseen in the diagnosis of the disease. These include cognitive deficits (Emre, 2004), autonomic dysfunction (Jost, 2003), depression and sleep disturbances (Zesiewicz et al., 2006).
1.5.2 Aetiology of PD

A single causing factor for PD remains unknown and identifying common factors between PD patients remains challenging. The clinical motor symptoms of PD are fairly characteristic, and the responsiveness to L-DOPA is a successful indicator of parkinsonism (Ward and Gibb, 1990), but definite diagnosis requires post-mortem analysis. There are several potential factors that have been postulated to induce PD including, environmental toxins, genetic factors, glutamate excitotoxicity and mitochondrial defects (Olanow, 2007). However, not all are consistent amongst patients and aetiology generally involves a combination of factors.

**Environmental factors**

The development of parkinsonism is associated with several environmental factors such as exposure to well water, trace metals (cyanide), organic solvents and living in rural areas (Olanow and Tatton, 1999). There is a 70% higher incidence of PD in populations exposed to low-levels of pesticides over long periods (Ascherio et al., 2006). Meanwhile, the most convincing evidence of environmental factors in the cause of PD was from heroin addicts in San Jose, California. Langston et al. (1983) observed the induction of a parkinsonian-like state following exposure to synthetic meperidine derivative, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983). MPTP is converted in astrocytes by monamine oxidase B (MAO-B) to active toxin 1-methyl-4-phenylpyridinium (MPP⁺) (Markey and Schmuff, 1986). MPP⁺ is selectively taken up by dopaminergic neurons through DAT in the SNc (Gainetdinov et al., 1997) and causes cell death. More specifically, MPP⁺ causes inhibition of complex I of the electron transport chain in mitochondria, leading to ATP depletion and increased free radical production (Sayre, 1989).

**Genetic factors**

Familial PD is reported to represent 5-10% of PD cases. However, it has been argued environmental toxin exposure may account for many familial PD cases (Calne et al., 1987). Nine dominant or recessive genetic loci mutations (PARK1-8 and PARK10) have been linked to familial PD (Samii et al., 2004). Single mutations in the α-synuclein gene were identified in familial PD patients of European origin but not in sporadic PD cases (Munoz et al., 1997; Polymeropoulos et al., 1997; Kruger et al., 1998; Bostantjopoulou et al., 2001). The α-synuclein protein is accumulated in Lewy Bodies, suggesting dysfunction of protein degradation mechanisms in PD (Spillantini et al., 1997). Indeed, gene mutation (PARK2) of
parkin encodes for a mutated ubiquitin-protein ligase protein, which suggests a deficiency in the ubiquitin-proteasome system (Samii et al., 2004). PARK2 has been found in autosomal recessive juvenile parkinsonism, with 77% incidence in patients at 20 years of age or below (Lucking et al., 2000). While single mutations in leucine-rich repeat kinase 2 (LRKK2) gene, a protein for cellular adaptation and programmed cell death, have also been reported in both sporadic (Floris et al., 2008) and familial PD cases (Nichols et al., 2005).

**Glutamate excitotoxicity**

Excessive glutamate *in vitro* and *in vivo* can induce neuronal cell death (Olney, 1969; Olney and Sharpe, 1969). This process has been implicated in the pathogenesis of PD due to excessive glutamatergic innervation, particularly from the STN, exacerbating loss of SNc dopaminergic neurons (Rodriguez et al., 1998; Meredith et al., 2009). The proposed mechanism for excitotoxic neurodegeneration is similar to the *in vitro* phenomenon ‘delayed excitotoxicity’. Following excessive exposure to glutamate, self-propagating neuronal death occurs approximately 6-12 hours after initial exposure. Excessive glutamate induces massive Na\(^+\) depolarisation of the postsynaptic neuron, leading to massive Ca\(^{2+}\) influx through ionotropic glutamate receptors and voltage-gated ion channels, activating calcium dependent proteins such as protein kinase C (Choi, 1992). In turn, passive influx of Cl\(^-\) causes build up of cytosolic osmotic pressure (oedema). Subsequently, membranes undergo lysis and mitochondria collapse, which leads to cell death by apoptosis and necrosis (Dugan and Choi, 1994). Furthermore, increased intracellular calcium activates nitric oxidase synthase (NOS) and leads to increased nitric oxide (NO) formation, which generates reactive free radicals targeting the electron transport chain (Dugan and Choi, 1994). Indeed, sustained exposure to nitric oxide, or nitric oxide donor, causes dysfunction of complexes I-IV of the electron transport chain (Bolanos et al., 1996).

**Oxidative stress**

Oxidative metabolism of dopamine produces both hydrogen peroxide (H\(_2\)O\(_2\)) and reactive oxygen species (ROS). Under oxidative stress, there is excess lipid hydroperoxidation and ROS formation, which ultimately leads to cell death by damaging mitochondrial structures. Inhibition of complex I of the mitochondrial electron transport chain can generate ROS (Hensley et al., 1998), which are normally reduced by cell scavenging mechanisms that use anti-oxidants such as glutathione (GSH).
In the parkinsonian state, cells exhibit signs of oxidative stress (Jenner, 1998). For example, in the SNc of PD patients there is increased lipid hydroperoxidation (Dexter et al., 1994), while levels of GSH are reduced, indicating reduced ROS scavenging (Sian et al., 1994). Oxidative stress in PD may be a result of several factors such as excessive dopamine metabolism, an inability to clear hydrogen peroxide (reduced GSH) (Sian et al., 1994) or increased iron promoting hydroxide formation (Dexter et al., 1991; Olanow and Tatton, 1999).

**Mitochondrial defects**
As previously mentioned, MPTP induces parkinsonism through active neurotoxin, MPP⁺. MPP⁺ causes inhibition of NADH-coenzyme Q reductase of complex I mitochondrial electron transport chain and decreases ATP levels, leading to cell death (Singer and Ramsay, 1990). The mechanism of MPP⁺ action has led to interest in mitochondrial defects in PD. Indeed, studies have shown that PD patients have 40% decreased mitochondrial complex I activity in the SNc (Schapira et al., 1989; Schapira et al., 1990). Deficiencies of mitochondrial complex I have also been found in platelets of PD patients (Parker et al., 1989; Krige et al., 1992); while deficiencies of complexes I, II and IV have been identified in skeletal muscle samples (Bindoff et al., 1989).

The aetiology of PD appears to be multifactorial, but is commonly linked to mitochondrial dysfunction, specifically complex I. For example, it has been found that chronic systemic exposure to pesticides (rotenone) leads to defects of mitochondrial complex I in rodents, leading to dopaminergic cell loss and formation of aggregates of α-synuclein protein (Betarbet et al., 2000). Furthermore, the autophagic destruction of dysfunctional mitochondrial is mediated by parkin protein (Narendra et al., 2008). This protein is found mutated at different loci in sporadic and familial PD, and may initiate unregulated cell death (Samii et al., 2004). Although many factors have been linked to the development of PD, a specific mechanism responsible for the progressive neurodegeneration of dopaminergic neurons remains elusive.

### 1.5.3 Animal models of PD
In the last 30 years, animal models have been commonly used in the study of PD and related basal ganglia disorders (Ungerstedt and Arbuthnott, 1970; Langston et al., 1984; Crossman, 1987; Cenci et al., 1998). Animal models have been invaluable in uncovering
the pathophysiological mechanisms in PD, which has helped identify therapeutic targets for symptomatic treatments. In the next section, the main animal models used in PD research will be discussed.

**MPTP-lesioned non-human primate (NHP) model of PD**

Parkinsonism can be induced in NHPs by administration of MPTP at a range of doses, commonly between 1-2 mg/kg, over several days (Pearce et al., 1995; Fox and Brotchie, 2010). However, there are inter-species and inter-animal variabilities with the effective dose of MPTP in reproducing the cardinal symptoms of PD. In *Macaca fascicularis*, MPTP is often administered over 5 consecutive days (Bezard et al., 1997c; Bezard et al., 2001b) at a cumulative dose of 3.0 ± 0.2 mg/kg, which causes ~80% loss of dopamine content in the caudate nucleus and putamen (Bezard et al., 2001b). Methods of MPTP administration include systemic injection into the intraperitoneal cavity, intravenous, intramuscular and intracarotid artery infusion (Przedborski et al., 2001). Unilateral intracarotid infusion induces an asymmetric parkinsonian state, which is advantageous in avoiding intensive post-operative care. The progressive loss of dopaminergic neurons found in PD can be modelled by chronic low dose MPTP treatment over a period of weeks (Przedborski et al., 2001). In this model, however, some species of NHPs such as marmosets are able recover after treatment cessation (Colosimo et al., 1992).

The main advantage of the MPTP-lesioned NHP model is the reproduction of identical motor symptoms such as bradykinesia, rigidity and tremor. However, tremor does not appear in all NHPs species, such as marmosets, following acute or chronic MPTP treatment regimen (Eslamboli, 2005). Like in PD patients, motor function is restored in MPTP-lesioned NHPs following initial dopaminergic replacement therapy (Burns et al., 1983). Moreover, similar to PD patients, long-term dopaminergic replacement therapy in MPTP-lesioned NHPs can induce motor complications such as dyskinesia, ‘wearing off’ and ‘on-off’ phenomena (Crossman et al., 1987; Jenner, 2003b; Fox and Brotchie, 2010).

Neurochemical changes induced by MPTP treatment in NHPs resembles that of PD patients, which has been used to explore the etiology and pathophysiology of PD (Mitchell et al., 1989; Henry et al., 2003; Jenner, 2003a; Fox and Brotchie, 2010). The MPTP-lesioned NHP model of PD shows similar pathological changes to PD patients such as loss of dopaminergic neurons in the SNc, decreased dopamine levels and reduced DAT binding
in the striatum (Bezard et al., 2001b). However, intraneuronal inclusions of α-synuclein aggregates that are found in MPTP-lesioned NHPs are non-fibrillar, making them different to Lewy bodies found in PD patients (Forno et al., 1988; Kowall et al., 2000).

There are several drawbacks of the MPTP-lesioned NHP model such as selective dopaminergic neuronal degeneration, which does not mimic the range of neurotransmitter systems affected in PD (Fox and Brotchie, 2010). The MPTP-lesioned NHP model is also limited by ethical constraints, expensive housing conditions and inter-species variability. However, this model remains the best PD model for testing novel pharmacological treatments, gene therapies and neural grafts for potential future clinical treatments.

**Reserpine-treated rat model of PD**

Reserpine-treated animals have been widely used for modelling clinical PD symptoms such as muscular rigidity, akinesia and hypokinesia (Carlsson et al., 1957; Goldstein et al., 1975). In 1957, Carlsson et al. used reserpine-treated rabbits to identify the effectiveness of L-DOPA on akinesia. Following systemic administration of reserpine, temporary motor deficiencies are induced because of catecholamine depletion from intracellular vesicular stores (Betarbet et al., 2002).

Behavioural tests are used to monitor reserpine-treated animals for motor deficiencies. The direction of locomotor activity is analysed in reserpine-treated rats for a therapeutic response. For example, following administration of a high dose of dopaminergic agents, reserpine-treated rats show increased locomotion in both horizontal and vertical directions, which are used as anti-parkinsonian and dyskinetic responses, respectively (Segovia et al., 2003). Indeed, vertical locomotor activity is reduced in reserpine-treated rats following administration of anti-dyskinetic drugs (Johnston et al., 2005).

The reserpine-treated rat model of PD is an effective model in reproducing motor deficits associated with PD (Colpaert, 1987). However, the main disadvantage of the reserpine-treated rat model of PD is that the induced hypokinetic state is reversible and, thus, varies with time (Goldstein et al., 1975). Reserpine treatment also causes non-selective catecholamine neurotransmitter depletion, which does not accurately model the pathogenesis of PD. Moreover, major pathological changes associated with PD, such as the
loss of dopaminergic neurons in the SNc, are not found in the reserpine-treated rat model of PD (Betarbet et al., 2002).

**MPTP-lesioned mouse model of PD**

In comparison to NHPs, rodents are less sensitive to MPTP toxicity (Schmidt and Ferger, 2001; Schober, 2004). Within species of rodents, rats are less sensitive to MPTP compared to mice (Boyce et al., 1984; Giovanni et al., 1994a; Giovanni et al., 1994b). Moreover, mice strains can differ in their susceptibility to MPTP, with the most sensitive strain being the C57/black6 mouse. MPTP-lesioned mice exhibit non-typical parkinsonian behaviours such as hypersalivation and convulsions (Schober, 2004). A single high dose of MPTP (40 mg/kg, i.p.) can induce transient motor deficits in mice but recover over eight days following initial treatment (Schmidt and Ferger, 2001).

The MPTP-lesioned mouse model of PD is often used to model the pathogenesis of neuronal degeneration. MPTP treatment induces oxidative stress, reducing GSH (Srirame et al., 1997) and increasing lipid peroxidation (Rios and Tapia, 1987). Different MPTP treatment regimens are used in mice to model four different stages of neuronal death in PD (Schmidt and Ferger, 2001; Schober, 2004).

1. Presymptomatic stage (Aubin et al., 1998).
2. Immediate onset PD – a model of rapid neuronal death (Jackson-Lewis et al., 1995).
3. Sub-chronic PD - a model of delayed neuronal degeneration (Tatton and Kish, 1997).
4. Chronic PD - a model of the progressive nature of idiopathic PD (Bezard et al., 1997a; Bezard et al., 1997b).

The MPTP-lesioned mouse model of PD is advantageous compared to other PD models because of fewer ethical constraints, easy maintenance of animals and cost effectiveness (Przedborski et al., 2001). However, the main drawback of the MPTP-lesioned mouse model of PD is that the motor deficiencies induced are often transient, which makes it difficult to test for symptomatic treatments.

**6-Hydroxydopamine (6-OHDA)-lesioned rat model of PD**

6-OHDA is a neurotoxin of the catecholamine transport system that causes degeneration of dopaminergic and noradrenergic neurons (Ungerstedt, 1968). The neurotoxic properties of
6-OHDA are attributed to the induction of oxidative stress (Sachs and Jonsson, 1975; Perumal et al., 1992). Unlike MPTP, which is highly lipophilic, 6-OHDA is not administered systemically because it cannot cross the blood brain barrier. Instead, for specific dopaminergic neuronal degeneration, stereotactic surgery is required. 6-OHDA can be administered into different brain regions such as the striatum, medial forebrain bundle (MFB) or SN for dopamine denervation (Faull and Laverty, 1969; Winkler et al., 2002; Grealish et al., 2008). Presurgical treatment is used with 6-OHDA to increase the efficiency and specificity of dopaminergic lesion. Pargyline and desipramine are commonly used to increase dopamine depletion by MAO-B inhibition and block uptake of 6-OHDA into noradrenergic neurons, respectively (Argenti and D’Mello, 1994; Murphy et al., 1998).

Severity of lesion depends on the dose of 6-OHDA administered (Winkler et al., 2002). Most studies use optimal doses of 6-OHDA, which cause almost immediate dopamine neuronal degeneration. Administration of 6-OHDA into the SN causes neuronal death after ~12 hours, which continues over 31 days (Jeon et al., 1995), while striatal dopamine levels fall to 40% within 3 days (Faull and Laverty, 1969). Administration of high doses of 6-OHDA into the MFB (12 µg) or striatum (28 µg) causes rapid retrograde neuronal degeneration of nigral TH⁺ cells and abolishes striatal TH⁺ cells within 14 days (Grealish et al., 2008). In contrast, the progressive nature of PD is modelled by administration of 6-OHDA into the striatum at a low dose, which induces slow retrograde neuronal degeneration (Przedborski et al., 1995; Winkler et al., 2002). This progressive degenerative model of PD has been used to test neuroprotective strategies (Georgievsk et al., 2002).

In reproducing this model, a unilateral 6-OHDA lesion is preferred to a bilateral lesion to avoid intense post-operative care (Cenci et al., 2002). Although a bilateral lesion mimics the anatomy of PD pathogenesis more accurately, unilateral lesions are used more often because of characterised, quantifiable motor behaviours (Ungerstedt and Arbutnott, 1970). For example, unilateral 6-OHDA-lesioned rats display characteristic rotations following dopamine receptor stimulation. The direction of rotation following dopaminergic challenge is indicative of different stimulatory effects. For instance, following dopamine agonist treatment, unilateral 6-OHDA-lesioned rats display contraversive rotations to lesioned hemisphere due to activation of supersensitive dopamine receptors in the lesioned striatum. These contraversive rotations only occur following ~95% striatal dopamine depletion and
were originally classed as an anti-parkinsonian response (Hudson et al., 1993; Papa et al., 1994).

Rotation towards the ipsilateral side of the 6-OHDA-lesion is induced following administration of dopamine releasing agents such as amphetamine (Ungerstedt and Arbuthnott, 1970). Amphetamine is an indirect dopamine agonist that causes extracellular increase of dopamine in the unlesioned striatum (Langeloh et al., 1987). The ipsilateral rotations induced following amphetamine treatment in unilateral 6-OHDA-lesioned rats is often used to indicate presence of a partial lesion (~50% striatal dopamine denervation) (Hefti et al., 1980; Hudson et al., 1993).

The 6-OHDA-lesioned rat model of PD is used to test for novel symptomatic treatments and neuroprotective strategies. The main advantages of this model are that motor deficits are simple to measure and quantifiable (Ungerstedt and Arbuthnott, 1970; Lundblad et al., 2002; Dekundy et al., 2007). Furthermore, in molecular studies, the unlesioned hemisphere can be used as a control to study changes induced following dopamine denervation (Cenci et al., 1998; Geurts et al., 2003; Konradi et al., 2004). However, this model is limited because the motor behaviours induced following unilateral 6-OHDA lesion, i.e. rotational bias, do not occur in PD patients and require a certain level of interpretation (Marin et al., 2006). Furthermore, the pathogenesis of PD is not accurately modelled, with lack of Lewy body formation and rapid neuronal degeneration.

1.5.4 Pathophysiology of basal ganglia in PD
The classic model of the basal ganglia is often used to describe the pathophysiology of PD (Figure 4; Obeso et al., 2000; Wichmann et al., 2011). The degeneration of nigrostriatal dopamine neurons causes an imbalance of both the direct and indirect pathways of the basal ganglia. With loss of dopamine, basal ganglia motor nuclei are overinhibited due to underactivity of the direct pathway and hyperactivity of the indirect pathway (Albin et al., 1989). Thus, both pathways contribute to reduced feed-back to the motor cortices, thereby reducing movement.

The pathogenesis of PD remains complex but the use of animal models has greatly helped our understanding of this phenomenon. For example, loss of nigrostriatal dopaminergic neurons in PD occurs in a somewhat selective manner. Dopaminergic projections from the
ventral tier of the SNc lack calbindin D28K protein and are more sensitive to neuronal degeneration (Gibb and Lees, 1991; Iravani et al., 2005; Smith and Villalba, 2008). These dopaminergic neurons innervate the sensorimotor regions of the striatum (Haber et al., 1995b).

The striatum undergoes maladaptive changes following dopaminergic neuronal degeneration, to compensate for loss of striatal dopamine innervation. For example, intrastriatal dopaminergic neurons increase in the parkinsonian state (Mura et al., 1995; Meredith et al., 1999; Porritt et al., 2000; Darmopil et al., 2008). Moreover, striatal dopamine D1 receptor responses are sensitised following dopamine depletion (Pifl et al., 1992a; Pifl et al., 1992b; Tong et al., 2004), which may be due to up-regulation of G-protein Gαolf subunits in the striatum (Corvol et al., 2004). The loss of dopamine regulation on corticostriatal glutamatergic projections leads to impaired LTP but this can be restored following dopamine replacement therapy (Centonze et al., 1999; Pisani et al., 2005a). Overall, the loss of dopamine innervation to the striatum causes an imbalanced, unstable system that manifests itself in the clinical features seen in PD.

Striatal mRNA expression of PPE-A and PPE-B have been characterised in the parkinsonian state and indicate the activity of striatofugal pathways. In PD, up-regulated striatal PPE-A mRNA suggests overactivation of striatopallidal neurons, while reduced striatal PPE-B mRNA indicates underactivation of striatonigral projections (Gerfen et al., 1991; Henry et al., 1998; Ravenscroft et al., 2004). Moreover, in the 1980s, the neuronal metabolic marker, 2-deoxyglucose (2-DG) was used to elucidate the activity of basal ganglia subnuclei in the parkinsonian state. Experimental evidence in MPTP-lesioned NHPs showed increased uptake of 2-DG in the GPi, ventral anterior and lateral thalamic nuclei, reflecting a hyperinhibited state (Crossman et al., 1985; Mitchell et al., 1986; Mitchell et al., 1989), while 2-DG was decreased in the STN suggesting a hyperactive state (Mitchell et al., 1989). These findings were later supported from in vitro studies in brain sections of MPTP-lesioned NHPs that showed increased mitochondrial activity of neurons in the STN and GPm/ SNr (Vila et al., 1997).

In addition, electrophysiological recording of STN/ GPm neurons showed increased activity following MPTP treatment in NHPs (Filion et al., 1991; Bergman et al., 1994; Soares et al., 2004). Furthermore, behavioural studies demonstrated that the STN
contributed to the expression of parkinsonian motor symptoms. Indeed, following surgical or neurochemical (muscimol or kainic acid) induced lesions of the STN, PD motor symptoms were dramatically ameliorated in MPTP-lesioned NHPs (Bergman et al., 1990; Aziz et al., 1991; Wichmann et al., 1994; Guridi et al., 1996). These past in vivo and in vitro data has helped revolutionise the understanding of the pathophysiology of PD.
Pathophysiology of PD

**Figure 4.** A schematic diagram illustrating the pathophysiology of Parkinson’s disease (PD). Loss of dopamine input leads to underactivation of the ‘direct’ pathway and overactivation of the ‘indirect’ pathway, indicated by reduced prodynorphin and increased enkephalin expression levels, respectively. Subsequent hyperinhibition of thalamic motor nuclei leads to reduced feed-back through the motor cortex and reduced movement. Arrow size indicates neuronal activity. Dopamine has opposite effects on the dopamine receptors. D₁R, dopamine D₁ receptor; D₂R, dopamine D₂ receptor; Enk, enkephalin; Dyn, prodynorphin; STR, striatum; GPI, lateral globus pallidus; GPm, medial globus pallidus; STN, subthalamic nucleus; SNr, substantia nigra pars reticulata; SNC, substantia nigra pars compacta; VL thalamus, ventrolateral thalamic motor nucleus.
1.6 Treatments for PD

1.6.1 L-DOPA

Following discovery of its anti-akinetic effects in the early 1960s (Carlsson et al., 1957; Friedhoff et al., 1963), L-DOPA remains the most effective symptomatic treatment for PD (Rascol et al., 2002). Unlike its decarboxylated product, 3,4-dihydroxyphenylethylamine or dopamine, L-DOPA readily crosses the blood brain barrier and is suitable for systemic administration. However, to avoid peripheral metabolism and several peripheral side-effects, such as gastrointestinal disturbances and emesis, L-DOPA is administered with peripheral decarboxylase inhibitors carbidopa (Sinemet®) or benserazide (Madopar). L-DOPA is catabolised to dopamine by DOPA decarboxylase and restores striatal dopamine levels.

Despite the loss of dopaminergic neurons in PD, L-DOPA is still converted to dopamine by the serotonergic system (Cenci et al., 2009). However, a combination of unregulated dopamine release from serotonergic terminals and reduced dopamine clearance from loss of DATs in the striatum, can lead to maladaptive changes in pre- and postsynaptic neurons (Cenci and Lundblad, 2006; Cenci and Lindgren, 2007).

L-DOPA-induced motor complications

The concept of ‘priming’ refers to the process of sensitisation following repeated dopaminergic treatment, which leads to abnormal responses to further treatments. For example, long-term L-DOPA treatment in PD patients prime for motor complications, which tend to increase in severity over time (Fahn et al., 2004). These motor complications often take the form of dyskinesia, ‘on-off’ phenomenon, ‘wearing off’ and a range of non-motor fluctuations (Fox and Lang, 2008). There are three main risk factors that increase the chance of developing LID in PD pateints, which are (1) severe parkinsonism, (2) young onset PD and (3) long-term high dose L-DOPA treatment (Ballard et al., 1985; Schrag and Quinn, 2000; Fahn et al., 2004).

Cumulative data over three decade’s show approximately 40% of PD patients develop LID over 4-6 years of L-DOPA treatment, and this rises to 69% after 9-15 years of treatment (Ahlskog and Muenter, 2001). LID commonly appears in an idiosyncratic mixture of chorea (irregular flow of muscular movements in rapid and slow phases) and dystonia (slow twisted movements from abnormal muscular contractions) (Fahn, 2000). PD patients
can exhibit LID at different stages of their L-DOPA dose cycle. For example, ‘peak-dose dyskinesia’ refers to appearance of LID when plasma concentrations of dopamine are highest (Nutt, 1990; Fabbrini et al., 2007). ‘Square wave dyskinesia’ refers to dyskinesia throughout the L-DOPA dose cycle (Fox and Lang, 2008), while ‘disphasic dyskinesia’ refers to the appearance of dyskinesia at the beginning and end of dose cycle, and all are linked to a reduced quality of life (Chapuis et al., 2005).

‘Wearing off’ refers to the reduced therapeutic response to dopaminergic treatments and, thus, cardinal symptoms of PD reappear within hours of treatment (Shoulson et al., 1975). PD patients fluctuate between an ‘on’ state, when PD symptoms are alleviated, and an ‘off’ state, when PD symptoms are present. As a result, the frequency of medication taken is often increased. The ‘wearing off’ effect may be due to the progressive degeneration of dopaminergic neurons found in idiopathic PD (Obeso et al., 2008). Thus, ‘wearing off’ may occur because of the reduced capacity of remaining presynaptic terminals to catabolise dopamine (Papa et al., 1994).

6-OHDA-lesioned rat model of LID
The MPTP-lesioned NHP is the gold-standard model for studying LID as, similar to PD patients, choreiform and dystonic movements are induced following repeated L-DOPA treatment (Crossman, 1987). In the late 1990s, Cenci et al. (1998) characterised dyskinetic-like motor behaviours in L-DOPA-treated unilateral 6-OHDA-lesioned rats. Since then, the unilateral 6-OHDA-lesioned rat model of LID has been widely used for uncovering pathophysiological mechanisms and testing for novel anti-dyskinetic treatments.

Chronic low dose L-DOPA treatment in unilateral 6-OHDA-lesioned rats induce abnormal involuntary movements (AIMs), which are described as purposeless stereotypical movements affecting the jaw, trunk and limbs (Cenci et al., 1998; Lundblad et al., 2002; Dekundy et al., 2007). AIMs are used to model LID in PD patients and are reduced following administration of clinically used anti-dyskinetic drugs (Luginger et al., 2000; Dekundy et al., 2007). However, unilateral 6-OHDA-lesioned rats often show AIMs after the first treatment with L-DOPA, which does not accurately model the clinical scenario. This discrepancy may be due to severe dopamine denervation (>80%) in unilateral 6-OHDA-lesioned rats (Winkler et al., 2002), which lowers the threshold for induction of AIMs (Cenci and Lundblad, 2006). Although AIMs severity can increase with repeated L-
DOPA treatment (Cenci et al., 1998), approximately 20% of animals do not develop AIMs and are used to model stable L-DOPA responders (Konradi et al., 2004).

The unilateral 6-OHDA-lesioned rat model of LID has been used to investigate the pathophysiological state of dyskinetic PD patients (Konradi et al., 2004). One of the best characterised molecular markers for LID is up-regulation of striatal PPE-B mRNA, or its cleaved opioid product prodynorphin (Cenci et al., 1998; Henry et al., 2003). Up-regulation of these molecular markers is found in the lesioned striatum of the unilateral 6-OHDA-lesioned rat model of LID (Cenci et al., 1998; Henry et al., 1999; Ravenscroft et al., 2004). Other pathophysiological changes such as increased striatal dopamine efflux (de la Fuente-Fernandez et al., 2004), also occur in the lesioned striatum of the unilateral 6-OHDA-lesioned rat model of LID (Lindgren et al., 2010).

1.6.2 Dopamine agonists

Dopamine agonists activate postsynaptic receptors and mimic dopaminergic neurotransmission without the need for catabolism in presynaptic neurons. Clinically used dopamine agonists have notably longer plasma half-lives than L-DOPA (Kvernmo et al., 2006) and are used to treat young-onset PD patients because of a lower tendency to induce dyskinesia (Rascol, 2000). However, the anti-parkinsonian efficacy of dopamine agonist treatment decreases after 1-3 years (Montastruc et al., 1993). Thus, PD patients are often introduced to L-DOPA within 5 years of dopamine agonist treatment (Rascol et al., 2000; Kondo, 2002).

Dopamine agonists are classed into two categories; ergot derivatives (bromocriptine, pergolide and cabergoline) and non-ergot derivatives (pramipexole and ropinirole). Both types of drugs display similar levels of motor improvements in newly diagnosed PD patients (Bonuccelli, 2003). However, the clinical use of ergot derived dopamine agonists is limited due to risk of cardiac valvulopathy (Pritchett et al., 2002; Antonini and Poewe, 2007).

PD patients treated long-term with dopamine agonists have lesser risk of developing motor complications (Kondo, 2002). For example, ropinirole, a selective dopamine D2 receptor agonist, reduced the incidence of dyskinesia compared to L-DOPA, and delayed the onset of dyskinesia by 3 years in PD patients (Rascol et al., 2000). Following long-term
pramipexole treatment in newly diagnosed PD patients, dyskinesia were less common compared to L-DOPA-treated PD patients, with reduced parkinsonism as measured by the unified Parkinson’s disease ratings scale (UPDRS) (Parkinson’s, 2009). In advanced PD patients, pramipexole is given as a supplement to reduce the therapeutic dose of L-DOPA, which reduces dyskinesia and daily ‘off time’ (Bennette and Piercey, 1999). Furthermore, pramipexole has neuroprotective effects on dopaminergic SNc neurons against MPTP toxicity in NHPs (Iravani et al., 2006a). Thus, these data support the use of dopamine agonists for initial therapeutic treatment in early diagnosed PD patients (Kondo, 2002).

1.6.3 Catechol-O-methyltransferase (COMT) inhibitors
In the brain, dopamine is metabolised by COMT. COMT catalyzes the 3-O-methylation of dopamine to 3-methoxytyramine, which is then deaminated by monoamine oxidase to homovanillic acid (HVA). COMT inhibitors (entacapone and tocapone) are administered in conjunction with L-DOPA to prolong L-DOPA half-life. Sustained plasma levels of L-DOPA induced by COMT inhibition extends ‘on time’, and reduces ‘off time’, in PD patients (Najib, 2001; Brooks and Sagar, 2003). However, PD patients that receive combined COMT inhibitors and L-DOPA treatment regimens remain at risk to L-DOPA-induced motor complications (Kaakkola, 2000; Deane et al., 2004). Furthermore, COMT inhibitors can cause autonomic dysfunctions such as diarrhea and heptotoxicity (Kaakkola, 2000). Tolcapone can also cause acute fulminant liver damage and, thus, is often avoided for treatment of PD (Olanow, 2000).

1.6.4 Monoamine oxidase B inhibitors
An alternative route of dopamine metabolism is via monoamine oxidase (MAO) enzymes. Dopamine is deaminated by MAO to 3,4-dihydroxyphenylacetic acid (DOPAC), which is oxidized by COMT to HVA. Two subtypes of MAO enzymes are localised in different neuronal regions; MAO-A is found in the mitochondria of the cytosol of presynaptic neuron, while MAO-B resides in mitochondria of astrocytes. Treatment with MAO-B inhibitors (selegeline and rasagiline) in PD patients reduces ‘off time’ and increases ‘on time’ (Calesnick, 1990; Parkinson’s, 2005; Rascol et al., 2005). Furthermore, monotherapy with rasagiline has proved beneficial in PD patients (Stern et al., 2004; Rascol, 2005). Less commonly used treatments are non-selective MAO inhibitors, which can block peripheral MAO enzymes in the gut and cause the ‘cheese effect’. This effect is the build up of
tyramine, a monoamine neurotransmitter, which can be released by noradrenergic synaptic terminals and cause hypertension and hemorrhages (Fahn, 2008).

### 1.6.5 Non-dopaminergic treatments in PD

Potential non-dopaminergic treatments for PD are an area of high interest because of motor complications associated with dopamine replacement therapy (Schapira et al., 2006; Fox et al., 2008a; Fox et al., 2008b). Studies have shown that pharmacological manipulation of several different non-dopaminergic neurotransmitter systems are effective in alleviating PD motor symptoms in patients (Meco et al., 2003; Stocchi et al., 2004; Nutt et al., 2008).

**Acetylcholine in PD**

The striatum has high acetylcholine content compared to other brain regions, despite having a low percentage of cholinergic interneurons (Izzo and Bolam, 1988). The striatal acetylcholine-dopamine balance is important for normal function (Barbeau, 1962) and cellular learning (Calabresi et al., 2007). Following the discovery that dopamine loss occurs in PD patients (Ehringer and Hornykiewicz, 1960), anti-cholinergic agents were suggested for treatment (Barbeau, 1962). Anti-cholinergic treatments, such as benzhexol, also known as trihexyphenidyl, are as effective as L-DOPA in PD patients for reducing tremor (Koller, 1986) but not akinesia (Parkes et al., 1974).

In NHPs, striatal cholinergic interneurons become more synchronised following MPTP treatment (Raz et al., 1996; Raz et al., 2001), which may cause increased release of acetylcholine in the striatum. Recent data in the unilateral 6-OHDA-lesioned rat model of PD, suggested that M$_4$-muscarinic cholinergic autoreceptors were overinhibited by RGS4 proteins, which may cause unregulated acetylcholine release in the striatum (Ding et al., 2006).

However, treatment with anti-cholinergic agents in PD patients is limited because of side-effects such as memory impairment, urinary retention and even motor deterioration (Fabbrini et al., 2002; Schapira, 2007).

**Glutamate in PD**

As mention earlier, the pathophysiology of PD describes hyperactivity of the STN that signals via excitatory glutamatergic transmission to different subnuclei of the basal ganglia.
and mediates expression of motor symptoms (Mitchell et al., 1989; Aziz et al., 1991; Guridi et al., 1996; Vila et al., 1997).

Amantadine, a non-competitive NMDA receptor antagonist, is clinically used to treat PD patients and effectively reduces rigidity, tremor and akinesia in PD patients (Parkes et al., 1974; Koller, 1986; Goetz et al., 2005). Although amantadine can increase dopaminergic neurotransmission (Heikkila and Cohen, 1972; Mizoguchi et al., 1994), the anti-parkinsonian effects of amantadine are mainly attributed to its anti-glutamatergic properties. *In vitro* studies have shown amantadine can antagonise NMDA receptors at distinct sites. For example, amantadine can bind to the phencyclidine site within the cation channel (Kornhuber et al., 1991; Parsons et al., 1996), or the sigma-1 site located outside the cation channel of the NMDA receptor (Kornhuber et al., 1993), stabilising the closed channel state (Blanpied et al., 2005).

NMDA receptors have been implicated in the pathogenesis of PD (Hallett and Standaert, 2004). Post-mortem studies of PD patients showed increased binding to NR1/ NR2B subunits of the NMDA receptor in the putamen (Calon et al., 2003). In MPTP-lesioned NHPs there was decreased protein levels of NR1 and NR2B subunits, while NR2A subunits were unchanged in synaptosomal membrane fractionates (Hallett et al., 2005). In behavioural studies, administration of CP-101606, a selective antagonist of NR2B subunit, reduced parkinsonian motor symptoms in both rodent and NHP models of PD (Steece-Collier et al., 2000). However in PD patients, CP-101606 did not improve parkinsonism but reduced severity of LID by 30% (Nutt et al., 2008). While other anti-glutamatergic drugs such as glutamate release inhibitor safinamide showed modest improvements (~16%) on motor ability in clinical trials (Stocchi et al., 2004).

**1.6.6 Neurosurgical treatments in PD and LID**

**Ablative procedures**

Ablative surgery of the globus pallidus in PD patients was commonly used for symptomatic treatment of parkinsonism from the 1940s, until the introduction of L-DOPA in the 1960s. In the early 1990s, behavioural data revealed that subthalamotomy dramatically improved motor symptoms of PD (Bergman et al., 1990; Aziz et al., 1991; Wichmann et al., 1994; Guridi et al., 1996), which restored interest in ablative procedures for treatment of PD.
Unilateral pallidotomy is effective in advanced PD patients, with long-term benefits on motor symptoms on the contralateral side (de Bie et al., 1999a; Samii et al., 1999; Fine et al., 2000; Yen et al., 2005). Advanced PD patients showed significant improvements on tremor, rigidity and bradykinesia on the contralateral side (Fine et al., 2000); while ipsilateral motor improvements were transient, if at all present (de Bie et al., 1999b).

Although opposite to the predictions made by the classic model of the basal ganglia (Figure 5), unilateral lesions of the GPm alleviates LID in MPTP-lesioned NHPs (Iravani et al., 2005), while there is no effect on LID following ablation of the GPI (Blanchet et al., 1994). Unilateral pallidotomy of the GPm is also effective in alleviating LID in PD patients, with 71-83% reduction of ‘on state’ dyskinesia scores (Samii et al., 1999; Fine et al., 2000). The location within the GPm (anteromedial, ventral or posteroventral areas) for optimal antidykinetic effects remains unclear (Hariz and Hirabayashi, 1997; Krauss et al., 1997; Gross et al., 1999). The use of pallidotomy is, however, limited because of frequent induction of severe side-effects such as cognitive and speech deficits, haemorrhages and fatality (Shannon et al., 1998; de Bie et al., 1999a; Yen et al., 2005).

**Deep brain stimulation (DBS)**

Initial reports showed that high frequency electrode stimulation of the GPm (Siegfried and Lippitz, 1994a; Siegfried and Lippitz, 1994b), subthalamic nucleus (Limousin et al., 1998; Rodriguez et al., 1998a; Deuschl et al., 2006) or thalamic nucleus (Benabid et al., 1987; Benabid et al., 1991; Ondo et al., 1998) was effective for alleviating tremor in PD patients. The effects of DBS, a procedure that requires stereotactic implantation of electrodes for high frequency stimulation, are reversible and can be modulated by implanted hardware (Benabid et al., 1991). Compared to ablative surgery, DBS has fewer associated side-effects when targeted in the same anatomical region, while having similar beneficial effects on PD symptoms such as tremor (Schuurman et al., 2000). Recent experimental evidence in MPTP-lesioned NHPs suggested that the PPN may be a target in DBS for alleviating akinesia in PD (Jenkinson et al., 2004; Jenkinson et al., 2006). The functional mechanism of DBS remains unclear but may include; the induction of virtual lesion, depolarization block and desynchronisation of neuronal pacemaker activity (Vitek, 2002).

DBS of the STN and GPm can also reduce motor complications associated with L-DOPA treatment in PD patients, such as on-off fluctuations and LID (Linazasoro et al., 2003;
Volkmann et al., 2004). Furthermore, the therapeutic dose of L-DOPA can be halved in PD patients following DBS, which, in turn, reduces LID (Limousin et al., 1998).

1.7 Pathophysiology of basal ganglia in LID

Using the classic basal ganglia model, early studies predicted the basal ganglia circuitry in LID to be opposite to the parkinsonian state (Crossman, 1990). Later, experimental evidence helped uncover complex pathophysiological mechanisms in the dyskinetic state.

In LID, hyperinhibition of basal ganglia output nuclei and subsequent disinhibition of thalamic motor nuclei leads to AIMS (Figure 5). These effects are attributed to a hyperactive direct pathway and hyperinhibited indirect pathway, which are induced following long-term L-DOPA treatment in PD. In support of this, dopamine agonist-induced dyskinetic MPTP-lesioned NHPs showed increased 2-DG uptake in the STN and GPm at peak-dose, reflecting a hyperinhibited state of these structures (Mitchell et al., 1992). These findings were later supported by recordings of GPm neurons in dyskinetic PD patients (Merello et al., 1999) and parkinsonian monkeys (Papa et al., 1999), which showed reduced neuronal discharge. While the basal ganglia target nuclei, the ventral anterior and ventral lateral thalamic complex, showed reduced 2-DG uptake in dyskinetic MPTP-lesioned NHPs, which suggested these structures were in a hyperactive state (Mitchell et al., 1992). Subsequent studies that employed thalamotomy in dyskinetic MPTP-lesioned NHPs found the severity of dyskinesia was reduced (Page et al., 1993).

Expression of striatal opioids associated with striatofugal pathways have been characterised in dyskinetic PD patients and in animal models of LID (Cenci et al., 1998; Henry et al., 1999; Henry et al., 2003). In particular, PPE-B expression is localised to the striatonigral neurons of the direct pathway and is specifically increased in LID (Cenci et al., 1998; Henry et al., 2003; Lundblad et al., 2004; Ravenscroft et al., 2004; Aubert et al., 2007). PPE-B is cleaved to opioid peptides (prodynorphin, leu-enkephalin and α-neoendorphin) (Dhawan et al., 1996), which stimulate μ-opioid receptors (You et al., 1996). As a result, μ-opioid receptors become overstimulated in LID (Chen et al., 2005). In support of this, behavioural studies in dyskinetic MPTP-lesioned NHPs showed administration of μ-opioid receptor antagonists reduced LID (Henry et al., 2001).
Another GPCR closely associated with appearance of LID is the dopamine D₁ receptor. Although selective dopamine D₁ receptor agonists can elicit full anti-parkinsonian effects in PD patients (Rascol et al., 2001b) and MPTP-lesioned NHPs (Grondin et al., 1999b), these compounds induce dyskinesia to a similar extent to L-DOPA. Following co-administration of selective dopamine D₁ receptor antagonist, SCH23390, and L-DOPA in MPTP-lesioned NHPs LID is reduced but at the expense of anti-parkinsonian effects (Grondin et al., 1999a). These data suggest overstimulation of dopamine D₁ receptors occurs in the pathogenesis of LID. In contrast, dopamine D₂ receptor agonists, such as ropinirole, are effective anti-parkinsonian agents and have reduced risk of dyskinesia (Rascol et al., 2006). Indeed, chronic treatment with dopamine D₂ receptor agonists in the unilateral 6-OHDA-lesioned rat model of PD mediates anti-parkinsonian effects, without the up-regulation of PPE-B mRNA (Henry et al., 1999; Ravenscroft et al., 2004).

Although reports into the changes of dopamine receptor expression in LID have been relatively inconsistent in PD patients (Rinne et al., 1991; Turjanski et al., 1997), MPTP-lesioned NHPs (Goulet et al., 1997; Grondin et al., 1999b), and 6-OHDA-lesioned rats (Gerfen et al., 1990; Konradi et al., 2004; Nadjar et al., 2006), the abnormalities in downstream signalling has helped elucidate abnormal dopamine signalling in PD and LID. For example, supersensitive dopamine D₁ receptors in PD are likely to be mediated by increased levels of Gαolf subunits, which are found in PD patients (Corvol et al., 2004) and 6-OHDA-lesioned rats (Herve et al., 1993). In LID, brain sections of dyskinetic MPTP-lesioned NHPs showed supersensitive dopamine D₁ receptor-mediated responses in in vitro GTPγS binding (Aubert et al., 2005). These findings are supported by abnormally high levels of second messenger signals downstream of the dopamine D₁ receptor signalling pathway, such as DARPP-32 and cdk5, in the dyskinetic state (Oh and Chase, 2002; Picconi et al., 2003; Aubert et al., 2005; Guan et al., 2007).
Pathophysiology of LID

Figure 5. A schematic diagram illustrating the pathophysiology of L-DOPA-induced dyskinesia (LID). The loss of dopamine input and repeated non-physiological stimulation of dopamine receptors leads to an overactive ‘direct’ pathway and underactive ‘indirect’ pathway. Subsequent disinhibition of motor nuclei leads to increased excitatory input to motor cortical regions producing abnormal involuntary movements. Arrow size indicates neuronal activity. Dopamine has opposite effects on the dopamine receptors. \( D_1 \)R, dopamine \( D_1 \) receptor; \( D_2 \)R, dopamine \( D_2 \) receptor; Enk, enkephalin; Dyn, prodynorphin; STR, striatum; GPI, lateral globus pallidus; GPM, medial globus pallidus; STN, subthalamic nucleus; SNr, substantia nigra pars reticulata; SNC, substantia nigra pars compacta; VL thalamus, ventrolateral thalamic motor nucleus.
1.7.1 Limitations of the basal ganglia model

In the classic model of the basal ganglia, the segregation of the direct and indirect pathways is an area of debate (Nadjar et al., 2006). Retrograde tracing studies in squirrel monkeys have shown consistent striatofugal projections to both GPM and GPI (Levesque and Parent, 2005). Furthermore, studies in rat striatal tissue found a high percentage of MSNs express functional dopamine D₁ and D₂ receptors (Surmeier et al., 1996; Yung et al., 1996; Aizman et al., 2000). Thus, these data suggest non-segregated direct and indirect pathways of the basal ganglia (Yung et al., 1996). In contrast, studies in bacterial artificial chromosome (BAC) transgenic mice show only a small percentage (5-6%) of MSNs co-express dopamine D₁ and D₂ receptors in the dorsolateral striatum, but a higher percentage (~17%) was found in the nucleus accumbens (Bertran-Gonzalez et al., 2008; Bertran-Gonzalez et al., 2010).

The circuitry in the classic model of the basal ganglia remains oversimplified (Alexander and Crutcher, 1990). For example, extensive dopaminergic projections to basal ganglia subcortical nuclei (GPI, GPM, STN and SNr) are often overlooked (Joel and Weiner, 2000; Prensa et al., 2000; Smith and Kieval, 2000; Sanchez-Gonzalez et al., 2005; Smith and Villalba, 2008; Rommelfanger and Wichmann, 2010). Such dopaminergic projections can directly modulate the neuronal activity of the basal ganglia subnuclei and by-pass feed-forward activity from striatum (Kreiss et al., 1996; Francois et al., 2000; Jan et al., 2000).

Other inconsistencies of the basal ganglia model were revealed from behavioural studies in animal models following ablative surgery. Pallidotomy of the GPM reduced dyskinesia in dyskinetic MPTP-lesioned NHPs (Iravani et al., 2005), which is opposite to the expected outcome predicted from the classic model of the basal ganglia (Marsden and Obeso, 1994; Obeso et al., 2011; Wichmann et al., 2011). Moreover, lesions of motor thalamic nuclei in PD patients do not worsen hypokinesia, which again is inconsistent with the model (Marsden and Obeso, 1994). Thus, information that is processed for motor behaviour in the basal ganglia is much more complex than originally thought (Alexander et al., 1986). It is suggested that motor programmes are dependent on neuronal synchronicity of firing patterns between basal ganglia subnuclei (Soares et al., 2004) and such neuronal activity is modulated by dopamine (Brown et al., 2001). Interestingly, in PD patients, macroelectrode recordings of local field potentials from STN and GPM neurons show increased neuronal synchronicity (Brown et al., 2001). These neurons show specific bands of frequency in
different motor states. For example, when PD patients are in the ‘off state’, STN and GPm neurons fire below 30 Hz (β-band); while in the ‘on state’ neurons fire at 60-80 Hz (γ-band) (Brown et al., 2001; Alonso-Frech et al., 2006). In dyskinetic PD patients, STN neurons exhibit neuronal activity at ~6 Hz (θ-band) (Alonso-Frech et al., 2006). It is speculated that subthalamotomy or DBS of the STN may elicit anti-dyskinetic effects by ‘re-setting’ neuronal firing patterns (Vitek, 2002; Brown and Eusebio, 2008).

1.8 L-DOPA treatment induced cellular changes

In normal basal ganglia physiology, dopamine receptors are stimulated in a continuous manner (Grace, 2008). This is a resultant effect of tonic firing of dopaminergic neurons, which holds steady-state dopamine levels in the striatum (Venton et al., 2003). In comparison, during phasic dopaminergic neuronal firing, local dopamine concentrations in the striatum are fluctuated (Venton et al., 2003).

In healthy brains, treatment with L-DOPA is efficiently buffered, cleared and stored by presynaptic neurons (Rodriguez et al., 2007). However, following loss of dopaminergic neurons, there is disruption of the buffering capacity and vesicle storage of dopamine, which lowers the threshold for induction of LID (Cenci and Lundblad, 2006). Indeed, positron emission tomography (PET) scans in dyskinetic PD patients have shown large extracellular dopamine levels are inefficiently cleared after L-DOPA treatment, which is due to reduced uptake of dopamine following loss of nigrostriatal terminals (Troiano et al., 2009). L-DOPA is also catabolised to dopamine by the serotonergic system. However, in the dopamine-denervated state, serotonergic neurons release dopamine in an unregulated manner due to the absence of dopamine autoreceptor regulation on presynaptic terminals (Arai et al., 1995; Maeda et al., 2005; Cenci and Lundblad, 2006).

Intermittent L-DOPA treatment in PD stimulates dopamine receptors in a non-physiological pulsatile manner, which primes for dyskinesia. The short L-DOPA half-life (60-90 mins) means that dopamine receptors fluctuate from high to low cycles of activation following treatment (Nutt et al., 1985; de la Fuente-Fernandez et al., 2004). In LID, high extracellular striatal dopamine levels correlate to expression of dykinesia in PD patients (de la Fuente-Fernandez et al., 2004). Behavioural studies in MPTP-lesioned NHPs have shown that pulsatile dopamine receptor stimulation mediates induction of dyskinesia. For example, intermittent administration, but not continuous infusion, of short acting dopamine
D₁ or D₂ agonists induce dyskinesia (Blanchet et al., 1995; Goulet et al., 1996; Grondin et al., 1999b) and cause up-regulation of striatal PPE mRNA expression in MPTP-lesioned NHPs (Morissette et al., 1997). Comparisons between intermittent and continuous L-DOPA treatment have also been made in PD patients (Stocchi et al., 2005) with less dyskinesia induced following continuous L-DOPA infusion. Thus, continuous dopaminergic stimulation (CDS) is beneficial in avoiding induction of motor complications (Olanow et al., 2006). However, CDS has been linked with a risk of psychosis and tolerance in PD patients (Vaamonde et al., 1991).

Long-term intermittent L-DOPA treatment in PD mediates persistent fluctuations of striatal dopamine receptor activity, leading to maladaptive cellular changes associated with LID (Cenci and Lindgren, 2007; Cenci and Konradi, 2010). Both pre- and postsynaptic plasticity-induced changes have been identified in animal models of LID (Cenci and Lundblad, 2006). Chronic L-DOPA treatment in unilateral 6-OHDA-lesioned rats causes increased striatal dopamine D₁ receptor mRNA levels (Visanji et al., 2009). Moreover, dopamine D₁ receptors undergo abnormal subcellular trafficking in LID, which leads to increased recruitment to the plasma membrane and cytoplasm (Guigoni et al., 2007; Berthet et al., 2009). The abnormal activation of dopamine D₁ receptors in LID also leads to increased second messenger signals such as DARPP-32 (Picconi et al., 2003; Aubert et al., 2005; Guan et al., 2007) and ERK1/2 (Pavon et al., 2006; Santini et al., 2007; Westin et al., 2007).

Second messenger signalling pathways associated with dopamine receptors have major implications in the pathophysiology of LID (Santini et al., 2008). For example, genetic manipulation of signalling factors downstream of dopamine D₁ receptors can prevent induction of dyskinesia. In 6-OHDA-lesioned DARPP-32 knockout mice, AIMS are expressed at lower levels compared to wild-type following L-DOPA treatment (Santini et al., 2007). Moreover, both dopamine D₁ and NMDA receptor activation in LID cause increased phosphorylation of ERK1/2 (Calabresi et al., 2008), which positively correlates to AIMS in rodent models of LID (Pavon et al., 2006; Santini et al., 2007; Westin et al., 2007). The activation of ERK1/2 leads to phosphoprylation of mitogen- and stress-activated kinase-1 (MSK-1) (Santini et al., 2008). In turn, MSK-1 activates CREB (Carlezon et al., 2005), which increases expression of immediate early genes (IEGs) such as c-fos, jun and fosB in LID (Andersson et al., 1999; Cenci et al., 1999; Berton et al., 2009; Cao et al.,
2010). Specific IEGs, fos and jun, form the activating protein 1 (AP1) heterotrimer that binds to CREB/AP-1 binding sites in the promoter elements of prodynorphin gene. Moreover, IEG subtypes ΔfosB/fosB are up-regulated in the striatum of unilateral 6-OHDA-lesioned rats following chronic L-DOPA treatment and, in turn, specifically up-regulate prodynorphin mRNA (Andersson et al., 1999; Andersson et al., 2003). In behavioural studies using the unilateral 6-OHDA-lesioned rat model of LID, a causal role of ΔfosB has been identified. Knockdown of fosB via intrastriatal administration of antisense oligonucleotides attenuated AIMs and inhibited prodynorphin mRNA up-regulation (Andersson et al., 1999). Further studies showed overexpression of ΔfosB induced AIMs in treatment naïve unilateral 6-OHDA-lesioned rats (Cao et al., 2010). In MPTP-lesioned NHPs, ΔfosB is also found up-regulated in the motor striatum and can be inhibited by viral-mediated overexpression of ΔJunD proteins, which attenuates dyskinesia (Berton et al., 2009).

As mentioned above, abnormal overactivation of dopamine D1 and NMDA receptor signalling pathways in LID leads to synaptic plasticity-induced changes (Calabresi et al., 2008; Santini et al., 2008; Cenci and Konradi, 2010). An example of this is the inability of corticostriatal synapses to depotentiate following induction of LID (Picconi et al., 2003). This pathophysiological feature may reflect an inability of neurons to ‘de-select’ or suppress unwanted motor programmes initiated in the basal ganglia motor circuit.

Dopamine D2-mediated signalling pathways are less well characterised in LID. Although dopamine D2 receptor signalling opposes the G αolf/ cAMP/ PKA signalling pathway, repeated dopamine D2 receptor agonist (U91356A) treatment can still induce dyskinesia (Blanchet et al., 1995; Morissette et al., 1997). Recent data suggests factors of the non-canonical dopamine D2 receptor signalling pathway may play pathophysiological roles in LID. For example, in dyskinetic MPTP-lesioned NHPs, phosphorylated non-canonical signalling molecules downstream of dopamine D2 receptors, Akt (Ser473) and GSK3β (Ser9), are positively correlated to the severity of dyskinesia (Morisette et al., 2010). Moreover, overexpression of RGS9-2, an endogenous regulator of dopamine D2 receptors, attenuated dyskinesia in MPTP-lesioned NHPs (Gold et al., 2007).
1.8.1 Non-dopaminergic systems in LID

**Glutamate**

Anti-glutamatergic treatments have shown anti-dyskinetic effects in LID. In PD patients, administration of riluzole, a glutamate release inhibitor, reduced dyskinesia severity by up to 30% (Merims et al., 1999), while amantadine, a non-selective NMDA receptor antagonist, is often clinically used for dyskinesia and can reduce severity by up to 50% in PD patients (Metman et al., 1999; Luginger et al., 2000). Unfortunately, the therapeutic efficacy of amantadine lasts on average 4.9 months (Thomas et al., 2004) and causes side-effects such as cognitive decline and psychosis (Luginger et al., 2000; Thomas et al., 2004).

NMDA ionotropic glutamate receptors show pathophysiological changes in LID. In the unilateral 6-OHDA-lesioned rat model of PD, striatal NR2A/NR2B subunits are hyperphosphorylated at tyrosine residues (Menegoz et al., 1995) and are further phosphorylated following repeated L-DOPA treatment (Oh et al., 1998). In other studies using animal models of LID, striatal NR2A protein levels were found increased (Hallet et al., 2005; Gardoni et al., 2006); while results have been inconsistent for striatal NR2B proteins (Hallet et al., 2005; Hurley et al., 2005; Gardoni et al., 2006). In dyskinetic MPTP-lesioned NHPs, subcellular analysis of striatal synaptosomal membranes revealed that NR1/NR2B protein levels are increased to normal levels following L-DOPA treatment (Hallet et al., 2005), while in severely dyskinetic MPTP-lesioned NHPs, striatal NR1/NR2B NMDA receptor binding was increased compared to animals with low levels of dyskinesia (Hurley et al., 2005). Further studies have shown the involvement of NR2B subunits in LID may be attributed to downstream interactions with membrane-associated guanylate kinase (MAGUK). A pioneering study showed that peptide blockade of NR2B subunit interaction with MAGUK proteins in non-dyskinetic unilateral 6-OHDA-lesioned rats caused an immediate induction of AIMs (Gardoni et al., 2006).

Behavioural studies in MPTP-lesioned NHPs have shown repeated de novo treatment with NR1/NR2B antagonist (CI-1041) in combination with L-DOPA prevented the induction of dyskinesia (Morissette et al., 2006). Acute treatment with NR1/NR2B antagonists is also beneficial in attenuating dyskinesia in MPTP-lesioned NHPs (Blanchet et al., 1999) and PD patients (Nutt et al., 2008). The specific NR2B selective NMDA receptor antagonist, CP-101606 reduced LID by 30% in PD patients but caused cognitive deficits (Nutt et al., 2008). Studies have suggested a possible strategy to overcome such adverse effects by combining
treatment with AMPA receptor antagonists at sub-threshold doses. These treatment regimens have been tested in MPTP-lesioned NHPs and showed a synergistic effect on the reduction of dyskinesia (Bibbiani et al., 2005; Kobylecki et al., 2011).

Recent data in MPTP-lesioned NHPs showed AMPA receptor subunits GluR2/3 are up-regulated in the dyskinetic state (Silverdale et al., 2010). Moreover, Kobylecki et al. (2010) showed that antagonism of calcium permeable AMPA receptors, with specific antagonist IEM1460, reduced dyskinesia in MPTP-lesioned NHPs and unilateral 6-OHDA-lesioned rats. In the same study, de novo treatment with IEM1460 + L-DOPA prevented the induction of AIMs in unilateral 6-OHDA-lesioned rats (Kobylecki et al., 2010). A similar result was found following de novo treatment with MTEP, a metabotropic glutamate receptor 5 (mGluR5) antagonist, in unilateral 6-OHDA-lesioned rats (Mela et al., 2007; Rylander et al., 2009).

**Serotonin**

The serotonergic (5-hydroxytryptamine, 5-HT) system widely innervates the basal ganglia (Lavoie and Parent, 1990). As mentioned previously, serotonergic neurons catabolise L-DOPA to dopamine after loss of nigrostriatal dopaminergic neurons (Cenci and Lundblad, 2007; Carta et al., 2008). In this case, serotonergic afferent neurons release dopamine as a ‘false transmitter’ with serotonin in the striatum (Tanaka et al., 1999; Carta et al., 2007). This unregulated release of dopamine is linked to the development and expression of LID (Lindgren et al., 2010). Indeed, lesion of the serotonergic system in unilateral 6-OHDA-lesioned rats can prevent the induction of AIMs following repeated L-DOPA treatment (Carta et al., 2007).

Serotonergic autoreceptors are targets for modulating dopamine release, making them potential targets for treatment of LID (Carta et al. 2008; Fox et al., 2008b). Over 14 different 5-HT receptor subtypes have been identified in the central nervous system (Hoyer et al., 1994). With the exception of 5-HT$_3$, which forms an ion channel, all the known subtypes of 5-HT receptors are G-protein coupled. 5-HT receptors are located on the pre- and postsynaptic membranes of neurons in the basal ganglia (Rav-Acha et al., 2008). 5-HT$_{1A}$ receptors are located on both pre- and postsynaptic terminals (Kreiss and Lucki, 1994) and can mediate autoreceptor inhibition of neurotransmitter release (Invernizzi et al., 1991; Casanovas and Artigas, 1996; Beyer et al., 2004).
Several studies have investigated the pathophysiological roles of 5-HT$_{1A}$ in LID and data suggests that it may be a suitable therapeutic target. In the unilateral 6-OHDA-lesioned rat model of LID, AIMS are reduced following acute 5-HT$_{1A}$ agonist treatment (Carta et al., 2007; Dupre et al., 2007; Eskow et al., 2007; Dupre et al., 2008a). In dyskinetic MPTP-lesioned NHPs, treatment with selective 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, reduced LID but compromised the anti-parkinsonian effects of L-DOPA (Iravani et al., 2006b), while in dyskinetic PD patients, treatment with sarizotan, a combined 5-HT$_{1A}$ agonist and dopamine D$_2$/D$_4$ receptor antagonist, reduced LID and increased ‘on time’ (Olanow et al., 2004). In smaller clinical trials, partial 5-HT$_{1A}$ agonists, buspirone and clozapine effectively reduced dyskinesia in PD patients during several weeks of treatment without compromising the beneficial effects of L-DOPA (Bonifati et al., 1994; Pierelli et al., 1998).

**Cannabinoids**

The striatal endocannabinoid system shows profound neurochemical changes in parkinsonian patients (Pisani et al., 2005b; Pisani et al., 2010) and animal models (Gubellini et al., 2002; Van der Stelt et al., 2005). Levels of endogenous cannabinoids, anandamide (N-arachidonoylthanolamine, AEA) and 2-arachidonoyl-glycerol (2-AG) are drastically increased (49% and 88%, respectively) in the striatum of untreated MPTP-lesioned NHPs (Van der Stelt et al., 2005), while in PD patients, AEA levels in the cerebrospinal fluid were found 2-fold greater compared to control levels (Pisani et al., 2005b; Pisani et al., 2010). Interestingly, in a recent study, AEA levels were normalised following chronic dopamine replacement therapy in PD patients (Pisani et al., 2010).

AEA and 2-AG activate CB$_1$ receptor (Howlett et al., 2010), a GPCR expressed at high densities in the basal ganglia (Herkenham et al., 1991; Maileux et al., 1992). In vitro studies using striatal sections of MPTP-lesioned NHPs showed increased [$^{35}$S]GTP$_{γ}$S binding after CB$_1$ agonist (WIN55,212-2) activation, which suggests hyper-responsive of CB$_1$ receptors in the PD state (Lastres-Becker et al., 2001). This overactivation of CB$_1$ receptors is, however, normalised after chronic L-DOPA treatment (Lastres-Becker et al., 2001). 

Behavioural studies in MPTP-lesioned NHPs have shown treatment with rimonabant, an inverse CB$_1$ receptor agonist, dose-dependently reduced parkinsonism and dyskinesia (Van
der Stelt et al., 2005). Furthermore, administration of synthetic cannabinoid agonist, nabilone reduced dyskinesia and potentiated the anti-parkinsonian effects of L-DOPA in MPTP-lesioned NHPs (Fox et al., 2002). In a small pilot study, nabilone reduced dyskinesia from ‘marked’ to ‘moderate’ severity in PD patients but caused sedation and hallucinations (Sieradzan et al., 2001). It has been suggested that the anti-parkinsonian and anti-dyskinetic effects of cannabinoid agonists may, at least partly, be due to a reduction of glutamatergic transmission in the striatum (Gerdeman and Lovinger, 2001).

**Noradrenaline**

Noradrenergic receptors, subtypes α2a, α2c and β1, are highly expressed in the dorsal striatum (Nicholas et al., 1996; Holmberg et al., 1999). Specifically, noradrenergic α2c receptors are located on MSNs of the striatonigral pathway, and may contribute to the overactivation of direct pathway in LID (Figure 5; Fox et al., 2008a). Selective antagonism of α2c adrenergic receptors reduced dyskinesia in PD patients (Rascol et al., 2001a), MPTP-lesioned NHPs (Grondin et al., 2000) and unilateral 6-OHDA-lesioned rats (Lundblad et al., 2002). Treatment with α2a/α2c adrenergic receptor antagonist, fipamezole in MPTP-lesioned NHPs reduced dyskinesia severity and extended L-DOPA ‘on time’ (Savola et al., 2003). Although the beneficial effects of selective α2c adrenergic receptor antagonist, idazoxan has been, albeit, inconsistent in two pilot studies in dyskinetic PD patients (Manson et al., 2000; Rascol et al., 2001a), treatment with this compound is unsuitable because of associated side-effects such as headaches, nausea and vomiting (Manson et al., 2000).

**Opioids**

As mentioned above, induction of LID is associated with up-regulation of striatal PPE-B mRNA (Henry et al., 1999; Henry et al., 2003; Ravenscroft et al., 2004). Pharmacological modulation of opioid receptors (μ, δ, and κ) mediates varied effects on LID in clinical and preclinical studies (Henry et al., 2001; Fox et al., 2004; Cox et al., 2007). For example, non-selective opioid antagonist, naloxone was not effective in reducing LID in PD patients, but increased duration of L-DOPA action (Fox et al., 2004). Specific κ-receptor antagonist U50,488 reduced LID in MPTP-lesioned NHPs but compromised the anti-parkinsonian effects of L-DOPA (Cox et al., 2007), while the δ-opioid receptor antagonist, naltrindole reduced dyskinesia in MPTP-lesioned NHPs, with no effect on the anti-parkinsonian effects of L-DOPA (Henry et al., 2001). It has been suggested that the anti-dyskinetic
effects induced by natrindole may be a result of blockade of δ-opioid receptors in the globus pallidus (McCormick and Stoessl, 2002).

The μ-opioid receptors are co-expressed with prodynorphin (Guttenberg et al., 1996) and may be a therapeutic target to dampen the hyperactivity of the direct pathway in LID (Figure 5). Administration of μ-opioid receptor antagonist, cyprodine in dyskinetic MPTP-lesioned NHPs reduced dyskinesia (Henry et al., 2001). Interestingly, administration of μ-opioid receptor agonist, morphine also induced anti-dyskinetic effects in L-DOPA-primed MPTP-lesioned NHPs (Samadi et al., 2004). It remains unknown how both agonist and antagonistic actions on μ-opioid receptors can lead to anti-dyskinetic effects (Henry et al., 2001; Samadi et al., 2004). Speculatively, the overall behavioural response may depend on the pre- or postsynaptic μ-receptors that are stimulated in different brain regions. Recent data suggests that RGS proteins play a major role in the behavioural responses of μ-opioid receptors, which can be opposite depending on the anatomical location of RGS proteins in the brain (Han et al., 2010).

1.9 RGS proteins in PD and LID

GPCRs such as dopamine, serotonin, metabotropic glutamate, adrenergic and opioid receptors, initiate abnormal neurotransmitter signals in PD and LID. Many of these GPCRs are regulated by endogenous RGS proteins in the basal ganglia. Thus, RGS proteins may have pathophysiological roles in PD and LID.

The first study that showed changes in RGS proteins in PD was conducted in post-mortem analysis of protein levels in the striatum of PD patients (Tekumalla et al., 2001). Authors of this study found increased RGS9-2 and ΔfosB protein levels in L-DOPA-treated PD patients. However, results were inconclusive as to whether these changes were due to parkinsonism or dyskinesia (Tekumalla et al., 2001). A later study conducted in dyskinetic and non-dyskinetic MPTP-lesioned NHPs showed striatal RGS9-2 protein levels remained unchanged (Gold et al., 2007). Interestingly, behavioural data from studies conducted in animal models have indicated that RGS9-2 proteins are implicated in the expression of AIMs. Kovoor et al. (2005) showed that AIMs were rapidly induced in reserpine-treated RGS9-2 KO mice following treatment with apomorphine or quinpirole. Meanwhile, a study in MPTP-lesioned NHPs showed that viral-mediated overexpression of RGS9-2 reduced dyskinesia, without compromising the anti-parkinsonian effects of L-DOPA (Gold et al.,
The author’s of this paper suggested these anti-dyskinetic effects were attributed to dampening dopamine D2 receptors, as the acute anti-parkinsonian effects of dopamine D2 agonist, ropinirole were compromised following overexpression of striatal RGS9-2 (Gold et al., 2007).

Other RGS protein subtypes have been linked to the expression of motor symptoms in PD (Geurts et al., 2003; Ding et al., 2006). In the unilateral 6-OHDA-lesioned rat model of PD, mRNA levels of RGS proteins 2, 5, 8 were elevated, while RGS4 and 9 mRNA levels were reduced in the striatum (Geurts et al., 2003). The functional relevance of these changes in RGS protein mRNA levels are yet to be understood (Herrera-Marschitz et al., 2010). Of particular interest, RGS4 proteins appear to mediate motor behaviours, as the only behavioural phenotype displayed in RGS4 KO mice is a sensorimotor deficit (Grillet et al., 2005).

RGS4 mRNA is found in high ChAt regions (Ding et al., 2006; Ebert et al., 2006). In the striatum, such regions are classed as the matrix compartments (Pert et al., 1976; Graybiel et al., 1986; Gerfen, 1992a) and are part of the basal ganglia motor circuit, receiving inputs from sensorimotor cortical associated areas (Gerfen, 1989; McGeorge and Faull, 1989; Gerfen, 1992b).

In 2006, Ding et al. showed that RGS4 mRNA was increased in striatal intercholinergic neurons in the reserpine-treated and unilateral 6-OHDA-lesioned rat models of PD. Using in vitro techniques, authors showed up-regulation of RGS4 caused inhibition of M4-cholinergic autoreceptors, which was suggested to mediate the increased striatal acetylcholine levels in PD (Ding et al., 2006). Given that RGS4 proteins modulate a range of GPCRs affected in PD and LID, such as mGluR5, 5-HT1A, dopamine D2, M4-cholinergic and µ-opioid receptors (Beyer et al., 2004; Ghavami et al., 2004; Garzon et al., 2005c; Ding et al., 2006; Ho et al., 2007; Schwendt and McGinty, 2007), the pathophysiological roles of RGS4 proteins are likely to extend beyond a single receptor.

In summary, data published to date suggests that RGS proteins are implicated in the pathophysiology of PD and LID. However, further studies are needed to elucidate the functional mechanisms of RGS proteins in PD and LID, which may help identify novel therapeutic targets for future treatments.
1.10 **Overall aims and objectives**

We hypothesise that various RGS protein subtypes play pathophysiological roles in PD and LID and contribute to abnormal motor function. Therefore, using the unilateral 6-OHDA-lesioned rat model of PD and LID, we aim to:

1) Characterise the spatio-temporal gene expression profiles of several RGS protein subtypes in the striatum.

2) Characterise the acute effects of RGS protein inhibition on AIMS and motor ability following combined treatment with L-DOPA.

3) Determine the effects of combined *de novo* treatment with RGS protein inhibitors and L-DOPA on the induction of AIMS.

4) Elucidate the specific mechanisms that underlie the roles of RGS proteins in LID.
Chapter 2

The spatio-temporal distribution of RGS protein gene expression in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease and L-DOPA-induced dyskinesia
2.1 Introduction

The most common pharmacological treatment for PD is L-DOPA, the immediate metabolic precursor of dopamine, which effectively restores motor function (Friedhoff et al., 1963; Barbeau, 1969). However, long-term L-DOPA treatment can lead to debilitating side-effects such as LID (Cotzias et al., 1969). These side-effects occur in 40% of PD patients after 4-6 years of L-DOPA treatment (Ahlskog and Muenter, 2001) and significantly reduce quality of life (Chapius et al., 2005). The underlying cause of LID remains unknown. However, evidence suggests that non-physiological, pulsatile dopamine receptor stimulation contributes to the induction of LID (Blanchet et al., 1995; Stocchi et al., 2005).

In the striatum, GPCRs such as dopamine receptors are regulated by certain endogenous proteins (RGS proteins) (Zachariou et al., 2003; Kovoor et al., 2005; Celver et al., 2010). These RGS proteins negatively modulate neurotransmitter receptor signalling mechanisms (Tesmer et al., 1997a; Ghavami et al., 2004). In response to acute non-physiological dopamine receptor stimulation, RGS protein mRNA is immediately regulated (Geurts et al., 2002; Taymans et al., 2003) prior to protein translation (Schwendt and McGinty, 2007), which subsequently leads to modulation of G-protein signalling pathways.

In the parkinsonian state, RGS proteins in the striatum are susceptible to long-term changes following dopamine denervation (Tekumalla et al., 2001; Geurts et al., 2003; Ding et al., 2006). For example, striatal RGS9-2 protein levels are reduced in animal models of PD (Postashkin et al., 2007; Yin et al., 2010). These protein levels are elevated in L-DOPA-treated PD patients (Tekumalla et al., 2001), which indicates an intrinsic compensatory mechanism in response to dopaminergic replacement therapy. The RGS9-2 protein may be a therapeutic target to dampen non-physiological dopamine receptor stimulation (Neubig and Siderovski, 2002; Traynor and Neubig, 2005). Indeed, overexpression of RGS9-2 in the striatum inhibits dopamine D_{2} receptor signalling (Rahman et al., 2003), which reduces dyskinesia in MPTP-lesioned NHPs (Gold et al., 2007).

Other RGS protein subtypes are found expressed in the striatum (Gold et al., 1997) but their functional roles in PD and LID remain to be established. For example, RGS2 and RGS4 proteins are found co-localised with striatal dopamine D_{1} and D_{2} receptors, respectively (Taymans et al., 2004). These RGS proteins may have functional significance on the direct and indirect pathways of the basal ganglia (Herrera-Marschitz et al., 2010)
and could be targeted to improve motor function in PD and LID. Importantly, any abrupt changes in RGS protein levels following dopamine denervation or dopaminergic treatment could mediate neurotransmitter receptor sensitisation. Thus, RGS proteins may play major roles in the development and expression of motor symptoms in PD and LID. A previous study has shown that acute dopamine agonist treatment caused fluctuations in striatal RGS2 and RGS4 mRNA levels in unilateral 6-OHDA-lesioned rats (Taymans et al., 2004). These changes indicate early maladaptive responses to dopaminergic replacement therapy in PD, which may have important implications on the development of LID.

2.1.1 Aims and objectives

We hypothesize that RGS protein gene expression is abnormally expressed in PD and LID. Using the unilateral 6-OHDA-lesioned rat model of PD and LID, we aim to:

1) Characterise the spatio-temporal distribution of RGS2, RGS4 and RGS9-2 mRNA levels in the striatum.

2) Investigate whether any change in RGS protein mRNA correlates to the expression of AIMs or a molecular correlate of LID (PPE-B mRNA).
2.2 Materials and Methods
All animal work was carried out under the regulations of the Animals (Scientific Procedures) Act, 1986.

2.2.1 Animals
Male Sprague-Dawley rats (220-230g, Charles River, UK) were housed in a controlled environment with a 12 hour light/dark cycle (light periods 8:00 am – 8:00 pm), constant temperature (22 ± 1°C), humidity (relative, 30%) and free access to food (Standard pellets, B&K Universal) and water. Rats were housed with a maximum of four per cage. Rats were habituated to housing conditions for at least one week prior to surgery.

Unilateral 6-hydroxydopamine lesion of the right medial forebrain bundle
Unilateral 6-OHDA lesion of the right medial forebrain bundle was performed following methods as previously described (Henry et al., 1998). On the day of surgery rats weighed between 290-320g. Thirty minutes prior to surgery, pargyline (5 mg/kg; P8013, Sigma-Aldrich, UK) and desipramine (25 mg/kg; D3900, Sigma-Aldrich, UK), dissolved in sterile saline (0.9% w/v; Braun AG, Germany), was injected into the intraperitoneal (i.p.) cavity at a volume of 1 ml/kg. Pargyline reduces the metabolism of 6-OHDA by inhibition of monoamine oxidase and improves lesion efficacy (Marek et al., 1990; Kita et al., 1995), while desipramine inhibits the uptake of 6-OHDA into noradrenergic neurons (Taghzouti et al., 1991) and, thus, increases the specificity of the dopaminergic lesion. 6-hydroxydopamine with 0.1% ascorbic acid (6-OHDA; H116, Sigma-Aldrich, UK) was dissolved in sterile water (Braun AG, Germany) to a final concentration of 5 mg/ml. 6-OHDA was kept on ice and protected from light throughout the procedure to prevent oxidation.

Rats were anaesthetised with gaseous flow of 2% isoflurane mixed with oxygen and nitrous oxide. Hair above the incision site was shaved before rats were fixed into a stereotactic frame (David Kopf instruments, California, USA) using ear bars. The skin on the head was incised along the midline and a small burr hole was made through the skull. The dura matter was penetrated and 6-OHDA was manually injected into the right medial forebrain bundle at coordinates, relative to bregma; -2.8 mm anterior (A), +2.0 mm lateral (L) and 9 mm ventral (V). 6-OHDA was injected at a rate of 0.5 µl/minute over 5 minutes (min) using a Hamilton syringe (Gauge 22s, Hamilton, UK). The needle was left in place for a
further 5 min to allow the solution to dissipate and to prevent reflux along the needle track. The wound was closed with standard sutures (3-0 Mersilk®). Betadine® and EMLA® cream were applied over the sutured site for antisepsis and analgesia, respectively. Following surgery, rats received a subcutaneous (s.c.) injection of 10 ml Hartmann’s solution (Baxter, UK) and were placed in an incubator held at 29°C for recovery. Sham operations were performed using the same surgical procedure, but with sterile water (Braun AG, Germany) injected in place of 6-OHDA. Following surgical procedure and subsequent recovery, all animals were subject to treatment and behavioural testing as shown on the timeline in Figure 6.

2.2.2 L-DOPA/benserazide treatment

L-DOPA methyl ester hydrochloride (D1507, Sigma-Aldrich, UK) and benzerazide hydrochloride (B7283, Sigma-Aldrich, UK) were dissolved in sterile saline (0.9% w/v; Braun AG, Germany) and administered at a volume of 1 ml/kg.

Animals were allocated to the following treatment groups: (i) sham + vehicle, plus final vehicle at 1 h prior termination (n=10) (ii) 6-OHDA + vehicle, plus final vehicle at 1 h prior termination (n=10) (iii) 6-OHDA + L-DOPA, plus final L-DOPA dose administered 1 h prior termination (n=10) (iv) 6-OHDA + L-DOPA, plus final L-DOPA dose administered 24 h prior termination (n=10) (Figure 6). This chronic L-DOPA/benserazide treatment regimen, adopted from Lundblad et al. (2002), began four to five weeks post-lesion. L-DOPA/benserazide (6/15 mg/kg; i.p.) or vehicle was administered to animals once daily between 10:00 - 11:00 am for 21 days. On day 22, animals received a final dose of L-DOPA/benserazide or vehicle, and were killed 1 hour (h) or 24 h post-administration (Figure 6). These time points were used to assess the acute and long-standing effects of L-DOPA treatment on molecular correlates associated with dyskinesia (Westin et al., 2001; Westin et al., 2007).
Figure 6. The timeline of treatment and behavioural tests used for investigating the spatio-temporal gene expression profiles of regulators of G-protein signalling (RGS) protein subtypes in the striatum of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia (LID). AIMs, abnormal involuntary movements.
2.2.3 Behavioural analysis

Cylinder test
Forelimb akinesia was used to assess lesion extent as previously described (Schallert et al., 2000; Lundblad et al., 2002). Rats were placed in a clear perspex cylinder (20 cm diameter x 35 cm height) and recorded by a video camera in the dark. Each animal was tested for a maximum time of 2 min or until 20 weight-bearing paw contacts were made on the cylinder. Forelimb asymmetry was scored from video recordings, which was tested at three weeks post-lesion. Individual forelimb supporting contacts were counted to a total of 20 (Lundblad et al., 2002) and a percentage of contralateral forelimb contacts were calculated.

Assessment of AIMS
On day 21 of L-DOPA/benserazide treatment, AIMS were scored as previously described (Cenci et al., 1998; Dekundy et al., 2007) with minor modifications. AIMS assessment started between 10:00 - 11:00 am on the test day. Animals were placed in a clear Perspex observation box (22cm x 34cm x 20cm) for 10 min to acclimatise to experimental surroundings. Following L-DOPA/benserazide or vehicle treatment, animals were assessed for AIMS in 1 min intervals, every 30 min for 3 h.

Four subtypes of AIMS were scored according to the original rating scale (Cenci et al., 1998), which included:

- Locomotor (Lo) – increased contralateral rotations to the lesioned side.
- Limb (Li) - purposeless and repetitive movements of the forelimb contralateral to lesion.
- Axial (Ax) – twisted neck and upper body towards the side contralateral to the lesion.
- Orolingual (Ol) – excessive jaw movements and tongue protrusion.

Each AIM subtype was scored between 0-4 depending on duration presented (Lundblad et al., 2002; Dekundy et al., 2007):

- 0 = absent.
- 1 = infrequent, present for < half observation time.
- 2 = frequent, present for > half observation time.
• 3 = continuous, present throughout the observation time but can be suppressed by an external stimulus (repeated hand movement above the animal).
• 4 = continuous, present throughout observation time but cannot be suppressed by an external stimulus.

The sum of axial, limb and orolingual (ALO) AIMs score was calculated over the 3 hour assessment period and used for statistical analyses. Locomotor AIM scores were analysed separately as the relevance of this behaviour remains under debate (Marin et al., 2006). Animals that did not express any AIMs following L-DOPA treatment were not used for this study.

2.2.4 Tissue preparation

Following completion of behavioural assessments, rats were killed by exposure to a rising concentration of carbon dioxide. Rat brains were removed and immediately frozen in -45°C isopentane and stored at -80°C. Coronal sections were cryostat cut (Leica Microsystems, CM3050s, UK) to 20 µm thick sections at a chamber temperature of 18-20°C. Sections were thaw-mounted on gelatine/chrome-alum-coated slides (Henry et al., 1999) and left to air dry at room temperature before storage at -80°C. With respect to bregma (Paxinos and Watson, 1986), two series of three equivalent sections were cut rostral-caudal through the rat brain:

• Series one (12 slides collected) – rostral striatum, +1.70 mm
• Series three (12 slides collected) – caudal striatum, -0.26 mm

2.2.5 DAT binding

The extent of 6-OHDA lesion was assessed by quantification of DATs using cocaine analogue RTI-121 [3β-(4-iodophenyl) tropane-2β-carboxylic acid isopropyl ester] (Staley et al., 1995). Sections were pre-incubated in phosphate-buffered saline (PBS) (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 136 mM NaCl, 2.8 mM KCl, 10 mM NaI, pH 7.4) for 30 min at room temperature. Sections were then labelled with [¹²⁵I]-RTI (25 pmol) in PBS for 1 h at room temperature and washed twice with ice cold PBS buffer for 20 min. Slides were then rinsed with distilled water and dried with cool air. Sections were exposed to autoradiographic film (Kodak Biomax MR-1) with ¹²⁵I standards (radioactivity range: 1.4-
656 nCi/mg; Amersham) and placed in a fridge at 4°C. Films were developed after 24 h using an automatic film developer.

2.2.6 In situ hybridisation

**Tissue fixation**

Tissue sections from the striatum of the basal ganglia (series one and three) were fixed as previously described (Henry et al., 1999). Under RNAse free conditions, sections were equilibrated to room temperature for 30 min, submerged in 4% paraformaldehyde (BDH, UK) for 10 min, rinsed with diethylpyrocarbonate (DEPC)-treated water and incubated with 0.25% acetic anhydride (A-6404, Sigma-Aldrich UK) in triethanolamine (T-1502, Sigma-Aldrich UK) and 0.9% saline (pH 8.0) for 10 min. The tissue was then dehydrated in consecutive incubations with ascending concentrations of ethanol: 1 min in 70% ethanol, 1 min in 80% ethanol, 2 min in 95% ethanol, 1 min in 100% ethanol, 1 min in 95% ethanol and stored in 95% ethanol at 4°C until further use. All solutions were made with DEPC-treated water.

**Radiolabelling synthetic oligonucleotide probes**

Oligonucleotide probes (Invitrogen, UK) are listed in Table 3. Sequences are complementary to RGS2, RGS4, RGS9-2 (Geurts et al., 2002) and PPE-B (Henry et al., 1999) encoding mRNA in the rat.

The 3’-end of each oligonucleotide probe was labelled with [35S]-dATP (Perkin-Elmer, UK). In an autoclaved eppendorf tube, 16.8 µl DEPC-treated water, 8 µl terminal transferase buffer (Promega Madison, USA), 3.2 µl [35S]-dATP (20 µCi) and 2 µl terminal deoxynucleotidyl transferase (Promega Madison, USA) was added to each probe (4 pmol) and incubated at 37°C for 1 h. Reactions were stopped by heating eppendorfs to 70°C for 10 min. Labelled probes were then centrifuged through Biospin chromatography columns (Bio-Rad) at 1000 gAV for 4 min (Sigma 3K30) for purification. 200 µl of 1M dithiothreitol (DTT; D9779, Sigma-Aldrich UK) was added to the eluted purified probe. Labelling efficiency was estimated using a liquid scintillation counter (Tricarb, 1500 Packard) by mixing 1 µl of eluted probe mixture with 4 ml scintillation fluid.
Oligonucleotide/ mRNA hybridisation

Slides were removed from 95% ethanol and air-dried at room temperature. Air-tight hybridisation boxes containing paper towels soaked in 50% formamide were used to contain slides for incubation. Hybridisation buffer containing $^{35}$S-labelled oligonucleotide probe (with counts at approximately $3 \times 10^6$ cpm/ml) (Henry et al., 1999; Ravenscroft et al., 2004), 50% formamide (F-7508, Sigma), 10% dextran sulphate (D-8906, Sigma), 5x Denhart’s (D2532, Sigma), 10 mg/ml salmon testes DNA (D7656, Sigma), 10 mg/ml Poly-A (P9403, Sigma), 2% DTT (1M), 10% DEPC-treated water, 4x standard sodium citrate (SSC; S6639, Sigma) was used to maximise hybridisation. Approximately 50 µl of hybridisation buffer was pipetted onto each section before Parafilm® (Pechinery, W.I.) strips were placed over each slide and incubated in a hybridisation oven (Stuart Scientific, UK) at 42°C for 16-18 h.

Following incubation, Parafilm® strips were removed and slides were consecutively washed with the following solutions, in the following order; 1 x SSC for 30 min at room temperature, 1 x SSC for 30 min at calculated wash temperature (Table 3), 0.1 x SSC for 10 min at wash temperature, 70% ethanol for 2 min at room temperature, 90% ethanol for 2 min at room temperature. Sections were then air-dried.

Slides were then placed in a cassette and exposed to autoradiographic film sensitive to β-emitting isotopes (Kodak Biomax MR-1, Sigma-Aldrich, UK) for 3-4 weeks. In each cassette a $^{14}$C microscale standard (radioactivity range: 39.3-1117.5 nCi/g; Amersham) was included to determine the radioactivity range. Films were developed using an automatic film developer.

2.2.7 Experimental control

Competitive in situ hybridisation

A 25-fold excess of unlabelled antisense oligonucleotide probe was added with $^{35}$S-labelled oligonucleotide probe and procedures were performed as described.

RNAaese pretreatment

Following tissue fixation, selected slides were submerged in RNAase solution (ribonuclease A 20 μg/ml; Sigma-Aldrich, UK) diluted in DEPC-treated water for 30 min at 37°C before in situ hybridisation.
2.2.8 Image analysis

Autoradiographs were analysed with Image-Pro PLUS™ software (©1993-2004 Media Cybernetics Version 5.1.0.20) connected to a digital camera. The optical density (OD) was found for each tissue section. For the rostral and caudal striatum, the whole striatum and four separate sub-regions (dorsolateral, dorsomedial, ventrolateral and ventromedial) were analysed (Figure 7). The values for each tissue section were averaged over three coronal sections in each region. The OD of radioactive $^{14}$C microscale standards were used to construct a standard curve to convert OD values into tissue radioactivity (nCi/g).

2.2.9 Statistical analysis

Statistical analyses were carried out using Prism 5.0 (GraphPad Software, San Diego, CA). Cylinder test performances of treatment groups were compared using Student’s unpaired t-test. The cumulative AIMs scores were compared between different treatment groups using Kruskal-Wallis test followed by Dunn’s multiple comparison post hoc analysis. In situ hybridisation results were analysed using one-way ANOVA, followed by Tukey’s post hoc test for multiple comparisons. DAT binding was analysed with two-way ANOVA followed by Bonferroni multiple comparisons test, with ‘treatment’ and ‘side’ as given factors. Spearman’s rank correlation was used to analyse gene expression vs. cumulative AIMs scores. Pearson’s linear analysis was used to analyse correlations between gene expression and, cylinder test performance vs. DAT binding. A significance value of p<0.05 was used for all analyses.
Table 3. Synthetic oligonucleotide probes (30-50 bases) used for in situ hybridisation complementary to RGS protein and preproenkephalin-B mRNA sequences in the rat brain.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Accession No.</th>
<th>Nucleotide range</th>
<th>Sequence</th>
<th>Wash temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGS2</td>
<td>AF279918</td>
<td>221-249</td>
<td>5’-ATC AAG CCT TCT CCT GAG GAA GCG CTGC T-3’</td>
<td>50</td>
</tr>
<tr>
<td>RGS4</td>
<td>NM_017214</td>
<td>14-43</td>
<td>5’-CTT CTT TGC AGA GCA GAA GCA ACT GTG CTC-3’</td>
<td>49</td>
</tr>
<tr>
<td>RGS9-2</td>
<td>AF071475</td>
<td>5’-TCC TTT CCA GTC CGG TAC TGC TCT TTG GCC CGC ATG ACA AAA-3’</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>PPE-B</td>
<td>M10088</td>
<td>754-798</td>
<td>5’-GCT CCT CTT GGG GTA TTT GCG CAA AAA GCC GCC ATA GCG TTT GGC-3’</td>
<td>55</td>
</tr>
</tbody>
</table>

Table 3. Synthetic oligonucleotide probes (30-50 bases) used for in situ hybridisation complementary to RGS protein and preproenkephalin-B mRNA sequences in the rat brain.
**Figure 7.** Coronal sections of the (A) rostral (+1.70 mm, with respect to bregma) and (B) caudal striatum (-0.26 mm) in the rat brain showing the regions analysed for image analysis. DL, dorsolateral; DM, dorsomedial; VL, ventrolateral; VM, ventromedial, NAcO, Nucleus accumbens core; NAsH, Nucleus accumbens shell. Adapted from Paxinos and Watson (1986).
2.3 Results

2.3.1 Behavioural analysis

Cylinder test
There was a significant effect of lesion on cylinder test performance (p<0.001; Figure 8), which proved there was a significant effect of lesion. Unilateral 6-OHDA-lesioned rats showed reduced (43%) use of the contralateral forelimb compared to sham-operated animals (p<0.001).

AIMs
Following three weeks treatment, there was a significant effect of treatment group on sum of ALO AIMs (H=31.88; p<0.0001, Figure 9A), which were induced in 6-OHDA-lesioned L-DOPA-treated rats (18.00) compared to 6-OHDA-lesioned vehicle-treated (0.00; p<0.001) and sham-operated vehicle-treated rats (0.00; p<0.001).

Following three weeks treatment, there was a significant effect of treatment group on locomotor AIMs (H=21.65; p<0.0001, Figure 9B), which were induced in 6-OHDA-lesioned L-DOPA-treated rats (2.00) compared to 6-OHDA-lesioned vehicle-treated (0.00; p<0.001) and sham-operated vehicle-treated rats (0.00; p<0.001).
Figure 8. Cylinder test performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Percentage of left paw contacts made in unilateral 6-OHDA-lesioned and sham-operated animals three weeks post-surgery. Data expressed as mean ± SEM percentage left paw contacts made; sham-operated n=10, unilateral 6-OHDA-lesioned n=30. ***p<0.001 cf. sham-operated animals (unpaired t-test).
Figure 9. Abnormal involuntary movement (AIMs) scores on day 21 of three weeks, once daily vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. (A) Axial, limb and orolingual (ALO) AIMs scores and (B) locomotor scores displayed. Points represent data from individual animals. Bars represent median values from treatment group; sham-operated n=10; unilateral 6-OHDA-lesioned vehicle-treated n=10; L-DOPA-treated n=17. ***p<0.001 cf. sham-operated vehicle-treated, ####p<0.001 cf. unilateral 6-OHDA-lesioned vehicle-treated (Kruskal-Wallis test, Dunn’s multiple comparison post hoc analysis).
2.3.2 DAT binding

There was a significant effect of treatment ($F_{1,76}=147.39; p<0.0001$, Figure 10A and Figure 11), side ($F_{1,76}=200.05; p<0.0001$) and treatment x side interaction ($F_{1,76}=127.85; p<0.0001$) on $^{125}\text{I}$-RTI-121 binding, which was reduced in the operated side of the 6-OHDA-lesioned group compared to the unoperated side (84%, $p<0.0001$) and operated side of sham (83%, $p<0.0001$). There was no effect of sham-lesion on $^{125}\text{I}$-RTI-121 binding ($p>0.05$). Autoradiographic images from DAT binding in the striatum are shown in Figure 11.

There was a positive correlation between $^{125}\text{I}$-RTI-121 binding ipsilateral to the operated side and cylinder test score (Pearson correlation coefficient $r=0.8569; p<0.0001$; Figure 10B).
Figure 10. Assessment of 6-OHDA lesion efficacy by dopamine active transporter binding using $[^{125}\text{I}]$-RTI-121 autoradiography. (A) $[^{125}\text{I}]$-RTI-121 binding in unoperated (○) and operated (■) striatum. (A) Bars represent mean ± SEM; sham-operated n=10, unilateral 6-OHDA-lesioned n=30. ***p<0.001 cf. 6-OHDA-unoperated, ###p<0.001 cf. sham-operated (two-way ANOVA followed by Bonferroni multiple comparisons test). (B) Correlation analysis between cylinder test performance post-lesion and $[^{125}\text{I}]$-RTI-121 binding in the operated striatum (Pearson correlation coefficient r=0.8569; p<0.0001).
Figure 11. Pseudocolour transformations of autoradiographic images from dopamine active transporter (DAT) binding using $[^{125}]$I-RTI-121 autoradiography. Representative coronal sections of the (U) unoperated and (O) operated striatum in sham and unilateral 6-OHDA-lesioned rats.
2.3.3 In situ hybridisation for RGS protein gene expression in the striatum

RGS2

Rostral striatum

In the whole rostral striatum, there was a significant effect of treatment on RGS2 mRNA levels (F_{7,54}=26.08; p<0.0001, Figure 12A). RGS2 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration, compared to the operated sides of sham vehicle-treated (76%, p<0.001), 6-OHDA-lesioned vehicle-treated (90%, p<0.001) and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (60%, p<0.001). RGS2 mRNA levels were increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration compared to the unoperated side (59%, p<0.001). There was no significant difference between operated sides of sham vehicle-treated, 6-OHDA-lesioned vehicle-treated and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (p>0.05). There was no side-to-side difference in the sham-vehicle, 6-OHDA-vehicle and 6-OHDA L-DOPA-treated 24 h post-administration, treatment groups (p>0.05).

In the dorsolateral region of the rostral striatum, there was a significant effect of treatment on RGS2 mRNA levels (F_{7,48}=13.17; p<0.0001, Figure 13A). RGS2 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration, compared to the operated sides of sham vehicle-treated (77%, p<0.001), 6-OHDA-lesioned vehicle-treated (86%, p<0.001) and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (50%, p<0.001). RGS2 mRNA levels were increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration compared to the unoperated side (61%, p<0.001). There was no significant difference between operated sides of sham vehicle-treated, 6-OHDA-lesioned vehicle-treated and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (p>0.05). There was no side-to-side difference in the sham-vehicle, 6-OHDA-vehicle and 6-OHDA L-DOPA-treated 24 h post-administration, treatment groups (p>0.05).

In the dorsomedial region of the rostral striatum, there was a significant effect of treatment on RGS2 mRNA levels (F_{7,50}=5.515; p<0.0001, Figure 13B). RGS2 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration, compared to the operated sides of sham vehicle-treated (36%, p<0.01), 6-OHDA-lesioned vehicle-treated (51%, p<0.001) and 6-OHDA-lesioned L-
DOPA-treated rats at 24 h post-administration (36%, p<0.01). RGS2 mRNA levels were increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration compared to the unoperated side (35%, p<0.01). There was no significant difference between operated sides of sham vehicle-treated, 6-OHDA-lesioned vehicle-treated and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (p>0.05). There was no side-to-side difference in the sham-vehicle, 6-OHDA-vehicle and 6-OHDA L-DOPA-treated 24 h post-administration, treatment groups (p>0.05).

In the ventrolateral region of the rostral striatum, there was a significant effect of treatment on RGS2 mRNA levels (F_{7,52}=11.02, p<0.0001, Figure 13C). RGS2 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration, compared to the operated sides of sham vehicle-treated (50%, p<0.001), 6-OHDA-lesioned vehicle-treated (80%, p<0.001) and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (44%, p<0.001). RGS2 mRNA levels were increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration compared to the unoperated side (p<0.001). There was no significant difference between operated sides of sham vehicle-treated, 6-OHDA-lesioned vehicle-treated and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (p>0.05). There was no side-to-side difference in the sham-vehicle, 6-OHDA-vehicle and 6-OHDA L-DOPA-treated 24 h post-administration, treatment groups (p>0.05).

In the ventromedial region of the rostral striatum, there was no significant effect of treatment on RGS2 mRNA levels (F_{7,50}=1.901, p=0.0891, Figure 13D).

In the nucleus accumbens core, there was no significant effect of treatment on RGS2 mRNA levels (F_{7,54}=0.4017, p=0.8971, Figure 13E).

In the nucleus accumbens shell, there was no significant effect of treatment on RGS2 mRNA levels (F_{7,54}=0.5876, p=0.7631, Figure 13F).

Autoradiographic images of RGS2 mRNA expression in the rostral striatum are shown in Figure 15.
Figure 12. RGS2 mRNA expression in the (A) rostral and (B) caudal striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h or 24 h post-administration. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=7-8). ***p<0.001 cf. ipsilateral sham vehicle-treated group; ###p<0.001 cf. ipsilateral 6-OHDA vehicle-treated group; ^^p<0.001 cf. ipsilateral 6-OHDA L-DOPA-treated 1 h post-administration group; $$$p<0.001 cf. unoperated side (one-way ANOVA, Tukey’s post hoc analysis).
Figure 13. RGS2 mRNA expression in the sub-regions of the rostral striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h and 24 h post-administration. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6–8). ***p<0.001, **p<0.01 cf. ipsilateral sham vehicle-treated group; ###p<0.001, ##p<0.01 cf. ipsilateral 6-OHDA vehicle-treated group; ^^^p<0.001, ^^p<0.01 cf. ipsilateral 6-OHDA L-DOPA-treated 1 h post-administration group; $$$p<0.001, $$p<0.01 cf. unoperated side (one-way ANOVA, Tukey’s post hoc analysis).
**Caudal striatum**

In the whole caudal striatum, there was a significant effect of treatment on RGS2 mRNA levels ($F_{7,54}=26.97; \ p<0.0001$, Figure 12B). RGS2 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration, compared to operated side of sham vehicle-treated (60%, $p<0.001$), 6-OHDA-lesioned vehicle-treated (50%, $p<0.001$) and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (46%, $p<0.001$). RGS2 mRNA levels were increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration compared to the unoperated side (51%, $p<0.001$). There was no significant difference between operated sides of sham vehicle-treated, 6-OHDA-lesioned vehicle-treated and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration ($p>0.05$). There was no side-to-side difference in the sham-vehicle, 6-OHDA-vehicle and 6-OHDA L-DOPA-treated 24 h post-administration, treatment groups ($p>0.05$).

In the dorsolateral region of the caudal striatum, there was a significant effect of treatment on RGS2 mRNA levels ($F_{7,54}=18.92; \ p<0.0001$, Figure 14A). RGS2 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration, compared to the operated sides of sham vehicle-treated (87%, $p<0.001$), 6-OHDA-lesioned vehicle-treated (62%, $p<0.001$) and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (74%, $p<0.001$). RGS2 mRNA levels were increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration compared to the unoperated side (71%, $p<0.001$). There was no significant difference between operated sides of sham vehicle-treated, 6-OHDA-lesioned vehicle-treated and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration ($p>0.05$). There was no side-to-side difference in the sham-vehicle, 6-OHDA-vehicle and 6-OHDA L-DOPA-treated rats at 24 h post-administration, treatment groups ($p>0.05$).

In the dorsomedial region of the caudal striatum, there was a significant effect of treatment on RGS2 mRNA levels ($F_{7,54}=6.484; \ p<0.0001$, Figure 14B). RGS2 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration, compared to the operated sides of sham vehicle-treated (39%, $p<0.01$), 6-OHDA-lesioned vehicle-treated (38%, $p<0.01$) and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (52%, $p<0.001$). RGS2 mRNA levels were increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-
administration compared to the unoperated side (43%, \(p<0.001\)). There was no significant difference between operated sides of sham vehicle-treated, 6-OHDA-lesioned vehicle-treated and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (\(p>0.05\)). There was no side-to-side difference in the sham-vehicle, 6-OHDA-vehicle and 6-OHDA L-DOPA-treated 24 h post-administration, treatment groups (\(p>0.05\)).

In the ventrolateral region of the caudal striatum, there was a significant effect of treatment on RGS2 mRNA levels (\(F_{7,54}=43.28; p<0.0001\), Figure 14C). RGS2 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration, compared to the operated sides of sham vehicle-treated (123%, \(p<0.001\)), 6-OHDA-lesioned vehicle-treated (83%, \(p<0.001\)) and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (90%, \(p<0.001\)). RGS2 mRNA levels were increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration compared to the unoperated side (95%, \(p<0.001\)). There was no significant difference between operated sides of sham vehicle-treated, 6-OHDA-lesioned vehicle-treated and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (\(p>0.05\)). There was no side-to-side difference in the sham-vehicle, 6-OHDA-vehicle and 6-OHDA L-DOPA-treated 24 h post-administration, treatment groups (\(p>0.05\)).

In the ventromedial region of the caudal striatum, there was a significant effect of treatment on RGS2 mRNA levels (\(F_{7,54}=23.39; p<0.0001\), Figure 14D). RGS2 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration, compared to the operated sides of sham vehicle-treated (85%, \(p<0.001\)), 6-OHDA-lesioned vehicle-treated (51%, \(p<0.001\)) and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (92%, \(p<0.001\)). RGS2 mRNA levels were increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration compared to the unoperated side (55%, \(p<0.001\)). There was no significant difference between operated sides of sham vehicle-treated, 6-OHDA-lesioned vehicle-treated and 6-OHDA-lesioned L-DOPA-treated rats at 24 h post-administration (\(p>0.05\)). There was no side-to-side difference in the sham-vehicle, 6-OHDA-vehicle and 6-OHDA L-DOPA-treated 24 h post-administration, treatment groups (\(p>0.05\)).

Autoradiographic images of RGS2 mRNA expression in the caudal striatum are shown in Figure 15.
Figure 14. RGS2 mRNA expression in the sub-regions of the caudal striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h and 24 h post-administration. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral and (D) ventromedial striatum. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6-8). ***p<0.001, **p<0.01 cf. ipsilateral sham vehicle-treated group; ###p<0.001, ##p<0.01 cf. ipsilateral 6-OHDA vehicle-treated group; ^^^p<0.001 cf. ipsilateral 6-OHDA L-DOPA-treated 1 h post-administration group; $$$p<0.001 cf. unoperated side (one-way ANOVA, Tukey’s post hoc analysis).
Figure 15. Pseudocolour image transformations of autoradiographs from *in situ* hybridisation targeting RGS2 mRNA in the (U) unoperated and (O) operated sides of the rostral and caudal striata following 21 day, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Animals were killed at 1 hour post final vehicle or L-DOPA administration, or at 24 hours post final L-DOPA administration.
In the whole rostral striatum, there was a significant effect of treatment on RGS4 mRNA levels ($F_{7,52}=6.459; \ p<0.0001$, Figure 16A). RGS4 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated, at 1 h and 24 h post-administration, compared to operated side of sham vehicle-treated (44%, $p<0.01$ and 47%, $p<0.01$, respectively) and operated side 6-OHDA-lesioned vehicle-treated (43%, $p<0.01$ and 45%, $p<0.01$, respectively). There was no significant difference in RGS4 mRNA levels between the operated sides of sham and 6-OHDA vehicle-treated groups ($p>0.05$). There was no significant difference in RGS4 mRNA levels in the operated sides of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration ($p>0.05$). There was no side-to-side difference in any of the treatment groups ($p>0.05$).

In the dorsolateral region of the rostral striatum, there was a significant effect of treatment on RGS4 mRNA levels ($F_{7,50}=5.136; \ p=0.0002$, Figure 17A). RGS4 mRNA levels were significantly higher in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h post-administration compared to the operated side of sham vehicle-treated (41%, $p<0.01$). RGS4 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats, at 1 h and 24 h post-administration, compared to the operated side of 6-OHDA-lesioned vehicle-treated (53%, $p<0.001$ and 35%, $p<0.05$, respectively). There was no significant differences in RGS4 mRNA levels between the operated sides of sham and 6-OHDA vehicle-treated groups ($p>0.05$). There was no significant difference in the operated sides of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration ($p>0.05$). There was no side-to-side difference in any of the treatment groups ($p>0.05$).

In the dorsomedial region of the rostral striatum, there was no significant effect of treatment on RGS4 mRNA levels ($F_{7,50}=1.525; \ p=0.1807$, Figure 17B).

In the ventrolateral region of the rostral striatum, there was a significant effect of treatment on RGS4 mRNA levels ($F_{7,50}=6.562; \ p<0.0001$, Figure 17C). RGS4 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats, at 1 h and 24 h post-administration, compared to the operated side of sham vehicle-treated (49%, $p<0.01$ and 39%, $p<0.05$, respectively) and operated side of 6-OHDA-lesioned
vehicle-treated rats (59%, p<0.001 and 48%, p<0.01, respectively). There was no significant difference between the operated sides of sham and 6-OHDA vehicle-treated groups (p>0.05). There was no significant difference in the operated sides of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration (p>0.05). There was no side-to-side difference in any of the treatment groups (p>0.05).

In the ventromedial region of the rostral striatum, there was no significant effect of treatment on RGS4 mRNA levels (F_{7,50}=1.937; p=0.0832, Figure 17D).

In the nucleus accumbens core, there was no significant effect of treatment on RGS4 mRNA levels (F_{7,50}=1.057; p=0.4049, Figure 17E).

In the nucleus accumbens shell, there was a significant effect of treatment on RGS4 mRNA levels (F_{7,46}=3.073; p=0.0097, Figure 17F). RGS4 mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats, at 1 h and 24 h post-administration, compared to the operated side of sham vehicle-treated (31%, p<0.05 and 32%, p<0.05, respectively). There was no significant difference between the operated side of sham and 6-OHDA vehicle treated groups (p>0.05). There was no significant difference between the operated sides of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration (p>0.05). There was no side-to-side difference in any of the treatment groups (p>0.05).

Autoradiographic images of RGS4 mRNA expression in the rostral striatum are shown in Figure 19.
Figure 16. RGS4 mRNA expression in the (A) rostral and (B) caudal striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h or 24 h post-administration. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=7-8). **p<0.01 cf. ipsilateral sham vehicle-treated group; ##p<0.01 cf. ipsilateral 6-OHDA vehicle-treated group (one-way ANOVA, Tukey’s post hoc analysis).
Figure 17. RGS4 mRNA expression in the sub-regions of the rostral striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h and 24 h post-administration. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6-8). *p<0.01, *p<0.05 cf. ipsilateral sham vehicle-treated group; ###p<0.001, ##p<0.01, #p<0.05 cf. ipsilateral 6-OHDA vehicle-treated group (one-way ANOVA, Tukey’s post hoc analysis).
**Caudal striatum**

In the whole caudal striatum, there was no significant effect of treatment on RGS4 mRNA levels ($F_{7,54}=0.5754; p=0.7727$, Figure 16B).

In the dorsolateral region of the caudal striatum, there was no significant effect of treatment on RGS4 mRNA levels ($F_{7,52}=1.469; p=0.1988$, Figure 18A).

In the dorsomedial region of the caudal striatum, there was no significant effect of treatment on RGS4 mRNA levels ($F_{7,52}=0.5550; p=0.7886$, Figure 18B).

In the ventrolateral region of the caudal striatum, there was no significant effect of treatment on RGS4 mRNA levels ($F_{7,52}=1.212; p=0.3130$, Figure 18C).

In the ventromedial region of the caudal striatum, there was no significant effect of treatment on RGS4 mRNA levels ($F_{7,52}=1.024; p=0.4257$, Figure 18D).

Autoradiographic images of RGS4 mRNA expression in the caudal striatum are shown in Figure 19.
Figure 18. RGS4 mRNA expression in the sub-regions of the caudal striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h and 24 h post-administration. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=7-8) (one-way ANOVA, Tukey’s post hoc analysis).
Figure 19. Pseudocolour image transformations of autoradiographs from *in situ* hybridisation targeting RGS4 mRNA in the (U) unoperated and (O) operated sides of the rostral and caudal striata following 21 day, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Animals were killed at 1 hour post final vehicle or L-DOPA administration, or at 24 hours post final L-DOPA administration.
**RGS9-2**

**Rostral striatum**

In the whole rostral striatum, there was no significant effect of treatment on RGS9-2 mRNA levels ($F_{7,50}=1.305; p=0.2677$, Figure 20A).

In the dorsolateral region of the rostral striatum, there was no significant effect of treatment on RGS9-2 mRNA levels ($F_{7,50}=1.056; p=0.4055$, Figure 21A).

In the dorsomedial region of the rostral striatum, there was no significant effect of treatment on RGS9-2 mRNA levels ($F_{7,50}=0.4728; p=0.8496$, Figure 21B).

In the ventrolateral region of the rostral striatum, there was no significant effect of treatment on RGS9-2 mRNA levels ($F_{7,50}=0.9663; p=0.4658$, Figure 21C).

In the ventromedial region of the rostral striatum, there was no significant effect of treatment on RGS9-2 mRNA levels ($F_{7,50}=0.4376; p=0.8738$, Figure 21D).

In the nucleus accumbens core, there was no significant effect of treatment on RGS9-2 mRNA levels ($F_{7,52}=0.3830; p=0.9081$, Figure 21E).

In the nucleus accumbens shell, there was no significant effect of treatment on RGS9-2 mRNA levels ($F_{7,52}=1.267, p=0.2849$, Figure 21F).

Autoradiographic images of RGS9-2 mRNA expression in the rostral striatum are shown in Figure 23.
Figure 20. RGS9-2 mRNA expression in the (A) rostral and (B) caudal striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h or 24 h post-administration. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=7-8) (one-way ANOVA, Tukey’s post hoc analysis).
Figure 21. RGS9-2 mRNA expression in the sub-regions of the rostral striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h and 24 h post-administration. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=7-8) (one-way ANOVA, Tukey’s post hoc analysis).
Caudal striatum

In the whole caudal striatum, there was no significant effect of treatment on RGS9-2 mRNA expression levels ($F_{7,54}=0.431; p=0.8785$, Figure 20B).

In the dorsolateral region of the caudal striatum, there was no significant effect of treatment on RGS9-2 mRNA expression levels ($F_{7,54}=1.737; p=0.1198$, Figure 22A).

In the dorsomedial region of the caudal striatum, there was no significant effect of treatment on RGS9-2 mRNA expression levels ($F_{7,54}=0.5585; p=0.7860$, Figure 22B).

In the ventrolateral region of the caudal striatum, there was no significant effect of treatment on RGS9-2 mRNA expression levels ($F_{7,54}=1.784; p=0.1095$, Figure 22C).

In the ventromedial region of the caudal striatum, there was no significant effect of treatment on RGS9-2 mRNA expression levels ($F_{7,54}=1.471; p=0.1973$, Figure 22D).

Autoradiographic images of RGS9-2 mRNA expression in the caudal striatum are shown in Figure 23.
Figure 22. RGS9-2 mRNA expression in the sub-regions of the caudal striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h and 24 h post-administration. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral and (D) ventromedial striatum. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=7-8) (one-way ANOVA, Tukey’s post hoc analysis).
Figure 23. Pseudocolour image transformations of autoradiographs from in situ hybridisation targeting RGS9-2 mRNA in the (U) unoperated and (O) operated sides of the rostral and caudal striata following 21 day, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Animals were killed at 1 hour post final vehicle or L-DOPA administration, or at 24 hours post final L-DOPA administration.
**PPE-B**

**Rostral striatum**

In the whole rostral striatum, there was a significant effect of treatment on PPE-B mRNA levels ($F_{7,52}=10.27; p<0.0001$, Figure 24). PPE-B mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration, compared to the operated sides of sham-operated vehicle-treated (40%, $p<0.01$ and 47%, $p<0.01$, respectively) and 6-OHDA-lesioned vehicle-treated rats (80%, $p<0.001$ and 88%, $p<0.001$, respectively). PPE-B mRNA levels were increased in the operated sides of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration compared to the unoperated sides (53%, $p<0.001$ and 86%, $p<0.001$, respectively). There was no significant difference between the operated sides of sham and 6-OHDA-lesioned vehicle-treated ($p>0.05$). There was no side-to-side difference in the sham and 6-OHDA vehicle-treated groups ($p>0.05$).

In the dorsolateral region of the rostral striatum, there was a significant effect of treatment on PPE-B mRNA levels ($F_{7,51}=26.54; p<0.0001$, Figure 25A). PPE-B mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration, compared to the operated sides of sham-operated vehicle-treated (56%, $p<0.001$ and 80%, $p<0.001$, respectively) and 6-OHDA-lesioned vehicle-treated rats (86%, $p<0.001$ and 115%, $p<0.001$, respectively). PPE-B mRNA levels were increased in the operated sides of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration compared to the unoperated sides (82%, $p<0.001$ and 148%, $p<0.001$, respectively). There was no significant difference between the operated sides of sham and 6-OHDA-lesioned vehicle-treated ($p>0.05$). There was no side-to-side difference in the sham and 6-OHDA vehicle-treated groups ($p>0.05$).

In the dorsomedial region of the rostral striatum, there was a significant effect of treatment on PPE-B mRNA levels ($F_{7,45}=5.565; p=0.0001$, Figure 25B). PPE-B mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration, compared to the operated side of 6-OHDA-lesioned vehicle-treated (35%, $p<0.01$ and 26%, $p<0.05$, respectively). PPE-B mRNA levels were increased in the operated sides of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration compared to the unoperated sides (33%, $p<0.05$ and 37%, $p<0.01$, respectively). There was no significant difference between the operated sides of sham and
In the ventrolateral region of the rostral striatum, there was a significant effect of treatment on PPE-B mRNA levels ($F_{7,51}=16.15; p<0.0001$, Figure 25C). PPE-B mRNA levels were significantly increased in the operated side of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration, compared to the operated sides of sham-operated vehicle-treated (58%, $p<0.001$ and 87%, $p<0.001$, respectively) and 6-OHDA-lesioned vehicle-treated rats (92%, $p<0.001$ and 126%, $p<0.001$, respectively). PPE-B mRNA levels were increased in the operated sides of 6-OHDA-lesioned L-DOPA-treated rats at 1 h and 24 h post-administration compared to the unoperated sides (63%, $p<0.001$ and 105%, $p<0.001$, respectively). There was no significant difference between the operated sides of sham and 6-OHDA-lesioned vehicle-treated groups ($p>0.05$). There was no side-to-side difference in the sham and 6-OHDA vehicle-treated groups ($p>0.05$).

In the ventromedial region of the rostral striatum, there was no significant effect of treatment on PPE-B mRNA levels ($F_{7,50}=1.340; p=0.2518$, Figure 25D).

In the nucleus accumbens core, there was no significant effect of treatment on PPE-B mRNA levels ($F_{7,48}=2.171; p=0.536$, Figure 25E).

In the nucleus accumbens shell, there was no significant effect of treatment on PPE-B mRNA levels ($F_{7,50}=1.572; p=0.1656$, Figure 25F).

 Autoradiographic images of PPE-B mRNA expression in the rostral striatum are shown in Figure 26.
Figure 24. PPE-B mRNA expression in the rostral striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h or 24 h post-administration. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6-8). **p<0.01 cf. ipsilateral sham vehicle-treated group; ###p<0.001 cf. ipsilateral 6-OHDA vehicle-treated group; $$$p<0.001 cf. unoperated side (one-way ANOVA, Tukey’s post hoc analysis).
Figure 25. PPE-B mRNA expression in the sub-regions of the rostral striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h and 24 h post-administration. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6-8). ***p<0.001 cf. ipsilateral sham vehicle-treated group; ###p<0.001, ##p<0.01, #p<0.05 cf. ipsilateral 6-OHDA vehicle-treated group; $$$p<0.001, $$p<0.01, $p<0.05 cf. unoperated side (one-way ANOVA, Tukey’s post hoc analysis).
Figure 26. Pseudocolour image transformations of autoradiographs from \textit{in situ} hybridisation targeting PPE-B mRNA in the (U) unoperated and (O) operated sides of the rostral striatum following 21 day, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Animals were killed at 1 hour post final vehicle or L-DOPA administration, or at 24 hours post final L-DOPA administration.
Correlation between striatal RGS2 expression and AIMs
There was no significant linear correlation between RGS2 mRNA expression, in the operated rostral striatum at 1 h post L-DOPA administration, and the sum of ALO AIMs (Spearman’s r=-0.1429; 95% CI -1.601 to 0.7994; p=0.7520, Figure 27A).

Correlation between RGS2 and PPE-B mRNA expression in the whole rostral striatum
There was no significant linear correlation between RGS2 and PPE-B mRNA expression, in the operated striatum at 1 h post L-DOPA administration (Pearson’s r=-0.06775; 95% CI -3.439 to 3.002; p=0.8734, Figure 27B).

There was no significant linear correlation between RGS2 and PPE-B mRNA expression, in the operated striatum at 24 h post L-DOPA administration (Pearson’s r=-0.3138; 95% CI -0.4062 to 0.2248; p=0.4932, Figure 27C).
Figure 27. Correlation between the RGS2 mRNA expression in the operated side of the rostral striatum and the sum of axial, limb and orolingual (ALO) abnormal involuntary movements (AIMs) score, or preproenkephalin B (PPE-B) mRNA, in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with L-DOPA/benserazide (6/15 mg/kg; i.p.). (A) RGS2 mRNA at 1 h post L-DOPA administration vs. sum of ALO AIMs (n=8) (Spearman’s rank correlation); RGS2 mRNA vs. PPE-B mRNA at (B) 1 h and (C) 24 h post L-DOPA administration (n=7-8) (Pearson’s linear correlation).
Correlation between striatal RGS4 expression and AIMs
There was a significant positive linear correlation between RGS4 mRNA expression, in the operated rostral striatum at 1 h post L-DOPA administration, and the sum of ALO AIMs (Spearman’s r=0.9286; 95% CI 0.6236 to 2.814; p=0.0022, Figure 28A).

Correlation between RGS4 and PPE-B mRNA expression in the whole rostral striatum
There was a significant positive linear correlation between RGS4 and PPE-B mRNA expression, in the operated striatum at 1 h post L-DOPA administration (Pearson’s r=0.8555; 95% CI 1.440 to 5.845; p=0.0068, Figure 28B).

There was a significant positive linear correlation between RGS4 and PPE-B mRNA expression, in the operated striatum at 24 h post L-DOPA administration (Pearson’s r=0.9109; 95% CI 1.1215 to 3.858; p=0.0043, Figure 28C).
Figure 28. Correlation between the RGS4 mRNA expression in the operated side of the rostral striatum and the sum of axial, limb and orolinguinal (ALO) abnormal involuntary movements (AIMs) score, or preproenkephalin B (PPE-B) mRNA, in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with L-DOPA/benserazide (6/15 mg/kg; i.p.). (A) RGS4 mRNA at 1 h post L-DOPA administration vs. sum of ALO AIMs (n=8) (**p<0.01, Spearman’s rank correlation); RGS4 mRNA vs. PPE-B mRNA at (B) 1 h and (C) 24 h post L-DOPA administration (n=7-8) (**p<0.01, Pearson’s linear correlation).
Correlation between RGS4 and PPE-B mRNA expression in the sub-regions of the rostral striatum

In the dorsolateral striatum of the operated side, there was a significant positive linear correlation between RGS4 and PPE-B mRNA expression, at 1 h post L-DOPA administration (Pearson’s r=0.8730; 95% CI 1.129 to 5.377; p=0.0095, Figure 29A).

In the dorsomedial striatum of the operated side, there was a significant positive linear correlation between RGS4 and PPE-B mRNA expression, at 1 h post L-DOPA administration (Pearson’s r=0.8138; 95% CI 0.4783 to 2.861; p=0.0140, Figure 29B).

In the ventrolateral striatum of the operated side, there was a significant positive linear correlation between RGS4 and PPE-B mRNA expression at 1 h post L-DOPA administration (Pearson’s r=0.7859; 95% CI 0.5169 to 4.316; p=0.0208, Figure 29C).

In the ventromedial striatum of the operated side, there was no significant linear correlation between RGS4 and PPE-B mRNA expression at 1 h post L-DOPA administration (Pearson’s r=0.3264; 95% CI -1.964 to 4.038; p=0.4300, Figure 29D).
Figure 29. Correlation between RGS4 and PPE-B mRNA expression in the operated side of the rostral striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 h post-administration. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum (n=7-8) (*p<0.05, **p<0.01, Pearson’s linear correlation).
In the dorsolateral striatum of the operated side, there was a significant positive linear correlation between RGS4 and PPE-B mRNA expression, at 24 h post L-DOPA administration (Pearson’s r=0.8905; 95% CI 0.2827 to 1.663; p=0.0173, Figure 30A).

In the dorsomedial striatum of the operated side, there was no significant linear correlation between RGS4 and PPE-B mRNA expression, at 24 h post L-DOPA administration (Pearson’s r=0.4604; 95% CI -1.240 to 3.278; p=0.2986, Figure 30B).

In the ventrolateral striatum of the operated side, there was a significant linear correlation between RGS4 and PPE-B mRNA expression, at 24 h post L-DOPA administration (Pearson’s r=0.9418; 95% CI 0.7253 to 1.735; p=0.0015, Figure 30C).

In the ventromedial striatum of the operated side, there was no significant linear correlation between RGS4 and PPE-B mRNA expression, at 24 h post L-DOPA administration (Pearson’s r=0.6439; 95% CI -0.8888 to 5.742; p=0.1186, Figure 30D).
Figure 30. Correlation between RGS4 and PPE-B mRNA expression in the operated side of the rostral striatum in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with L-DOPA/benserazide (6/15 mg/kg; i.p.) at 24 h post-administration. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum (n=6-7) (*p<0.05, **p<0.01, Pearson’s linear correlation).
**Correlation between striatal RGS9-2 expression and AIMs**

There was no significant linear correlation between RGS9-2 mRNA expression, in the operated rostral striatum at 1 h post L-DOPA administration, and the sum of ALO AIMs (Spearman’s r=-0.04762; 95% CI -1.138 to 1.137; p=0.9349, Figure 31A).

**Correlation between RGS9-2 and PPE-B mRNA expression in the whole rostral striatum**

There was a significant positive linear correlation between RGS9-2 and PPE-B mRNA expression, in the operated striatum at 1 h post L-DOPA administration (Pearson’s r=0.7576; 95% CI 0.3064 to 4.094; p=0.0295, Figure 31B). There were, however, no significant correlations between RGS9-2 and PPE-B mRNA expression in any of the rostral striatal sub-regions (data not shown).

There was no significant linear correlation between RGS9-2 and PPE-B mRNA expression, in the operated striatum at 24 h post L-DOPA administration (Pearson’s r=–0.5854; 95% CI -1.939 to 0.4432; p=0.1674, Figure 31C).
Figure 31. Correlation between the RGS9-2 mRNA expression in the operated side of the rostral striatum and the sum of axial, limb and orolingual (ALO) abnormal involuntary movements (AIMs) score, or preproenkephalin B (PPE-B) mRNA, in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with L-DOPA/benserazide (6/15 mg/kg; i.p.). (A) RGS9-2 mRNA at 1 h post L-DOPA administration vs. sum of ALO AIMs (n=8) (Spearman’s rank correlation); RGS9-2 mRNA vs. PPE-B mRNA at (B) 1 h and (C) 24 h post L-DOPA administration (n=7-8) (*p<0.05, Pearson’s linear correlation).
Experimental control

Competitive in situ hybridisation

Autoradiograph signal was absent in the presence of 25-fold excess unlabelled probe for RGS2, RGS4 and RGS9-2 sequences in comparison to control (Figure 32).

RNAase pre-treatment

Autoradiograph signal was absent following RNAase pre-treatment in comparison to control (Figure 33).
Figure 32. Autoradiograph images displaying competitive *in situ* hybridisation utilising an oligonucleotide probe targeted against RGS9-2 mRNA in the caudal striatum. (A) Labelled probe only and (B) Labelled probe combined with 25-fold excess of unlabelled probe.
Figure 33. Autoradiograph images of in situ hybridisation utilising an oligonucleotide probe targeted against RGS9-2 mRNA in the caudal striatum. (A) Untreated tissue and (B) RNAase pre-treated tissue.
2.4 Discussion

The major findings from this study were that 21 days L-DOPA treatment increased RGS2 and RGS4, but not RGS9-2, mRNA expression in the striatum of the unilateral 6-OHDA-lesioned rat model of PD. RGS2 mRNA levels were increased in the rostral and caudal striatum at 1 hour post L-DOPA treatment, while RGS4 mRNA levels were increased specifically in the lateral regions of the rostral striatum at both 1 hour and 24 hours post L-DOPA treatment. These changes may have important implications in the expression of LID.

2.4.1 Methodological considerations

*Behavioural assessments of the unilateral 6-OHDA-lesioned rat*

The unilateral 6-OHDA-lesioned rat model of PD and LID was used to investigate changes in RGS protein gene expression. This model displays marked asymmetric motor deficits that can be easily quantified (Tillerson et al., 2001). For example, features of the parkinsonian syndrome, such as akinesia, can be measured in unilateral 6-OHDA-lesioned rats using the cylinder test (Schallert et al., 2000). The motor performance in the cylinder test negatively correlates with dopamine denervation (Winkler et al., 2002; Carta et al., 2006) and has been used as a behavioural measure to screen for full unilateral 6-OHDA lesions (Mela et al., 2007). The cylinder test also benefits from simplicity in application. However, it is limited when used repeatedly because of the rat’s nature to habituate to experimental conditions (Lundblad et al., 2002).

In this study, a low dose of L-DOPA (6 mg/kg) was administered within the clinical range used in PD patients (Rascol et al., 2000). Repeated L-DOPA treatment in 6-OHDA-lesioned rats induces AIMs (Lundblad et al., 2002; Dekundy et al., 2007) and also elevates various endogenous signalling factors, many of which are associated with the induction of AIMs, such as prodynorphin mRNA (Cenci et al., 1998; Henry et al., 1999; Konradi et al., 2004; Westin et al., 2007). In this study, animals were killed after L-DOPA treatment at 1 h and 24 h to measure the acute/long-standing and long-standing only effects of repeated L-DOPA treatment, respectively (Westin et al., 2001; Andersson et al., 2003; Westin et al., 2007).
**In situ hybridisation and molecular analysis**

The methods used for *in situ* hybridisation followed previous studies (Henry et al., 1999; Ravenscroft et al., 2004). Oligonucleotide probes complementary to RGS mRNA sequences in the rat were used (Henry et al., 1999; Geurts et al., 2003). These oligonucleotide probes were first tested in non-experimental rat brain tissue to ensure hybridisation could be achieved before experiments were started.

Each oligonucleotide probe was radioactively labelled and then processed for autoradiographic analysis, which required simple film exposure. The $^{35}$S radioisotope was chosen for radioactive labelling. Although $^{35}$S-dATP labelled probes produce images at a lower resolution (approximately 1 cell diameter) compared to $^3$H-labelled probes (Wilkinson et al., 1998), they give a semi-quantitative value for gene expression in a shorter period of time. Thus, results are produced more rapidly. In brain regions where target genes are highly expressed, $^{35}$S-dATP labelled probes produce a suitable degree of image resolution. However, its utility may be limited in regions with low gene expression. There are other disadvantages of $^{35}$S-dATP labelling which include; variability in radioactive labelling, radioactive exposure and expense.

There are several factors that can affect the autoradiographic signal in *in situ* hybridisation. These include the efficiency of oligonucleotide labelling, tissue preparation, age of tissue and exposure time to organic solvents, such as ethanol. The latter two factors change the permeability of cells by weakening the lipid membrane, which directly affects the number of oligonucleotide-mRNA duplexes formed during hybridisation (Wilkinson et al., 1998).

The specificity of RGS probe binding in this study was demonstrated by control experiments. Unlabelled oligonucleotide probes out-competed $^{35}$S-labelled probes for complementary mRNA sequences. Moreover, the absence of autoradiographic signal in RNAase-treated tissue confirms that oligonucleotide binding was specific to mRNA.

Oligonucleotide probes are more rigorous and less sensitive to RNAase compared to riboprobes (Wilkinson et al., 1998). Previous studies have used riboprobes to investigate RGS protein gene expression (Gold et al., 1997; Maple et al., 2007), which are more specific in detecting spatial gene expression (Wilkinson et al., 1998). However, this
technique is limited by extensive molecular cloning and specific plasmid generation. Unfortunately, these facilities and expertise are not available in our laboratory.

The spatial distribution of only a few RGS protein subtypes have been determined by using techniques such as immunohistochemistry (Waugh et al., 2005; Paspalas et al., 2009). Investigations into the cellular distribution of RGS proteins are generally limited, due to the lack of commercially available antibodies.

2.4.2 Spatio-temporal changes in RGS protein gene expression in the unilateral 6-OHDA-lesioned rat model of LID

RGS mRNA levels in the rostral striatum of unilateral 6-OHDA-lesioned rats following 21 days L-DOPA treatment are summarised in Table 4. The sensorimotor territories of the rostral striatum are located in the lateral quadrants, while non-sensorimotor territories are found in the medial striatal quadrants (McGeorge and Faull, 1989). Therefore, changes that are specific in the lateral striatum may have important implications on motor function. Both the acute and long-standing effects of repeated L-DOPA treatment are shown at 1 h post final L-DOPA administration, while only long-standing effects of L-DOPA treatment are shown after a 24 h washout period (Westin et al., 2007).

<table>
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<th>Rostral striatum</th>
<th>6-OHDA Vehicle</th>
<th>6-OHDA L-DOPA 1 h</th>
<th>6-OHDA L-DOPA 24 h</th>
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<td>DL</td>
<td>VL</td>
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<td>RGS2</td>
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<td>RGS4</td>
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<td>RGS9-2</td>
<td>NC</td>
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<td>PPE-B</td>
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Table 4. A summary of the changes in RGS protein mRNA expression in the operated rostral striatum of the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease following 21 days, once daily treatment with vehicle or L-DOPA/benserazide (6/15 mg/kg; i.p.) at 1 hour and 24 hours post-administration. Groups are compared to ipsilateral sham-operated and 6-OHDA-lesioned vehicle-treated groups. Arrows indicate magnitude of increase or decrease in mRNA levels. NC, no change; DL, dorsolateral; VL, ventrolateral; DM, dorsomedial; VM, ventromedial.
RGS2 and RGS4 gene expression is unaffected in PD

This study showed no change in RGS2, 4 or 9-2 mRNA levels following unilateral 6-OHDA lesion. These results are consistent with Taymans et al. (2004), who reported no change in RGS2 or RGS4 mRNA levels following unilateral 6-OHDA-lesion in rats. Previous studies have also demonstrated that protein levels of RGS2 and RGS4 remain unchanged in unilateral 6-OHDA-lesioned rats (Yin et al., 2010).

In contrast to these data, Geurts et al. (2003) reported increased RGS2, and decreased RGS4, mRNA levels in the striatum following unilateral 6-OHDA-lesion in rats. These discrepancies may have been caused by different surgical methods used to induce dopamine denervation. The manner of dopaminergic deafferentiation is dependent on the 6-OHDA lesion site and in the aforementioned study; the SN was targeted to cause anterograde degeneration of the nigrostriatal dopaminergic pathway (Ungerstedt, 1968; Ungerstedt and Abuthnott, 1970). In contrast, we administered 6-OHDA into the MFB to cause rapid loss of dopaminergic cells in the striatum, VTA and SNC (Grealish et al., 2008). These differences in surgical methods may have caused the discrepancies in results. Moreover, Geurts et al. (2003) performed in situ hybridisation three weeks post 6-OHDA-lesion, whereas our in vitro experiments were carried out seven weeks post-surgery. Therefore, it is possible initial changes in RGS protein mRNA levels may have resolved after the 6-OHDA-lesion stabilised for a longer period of time.

In a different study, RGS4 mRNA levels were found increased in the striatum following dopamine denervation in rodent models of PD (Ding et al., 2006). The authors reported RGS4 mRNA levels were elevated specifically in striatal intercholinergic neurons of unilateral 6-OHDA-lesioned and reserpine-treated rats (Ding et al., 2006). This change was not detected in the current study, which could be due to the difference in techniques used for experimental investigation. In the aforementioned study, single-cell reverse transcriptase PCR was used to detect mRNA in specific neuronal cells, while we used in situ hybridisation to investigate an overall change in mRNA levels in a large neuroanatomical region.

RGS9-2 gene expression is unaffected in PD

In the current study, there was no change in RGS9-2 mRNA levels following unilateral 6-OHDA-lesion. Previous studies have shown RGS9-2 protein levels decrease after four
weeks following unilateral 6-OHDA-lesion (Yin et al., 2010). However, these changes were not found in the MPTP-lesioned mouse model of PD, where initial decreased RGS9-2 protein levels resolved three weeks following MPTP-treatment (Potashkin et al., 2007). Moreover, no change in RGS9-2 protein levels were found in the striatum of the MPTP-lesioned NHP model of PD (Gold et al., 2007).

The functional role of RGS9-2 in PD may involve other protein factors, such as Gβ5 and R7BP, at the post-transcriptional level (Witherow et al., 2000; Anderson et al., 2010). R7BP has multiple roles associated with RGS9-2, such as stabilising RGS9-2 proteins (Anderson et al., 2007b), preventing its degradation by cysteine proteases and targeting RGS9-2 to the postsynaptic density of striatal neurons (Anderson et al., 2007a). Moreover, recent evidence suggests R7BP also plays a direct role in motor behaviour, with R7BP KO mice displaying sensorimotor deficits (Anderson et al., 2010). Future studies into the roles of RGS9-2 proteins in PD should consider other protein factors, particularly R7BP, that modulate its function in the striatum.

**RGS2 gene expression is up-regulated in LID**

This study showed that RGS2 mRNA levels were increased in the striatum at 1 h post final L-DOPA administration in the unilateral 6-OHDA-lesioned rat model of LID. Moreover, elevated RGS2 mRNA levels returned to control levels following a 24 h washout period.

Transient up-regulation of RGS2 proteins may have pathophysiological roles in LID. For example, RGS2 proteins may modulate supersensitive dopamine D₁ receptor signalling in the striatum following induction of LID (Aubert et al., 2005; Guigoni et al., 2007). More specifically, elevated levels of RGS2 proteins may negatively modulate endogenous signalling proteins, such as Gαs subunits (Tseng and Zhang, 1998; Roy et al., 2003; Roy et al., 2006) and adenylate cyclases (Sinnarajah et al., 2001; Salim et al., 2003; Roy et al., 2006), that are specifically activated as part of the dopamine D₁ receptor signalling pathway. Up-regulation of RGS2 proteins may represent a compensatory feed-back mechanism to reduce overstimulation of dopamine D₁ receptors in striatonigral neurons (Figure 5).

Previous studies have shown up-regulation of RGS2 mRNA in the striatum is dependent on dopamine D₁ receptor signalling (Geurts et al., 2002; Taymans et al., 2003). cAMP
produced following activation of dopamine D₁ receptors (Figure 2; Sibley et al., 1998) is likely to activate cAMP-response elements (CREs) located in the promoter region of the RGS2 gene, thereby causing increased transcription (Pepperl et al., 1998; Xie et al., 2011). Similarly, in the dyskinetic state, other cAMP-dependent proteins such as DARPP-32 are elevated as a result of overactivation of dopamine D₁ receptors (Santini et al., 2007; Santini et al., 2010). Our results show a transient up-regulation of RGS2 mRNA, which may reflect temporal inhibition of GPCR signalling mechanisms following L-DOPA administration in PD. Expression of RGS2 mRNA is regulated in a manner similar to immediate early genes, such as c-fos (Robinet et al., 2001; Kehrl and Sinnarajah, 2002). Thus, RGS2 proteins are fitting protein factors that could regulate rapid cellular signalling in response to non-physiological neurotransmitter receptor stimulation following dopamine denervation (Gerfen, 2000).

RGS4 gene expression is up-regulated in LID

This study demonstrated that RGS4 mRNA levels were increased in the dorsolateral and ventrolateral rostral striatum of unilateral 6-OHDA-lesioned rats following 21 days L-DOPA treatment. Increased RGS4 mRNA levels were seen at 1 h and 24 h post final L-DOPA administration. At the present time and to our knowledge, no other study has investigated the roles of RGS4 proteins in LID.

The discrete topographical regions where RGS4 mRNA levels were affected support the involvement of RGS4 proteins in LID. In the current study, RGS4 mRNA was increased in the lateral regions of the striatum, which are innervated by the sensorimotor areas of the cerebral cortex (McGeorge and Faull, 1989). Previous studies have shown RGS4 mRNA is expressed in high ChAT brain regions (Ding et al., 2006; Ebert et al., 2006). In the striatum, high ChAT regions correspond to the matrix compartments (Pert et al., 1976; Graybiel et al., 1986; Gerfen, 1992a), which receive inputs from sensorimotor cortical associated areas (Gerfen, 1989; Gerfen, 1992a). These data suggest RGS4 proteins have functional roles in motor behaviour. Indeed, RGS4 KO mice show a measurable sensorimotor deficit as their behavioural phenotype (Grillet et al., 2005).

In the current study, we found that the increased RGS4 mRNA levels positively correlated with cumulative AIMs scores. These data suggest RGS4 proteins are implicated in the expression of dyskinesia. Previous studies have shown RGS4 proteins do not directly
modulate the G_α subunits activated by dopamine D_2 receptors (Cabrera-Vera et al., 2004; Ghavami et al., 2004), but regulate the G_{αi/o/q} subunits activated by mGluR group I (mGluR 1 and 5 subtypes) (Saugstad et al., 1998; Schwendt and McGinty, 2007), μ-opioid (Georgoussi et al., 2006; Leontiadis et al., 2009; Han et al., 2010), 5-HT_{1A} (Beyer et al., 2004; Ghavami et al., 2004) and muscarinic M_4-cholnergic receptors (Ding et al., 2006). Many of these GPCRs have pathophysiological roles in LID. Thus, RGS4 proteins may play multiple roles in LID. For example, studies have shown RGS4 proteins negatively modulate mGluR5 receptors in the striatum (Schwendt and McGinty, 2007; Schwendt et al., 2011). The mGluR5 receptors are overstimulated in LID and administration of selective mGluR5 antagonists attenuates dyskinesia in animal models of PD (Dekundy et al., 2006; Mela et al., 2007; Levandis et al., 2008; Samadi et al., 2008; Rylander et al., 2009; Rylander et al., 2010a). Thus, it is possible that increased RGS4 proteins in the striatum reflect an endogenous mechanism to reduce overactivation of mGluR5 receptors in LID.

On the other hand, increased RGS4 protein levels may contribute to the expression of dyskinesia. This could be attributed to the inhibitory effects of RGS4 proteins on presynaptic serotonergic autoreceptors (Beyer et al., 2004; Gu et al., 2007). In LID, the up-regulation of RGS4 proteins may inhibit serotonergic 5-HT_{1A/B} autoreceptors and contribute to unregulated release of dopamine and its metabolites from presynaptic terminals (de la Fuente-Fernandez et al., 2004; Carta et al., 2006; Lindgren et al., 2010). As mentioned previously, unregulated release of dopamine into the striatum is a major factor in the development and the expression of L-DOPA-induced AIMs (Carta et al., 2007; Lindgren et al., 2010). Following administration of 5-HT_{1A} agonists, dyskinesia is reduced in unilateral 6-OHDA-lesioned rats (Dupre et al., 2007; Eskow et al., 2007), MPTP-lesioned NHPs (Bibbiani et al., 2001; Iravani et al., 2006b) and PD patients (Olanow et al., 2004; Bara-Jimenez et al., 2005). It is possible that inhibition of RGS4 proteins could enhance 5-HT_{1A} receptor activation, leading to reduced expression of dyskinesia. The therapeutic potential of RGS4 protein inhibition in LID is explored in Chapters 3 and 4.

The up-regulation of RGS4 mRNA in the striatum is dependent on dopamine D_2 receptor activation (Geurts et al., 2002; Taymans et al., 2003). Transient changes in striatal RGS4 mRNA levels have been shown to normalise over a 24 h washout period (Schwendt et al., 2006), while persistent changes beyond this time point are likely to involve long-term molecular adaptations. The upstream elements in the RGS4 promoter region have binding
sites for AP1 transcription factors (Zhang et al., 2005). The AP1 protein is a heterotrimer that consists of transcription factors; fos and jun. Subtypes of these transcription factors, such as ΔfosB and junB, are persistently up-regulated in the striatum following induction of LID (Andersson et al., 1999; Cenci et al., 1999; Westin et al., 2001). These transcription factors, particularly ΔfosB and junD, activate AP1 regulated genes in LID (Andersson et al., 2001), which may cause increased RGS4 mRNA levels. Moreover, fosB transcription factors also mediate the up-regulation of prodynorphin mRNA in the dyskinetic state (Andersson et al., 1999), which could explain the positive correlation between RGS4 and PPE-B mRNA levels in the rostral striatum.

A recent study has shown changes in RGS4 protein expression in the striatum parallels with mRNA levels (Schwendt and McGinty, 2007). Moreover, reports have shown RGS4 proteins are modulated in parallel with RGS4 mRNA in the rat striatum (Schwendt et al., 2006). Although in vitro cell studies have shown newly synthesised RGS4 proteins are rapidly degraded by proteasomes following ubiquitination, as part of the N-end rule, approximately 25% of these proteins remain stable (Davydov and Varshavsky, 2000).

**RGS9-2 gene expression is unaffected in LID**

This study demonstrated that there was no change in RGS9-2 mRNA levels following 21 days L-DOPA treatment in unilateral 6-OHDA-lesioned rats. To our knowledge, no other study has previously investigated the levels of RGS9-2 mRNA following induction of LID. However, RGS9-2 protein levels have been measured in LID. In dyskinetic MPTP-lesioned NHPs, RGS9-2 protein levels remain unchanged in the striatum (Gold et al., 2007), while in L-DOPA-treated PD patients it was reported that striatal RGS9-2 proteins are increased along with fosB proteins. However, no reference to expression of dyskinesia in these patients was documented in any part of the study (Tekumalla et al., 2001).

Experimental evidence suggests that RGS9-2 proteins have direct roles in the expression of motor behaviours (Rahman et al., 2003; Kovoor et al., 2005; Gold et al., 2007; Blundell et al., 2008). The production of RGS9-2 KO mice showed impaired motor function as measured on the rotorod (Blundell et al., 2008). Moreover, AIMs are rapidly induced in RGS9-2 KO mice following dopamine agonist treatment (Kovoor et al., 2005). The RGS9-2 protein is almost exclusively expressed in the rat striatum (Gold et al., 1997) and can attenuate µ-opioid (Zachariou et al., 2003; Psifogeorgou et al., 2007) and dopamine D₂
receptors (Gold et al., 2007). Behavioural experiments have shown RGS9-2 proteins primarily inhibit dopamine D₂ receptor expressing neurons (Kovoor et al., 2005; Gold et al., 2007). In the striatum, these neurons are the striatopallidal projection neurons (Bertran-Gonzalez et al., 2008) and form the indirect pathway of the basal ganglia. Interestingly, overexpression of RGS9-2 can attenuate LID in MPTP-lesioned NHPs but at the expense of dopamine D₂ receptor-mediated anti-parkinsonian responses (Gold et al., 2007).

In the current study, analysis of the RGS9-2 mRNA levels was insufficient to describe the roles of RGS9-2 proteins in LID. It is possible RGS9-2 proteins undergo post-translational modifications or recruit protein factors to mediate its role in dyskinesia. For example, expression and subcellular distribution of RGS9-2 is dependent on Gβ5 and R7BP (Chen et al., 2003; Anderson et al., 2007a; Anderson et al., 2007b). Interestingly, following loss of RGS9-2 there is translocation of, another RGS protein, RGS7 from intracellular sites to the plasma membrane (Anderson et al., 2009). Such protein interplay has functional implications on production of motor behaviour and psychostimulant-induced locomotor sensitisation (Anderson et al., 2010). These complex cellular mechanisms should be further investigated in order to develop novel pharmacological agents that specifically target RGS9-2 proteins for potential therapeutic use (Neubig and Siderovski, 2002; Traynor et al., 2009).
The potential mechanisms of RGS proteins in LID

**Figure 34.** A schematic diagram displaying the potential mechanisms of RGS proteins in L-DOPA-induced dyskinesia (LID). Activation of NMDA and dopamine D

1 receptors may cause up-regulation of RGS2 proteins in LID, leading to inhibition of several neurotransmitter receptors and cellular signals. Activation of dopamine D

2 receptors may cause the up-regulation of RGS4 proteins in LID, which may inhibit non-dopaminergic signalling pathways. RGS9-2 proteins may mediate inhibition of dopamine D

2 receptor-mediated signalling in LID. AC5, adenylate cyclase subtype 5; M

1/4 Ach, muscarinic acetylcholine receptor subtype 1/ 4; R7BP, R7 binding protein; mGluR5, metabotropic glutamate receptor subtype 5; 5-HT

1A, serotonin receptor subtype 1A. Black arrows represent stimulation; blue arrows represent inhibition.
Conclusion
Repeated L-DOPA treatment for 21 days in unilateral 6-OHDA-lesioned rats caused up-regulation of RGS2 and RGS4, but not RGS9-2, mRNA in the striatum. These changes in RGS protein mRNA showed specific spatio-temporal profiles. For example, RGS2 mRNA was elevated in both the rostral and caudal striatum, but only at 1 hour post L-DOPA administration. In contrast, RGS4 mRNA was up-regulated specifically in the lateral regions of the rostral striatum, at both 1 h and 24 h post L-DOPA administration. Furthermore, increased RGS4 mRNA levels positively correlated to behavioural and molecular markers of LID.

Expression of RGS4 is found higher in the lateral regions of the rostral striatum (Geurts et al., 2002), which are the sensorimotor regions (McGeorge and Faull, 1989). It has been demonstrated that RGS4 proteins have a general role in production of motor behaviours (Grillet et al., 2005). Thus, the increased RGS4 protein activity in LID may be directly associated with the expression of hyperkinetic motor behaviours. Selectively targeting RGS4 proteins may prove beneficial in reducing LID, without compromising the anti-parkinsonian effects of L-DOPA. In the next chapter, we investigate the acute behavioural effects of RGS4 protein inhibition in the unilateral 6-OHDA-lesioned rat model of LID.
Chapter 3

Behavioural pharmacology of RGS4 protein inhibitors in the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia
3.1 Introduction

Dopamine replacement therapy with the immediate metabolic precursor of dopamine, L-DOPA, remains the most common pharmacological treatment for PD. However, long-term L-DOPA treatment leads to severe motor complications such as ‘wearing off’ (Schoulson et al., 1975) and LID (Cotzias et al., 1969). A single causative factor for these motor complications remains to be established. However, large intermittent fluctuations of dopamine levels following L-DOPA treatment is thought to be a major contributing factor to the sensitisation of dopamine receptors and induction of dyskinesia in PD (Chase, 1998; Olanow and Obeso, 2000). In line with this hypothesis, dyskinetic PD patients show large increased extracellular dopamine levels in the striatum, in comparison to non-dyskinetic PD patients, following L-DOPA treatment (de la Feunte-Fernandez et al., 2004; Pavese et al., 2006). The unregulated release of dopamine in dyskinesia may, in part, be mediated by serotonergic (5-HT) neurons (Carlsson et al., 2007; Carta et al., 2007; Lindgren et al., 2010). Indeed, following L-DOPA administration, 5-HT neurons release dopamine as a false neurotransmitter into the dopamine-denervated striatum from their presynaptic terminals (Tanaka et al., 1999; Carta et al., 2008).

The raphe-striatal 5-HT system widely innervates the basal ganglia, with dense serotonergic input to the striatum (Lavoie and Parent, 1990). In animal models of dyskinesia, 5-HT neurons undergo morphological changes, such as an increased number of axonal varicosities (Rylander et al., 2010b; Zeng et al., 2010). 5-HT neurons can convert L-DOPA to dopamine and store dopamine in synaptic vesicles, via amino acid aromatic decarboxylase (AADC) and vesicular monoamine transporter 2 (VMAT2), respectively (Tison et al., 1991; Arai et al., 1994; Arai et al., 1995; Arai et al., 1996). 5-HT neurons compensate for dopaminergic neuron loss by catabolising L-DOPA to dopamine (Maeda et al., 2005), which is subsequently released into the extracellular space of the striatum (Tanaka et al., 1999). However, 5-HT neurons cannot efficiently clear dopamine following its release and are without the proper autoregulatory mechanisms for dopaminergic transmission (Carta et al., 2008). Thus, dopamine efflux is unregulated and imbalanced.

The 5-HT receptor family are potential therapeutic targets for treatments of dyskinesia (Nicholson and Brotchie, 2002; Fox et al., 2008b). In particular, somatodendritic 5-HT1A autoreceptors located in the dorsal raphe nucleus have been targeted (Hjorth and Sharp, 1991; Kreiss and Lucki, 1994; Knobelman et al., 2000). Stimulation of these receptors can
mediate autoreceptor inhibition of neurotransmitter release (Invernizzi et al., 1991; Casanovas and Artigas, 1996; Santiago et al., 1998). Indeed, administration of selective 5-HT\textsubscript{1A} receptor agonist, 8-OH-DPAT, in combination with L-DOPA, leads to reduced extracellular dopamine levels in unilateral 6-OHDA-lesioned rats (Kannari et al., 2001). In behavioural experiments, acute systemic administration of 8-OH-DPAT reduces dyskinesia in 6-OHDA-lesioned rats (Carta et al., 2007; Dupre et al., 2007; Eskow et al., 2007), MPTP-lesioned NHPs (Bibbiani et al., 2001; Iravani et al., 2006b) and PD patients (Olanow et al., 2004; Bara-Jimenez et al., 2005). More recent data suggests that a population of 5-HT\textsubscript{1A} receptors in the striatum are, at least partly, responsible for the anti-dyskinetic effects seen following 8-OH-DPAT treatment (Dupre et al., 2008a; Bishop et al., 2009; Dupre et al., 2011).

All known subtypes of 5-HT receptors, with the exception of 5-HT\textsubscript{3} which forms an ion channel, are G-protein coupled. GPCRs are negatively modulated by RGS proteins (Helper, 1999; Ross and Wilkie, 2000). \textit{In vitro} cell studies have shown that agonist-induced 5-HT\textsubscript{1A} receptor signalling is attenuated by several RGS proteins, which include RGS4, RGS10 and RGSZ1 (Ghavami et al., 2004). The short amphipathic N-terminal of RGS4 proteins mediates translocation to the plasma membrane and confers interaction with different GPCRs (Zeng et al., 1998; Bernstein et al., 2000), such as M\textsubscript{4}-cholinergic (Ding et al., 2006), mGluR5 (Schwendt and McGinty, 2007; Schwendt et al., 2011), \textmu\textsubscript{-}opioid (Georgoussi et al., 2006; Han et al., 2010) and 5-HT\textsubscript{1A} receptors (Beyer et al., 2004; Ghavami et al., 2004). The inhibitory effect of RGS4 proteins on GPCRs leads to changes in neurotransmission \textit{in vivo} (Beyer et al., 2004; Schwendt et al., 2011). For example, viral-mediated overexpression of RGS4 in the dorsal raphe nucleus attenuates somatodendritic 5-HT\textsubscript{1A} autoreceptor-mediated inhibition, leading to increased 5-HT release in the striatum (Beyer et al., 2004). Such inhibitory effects on 5-HT\textsubscript{1A} receptors may have functional consequences on movement (Nicholson and Brotchie, 2002). Indeed, RGS4 knockout mice show subtle sensorimotor deficits (Grillet et al., 2005). Moreover, within the striatum, RGS4 is predominantly expressed in the lateral regions of the striatum (Geurts et al., 2002), which receive sensorimotor projections from motor regions of the cortex (McGeorge and Faull, 1969). In the previous chapter, we found that RGS4 mRNA was specifically increased in lateral regions of the rostral striatum in the unilateral 6-OHDA-lesioned rat model of LID. It is possible that up-regulation of RGS4 proteins may hyperinhibit GPCRs, such as 5-HT\textsubscript{1A} receptors, and contribute to the expression of AIMs.
3.1.1 **Aims and objectives**

We hypothesize that RGS4 proteins are involved in the expression of AIMs given that up-regulation of RGS4 protein mRNA expression positively correlated to AIMs severity. These proteins may either; mediate a compensatory mechanism to inhibit GPCR signalling events for dampening of AIMs or, in contrast, positively contribute to the expression of AIMs. By assessing AIMs and general motor ability in the unilateral 6-OHDA-lesioned rat model of LID, we aim to:

1) Uncover the roles of RGS4 proteins in LID by characterising the acute behavioural effects of RGS4 protein inhibitor treatment, in combination with L-DOPA.

2) Characterise the behavioural responses following specific blockade of RGS4 proteins in the striatum using RGS4 protein inhibitors.

3) Characterise the behavioural effects during 7 days intrastriatal administration of RGS4 antisense oligonucleotides.
3.2 Materials and Methods
All animal work was carried out under the regulations of the Animals (Scientific Procedures) Act, 1986.

3.2.1 Animals
Animals were housed in husbandry conditions as described in Chapter 2 (2.2.1).

Unilateral 6-hydroxydopamine lesion of the right MFB
Male Sprague-Dawley rats received a unilateral 6-OHDA lesion of the right MFB as described in Chapter 2 (2.2.1). Rats weighed between 290-320g on the day of surgery. Briefly, thirty minutes prior to surgery, rats were injected with pargyline (5 mg/kg, i.p.; Sigma-Aldrich, UK) and desipramine (25 mg/kg, i.p.; Sigma-Aldrich, UK), dissolved in sterile 0.9% (w/v) sodium chloride (Braun AG, Germany), at a volume of 1 ml/kg. Under isoflurane anaesthesia, rats were fixed into a stereotactic frame (David Kopf instruments, California, USA) using ear bars. The skin on the head was incised along the midline and a small burr hole was made into the skull. The dura matter was penetrated and 2.5 µl of 6-OHDA hydrobromide (Sigma-Aldrich, 5 mg/ml dissolved in sterile water) was injected over 5 min (0.5 µl/min) into the right medial forebrain bundle using a Hamilton syringe (Gauge 22s, Hamilton, UK). The needle was left in place for a further 5 min to allow the solution to dissipate and prevent reflux along the needle track. The stereotactic coordinates were as follows, in mm relative to bregma: A = -2.8, L = -2.0, V = -8.6 (Paxinos and Watson, 1986). Following surgery, rats received a s.c. injection of 10 ml Hartmann’s solution (Baxter, UK) to prevent dehydration. Rats were then placed in an incubator held at 29°C until they regained consciousness.

3.2.2 Drug treatment
Drugs were administered systemically into the i.p. cavity at a volume of 1 ml/kg, unless otherwise stated. The baseline scores for each behavioural test was measured following L-DOPA or vehicle treatment. Novel compounds targeting RGS4 proteins were administered in combination with L-DOPA/benserazide or vehicle (please see ‘behavioural pharmacology of RGS4 protein inhibitors’ section below).
**L-DOPA/benserazide**

L-DOPA methyl ester hydrochloride (D1507, Sigma-Aldrich, UK) and benzerazide hydrochloride (B7283, Sigma-Aldrich, UK) were dissolved in sterile saline (0.9% w/v; Braun AG, Germany) and administered at a volume of 1 ml/kg.

L-DOPA priming began four to five weeks post-lesion in preparation for testing the acute effects of RGS4 protein inhibitors on AIMs expression (Figure 35A). Animals received one daily injection of L-DOPA/benserazide (6/15 mg/kg; i.p.) between 10:00 - 11:00 am for 21 days. This L-DOPA treatment regimen induces stable expression of AIMs in unilateral 6-OHDA-lesioned rats (Lundblad et al., 2002). Following 21 days L-DOPA treatment, animals received two L-DOPA/benserazide injections per week to maintain stable AIMs scores (Lee et al., 2000; Dekundy et al., 2007).

**RGS4 protein inhibitors**

RGS4-G_{\alpha0} interaction inhibitors, CCG-2046 (Tocris Bioscience, UK) and CCG-4986 (Chembridge, USA), were purchased for behavioural tests. For microinjection studies, each compound was dissolved in 0.4% (v/v) ethanol-sterile saline (0.9%, w/v) to final concentrations of 0.01, 0.02, 0.2 and 2 mM. A total volume of 10 µl was administered through an implanted cannula immediately prior to L-DOPA/benserazide injection.

Novel reversible RGS4 inhibitors, CCG-63802 and CCG-63808, were synthesised by Sequoia Research Products Ltd as described by Blazer et al. (2010). RGS4 protein inhibitors were dissolved in 10% (v/v) dimethyl sulfoxide (DMSO)-sterile saline (0.9%, w/v) at 0.003, 0.01, 0.03, and 0.1 mg/ml for systemic (i.p.) administration. Compounds were administered 30 min prior to L-DOPA/benserazide injection.

**3.2.3 Behavioural analysis**

**Assessment of AIMs**

AIMs were scored as previously described (Lundblad et al., 2002; Dekundy et al., 2007) with minor modifications. AIMs were assessed on days 1, 7, 14, and 21 during L-DOPA priming (Figure 35A). For each testing session, animals were placed in a clear Perspex box for 10 min to acclimatise to experimental surroundings. Following L-DOPA/benserazide injection, animals were assessed for AIMs in 1 min intervals, every 30 min for 3 h. AIMs assessments were carried out between 8:00 am and 5:00 pm.
Four subtypes of AIMs were assessed as previously described (Cenci et al., 1998). These included; locomotor, increased contralateral rotations to the lesioned side; limb, purposeless and repetitive movements of the forelimb contralateral to the lesion; axial, twisted neck and body posture towards the side contralateral to the lesion; and orolingual, excessive jaw movements and tongue protrusion.

Subtypes of AIMs were scored on a semi-quantitative scale, between 0-4, depending on duration (Cenci et al., 1998); 0 = absent; 1 = infrequent, present for less than half the observation time; 2 = frequent, present for over half the observation time; 3 = continuous, present throughout observation time, can be suppressed by external stimuli; 4 = continuous, present throughout observation time, cannot be suppressed by external stimuli.

The sum of ALO AIMs was calculated over the three hour testing period for each assessment session. A threshold level of ALO AIMs was used to eliminate non-dyskinetic rats (Carta et al., 2006) i.e. animals with sum of ALO AIMs <5 on day 21 of L-DOPA treatment were not used for this study. Locomotor AIMs were analysed separately as the relevance of this behaviour remains poorly understood (Lane et al., 2006; Marin et al., 2006). To closely assess the time course of ALO AIMs, scores at 0-1, 1-2 and 2-3 h post L-DOPA treatment were analysed.

**Rotorod training**

In preparation for motor ability assessment, rats were first trained on the rotorod similar to methods previously described (Rozas et al., 1997; Rozas and Labandeira, 1997). The rotorod unit consisted (Med Associates, Georgia, VT, USA) of a horizontal rotating beam, with four allocated lanes. Each rat was placed on the beam, with its head facing opposite to the direction of rotation. Rats were required to walk forward along the beam at a speed set by a standard PC. Beam rotations were set to constant (C.S.) and accelerating speeds given in rotations per min (r.p.m). Each trial run lasted for a maximum of 5 min. The rotorod score, defined as the time taken for each rat to fall, was automatically recorded by an infrared sensor at the base of the unit. Between each trial, rats were given a 5 min rest period to reduce stress and fatigue (Rozas et al., 1997).
Rotorod training was performed two weeks post-lesion, over four consecutive days. The rotorod training protocol was adapted from Bergquist et al. (2003), with minor modifications (Table 5).

<table>
<thead>
<tr>
<th>Trial speed rotations per minute (r.p.m)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant speed 8</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Constant speed 10</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Constant speed 12</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Constant speed 14</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Constant speed 16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Acceleration 4 to 40</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 5.** Unilateral 6-OHDA-lesioned rats were rotorod trained over four consecutive days at two weeks post-surgery. Each rotorod trial lasted a maximum of 5 minutes. Constant speed trials were set at different rotations per minute (r.p.m).
**Rotorod assessment post-training**

One week following rotorod training, rats were assessed on the rotorod. The post-training rotorod assessment took place over two consecutive days (Table 6). These tests were evaluated for stable rotorod performances, which indicate habituation to the experimenter and testing conditions (Rozas et al., 1997).

<table>
<thead>
<tr>
<th>Trial speed</th>
<th>Number of trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant speed 10</td>
<td>Day 1: 3, Day 2: 3</td>
</tr>
<tr>
<td>Constant speed 12</td>
<td>Day 1: 3, Day 2: 3</td>
</tr>
<tr>
<td>Constant speed 14</td>
<td>Day 1: 3, Day 2: 3</td>
</tr>
<tr>
<td>Constant speed 16</td>
<td>Day 1: 3, Day 2: 3</td>
</tr>
<tr>
<td>Acceleration 4 to 40</td>
<td>Day 1: -, Day 2: 3</td>
</tr>
</tbody>
</table>

**Table 6.** Post-training rotorod assessment took place over two consecutive days, one week after completion of rotorod training. Constant speed trials were set at different rotations per minute (r.p.m).

**Rotorod testing following drug treatment**

The rotorod acceleration trial was used to measure the effects of drug treatment on motor performance. During L-DOPA priming, rotorod tests were performed on treatment days 2, 8, 15 and 22 (Figure 35A). For testing the acute effects of RGS4 inhibitor treatment in combination with L-DOPA or vehicle treatment on motor ability, rats were assessed on the acceleration trial (4 to 40 r.p.m) every 30 min for 3 h (Figure 35B). Each trial run lasted a maximum of 5 min.

Average group rotorod performance over three hours was used for statistical analyses. ‘Peak’ rotorod performance, the rotorod score at 1 h post L-DOPA treatment, was analysed separately to assess the drug effects on the L-DOPA-induced anti-akinetic response (Dekundy et al., 2007; Kobylecki et al., 2011).
3.2.4 Behavioural pharmacology of RGS4 protein inhibitors

Systemic administration of RGS4 protein inhibitors

A cross over design study was employed to test the acute behavioural effects of RGS4 protein inhibition in unilateral 6-OHDA-lesioned rats primed with L-DOPA (Figure 35B). To do this, an RGS4 protein inhibitor or vehicle was systemically administered (i.p.) 30 min prior to L-DOPA/benserazide (6/15 mg/kg; i.p.) before behavioural assessment of AIMS or rotorod. The behavioural tests were assessed following treatment with the same dose of compound on separate days, with a minimum of two days between each assessment. Doses of the RGS4 protein inhibitor (0.003, 0.01, 0.03 and 0.1 mg/kg; i.p.) were randomised for each set of behavioural tests (Figure 35B). A minimum of one week washout was given between testing different RGS4 protein inhibitors. Baseline scores of AIMS and rotorod performance i.e. scores following vehicle + L-DOPA/benserazide treatment were regularly assessed to ensure values remained stable. Animals were kept for up to a period of 1 year to complete all behavioural tests.

Microinjection of RGS4 inhibitors into the lateral striatum

To investigate the acute behavioural effects of site-specific RGS4 protein inhibition in the striatum, a cross over design study was employed in unilateral 6-OHDA-lesioned rats that received cannula implantation targeting the lateral rostral striatum (Figure 35B). Unilateral 6-OHDA-lesioned rats were anaesthetised with gaseous flow of 2% isoflurane mixed with oxygen and nitrous oxide. Hair above the incision site was shaved and cleaned with 70% ethanol-distilled water solution. Rats were fixed into a stereotactic frame (David Kopf instruments, California, USA) using ear bars. An incision was made on the skin along the midline and a burr hole was made into the skull. The dura matter was penetrated and a 27-gauge stainless steel cannula (0.9 mm outer diamter) was inserted into the lateral striatum, ipsilateral to the lesioned hemisphere at the following stereotactic coordinates, in mm relative to bregma: A = +1.0, L = -3.5, V = -5.5 (Paxinos and Watson, 1986). Stainless steel stylets, 5 mm length (25 G) were inserted into each cannula to maintain patency. Two other burr holes were made into the skull where two small dental screws were fixed to hold the cannula in position. Dental cement (MPAIR1KG & L, Metrorodent, UK) was used to fix the cannula in an upright position, perpendicular to the skull. The wound was closed around the cannula using standard sutures (3-0 Mersilk®) and cleaned with Betadine® to maintain antisepsis. EMLA® cream was applied to the wound for analgesia. Animals were placed an incubator (29°C) until consciousness was regained. Baseline AIMS and rotorod
performances were re-tested two weeks post-cannulation to ensure that there were no significant effects of surgery on motor behaviour.

**Acute behavioural effects of CCG-2046 and CCG-4986 via microinjection**

The cross over design study in striatal cannulated unilateral 6-OHDA-lesioned rats primed with L-DOPA consisted of behavioural assessments of AIMs or rotorod, following acute drug treatment with RGS4 protein inhibitor (CCG-2046 or CCG-4986; intrastriatal administration) + L-DOPA/benserazide (systemic administration; Figure 35B). AIMs and rotorod were assessed following treatment with the same dose of compound on separate days, with a minimum of two days between each assessment. Doses of RGS4 protein inhibitor, CCG-2046 (0.02, 0.2 or 2 mM) or CCG-4986 (0.01, 0.02, 0.2 mM) for each set of behavioural tests were randomised (Figure 35B). A minimum of one week washout was left between testing each compound. Baseline scores of AIMs and rotorod performance i.e. scores following vehicle + L-DOPA/benserazide treatment were regularly assessed to ensure values remained stable. Animals were kept for up to a period of 1 year to complete all behavioural tests.

On the day of a behavioural assessment, styles were removed and a 30 G, 12 mm long, needle was inserted into the cannula. Manual injections of RGS4 protein inhibitor were made through the needle by using a 10 µl Hamilton syringe (26s G, Hamilton, UK) connected by flexible portex tubing (outer diameter 1.09 mm; Smiths Medical International, UK). A total volume of 10 µl vehicle or drug was injected through the cannula over 1 min. The needle was left in place for a further 30 seconds before removal. Following this, L-DOPA/benserazide (6/15 mg/kg) was immediately administered into the i.p. cavity.

**Eight days consecutive treatment with RGS4 antisense oligonucleotides**

Cannulated unilateral 6-OHDA-lesioned rats primed with L-DOPA were subject to eight days consecutive RGS4 antisense oligonucleotide treatment (5'-G*G*ACTTAGTTTAGAA*G*-3', *inserted phosphorothioate bonds; Integrated DNA technologies, Belgium) during the cross over design experiment. RGS4 antisense oligonucleotides were synthesised as previously described (Garzon et al., 2001). These were administered into the lateral striatum over eight consecutive days (2 nM/day), in combination with systemic L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. AIMs and
rotorod performances were assessed on alternate days during, and after, antisense treatment.

3.2.5 Validation of cannula injection site

Tissue preparation
Following the completion of all behavioural assessments, rats were killed by exposure to a rising concentration of carbon dioxide. Rat brains were removed and immediately frozen in -45°C isopentane and stored at -80°C. Coronal sections were cryostat cut (Leica Microsystems, CM3050s, UK) to 20 μm thick sections at a chamber temperature of 18-20°C. Sections were thaw-mounted on gelatine/chrome-alum-coated slides (Henry et al., 1999) and left to air dry before storage at -80°C. With respect to bregma (Paxinos and Watson, 1986), two series of three equivalent sections were cut rostral-caudal through the striatum of the rat brain:

- Series one (12 slides collected) – rostral striatum, +1.70 mm
- Series two (12 slides collected) – intermediate striatum, +1.0 mm

Cresyl violet staining
Cresyl violet staining was performed on coronal sections through the striatum. Tissue sections were removed from the -80°C freezer and left for 20 min to equilibrate to room temperature. Tissue sections were then dehydrated in consecutive incubations with descending concentrations of ethanol: 15 min in 95% ethanol, 1 min 70% ethanol and 1 min 50% ethanol. Tissue sections were then rinsed for 3 min in distilled water and incubated for 2 min in cresyl violet acetate (C5042, Sigma-Aldrich, UK), then rinsed for 1 min with distilled water. Tissue sections were then dehydrated with the ascending concentrations of ethanol: 1 min 50% ethanol, 2 min 70% ethanol, 3 min 90% ethanol and 1 min 100% ethanol. Sections were then treated with xylene (Genta, UK) and microscope cover-slips were mounted onto the slides with Depex mounting medium (BDH, UK). Sections were observed under a light microscope (Leica DMRB) at x1.6 magnification for confirmation of the cannula tract entering the striatum. Animals with cannula sites outside the striatum were removed from all analyses.
3.2.6 Statistical analysis
Statistical analysis was carried out by using Prism v5.0 (GraphPad Software). The AIMs assessment scores were analysed using Friedman’s non-parametric ANOVA followed by Dunn’s multiple comparisons test. Rotorod performances were analysed using one-way ANOVA repeated measures followed by Tukey’s post hoc analysis. In all tests, significance level of p<0.05 was used.
**Figure 35.** A timeline for testing the acute behavioural effects of RGS4 protein inhibitors in the unilateral 6-hydroxydopamine (6-OHDA)-lesioned rat model of L-DOPA-induced dyskinesia (LID). A. Unilateral 6-OHDA-lesioning and L-DOPA priming phase. B. Cross-over experimental design employed for testing acute effects of RGS4 protein inhibitors, via systemic or intrastrital injection, in combination with L-DOPA/benserazide. AIMs, abnormal involuntary movements.
3.3 Results

3.3.1 Behavioural analysis

*Induction of AIMs pre-cannulation*

Following repeated once daily L-DOPA treatment for 21 days, unilateral 6-OHDA-lesioned rats developed AIMs (Fr=10.60; p=0.0315, Figure 36A and B). Sum of ALO AIMs scores over 3 h post L-DOPA treatment were significantly increased (p<0.05) in 6-OHDA-lesioned rats post-cannulation, compared to day 1 of L-DOPA priming. There was no significant difference in ALO AIMs scores between days 1, 7, 14 or 21 of L-DOPA priming (p>0.05). There was no significant difference in locomotor AIMs scores following L-DOPA treatment on days 1, 7, 14, 21 or post-cannulation (Fr=6.536; p=0.1626, Figure 36B).

*Rotorod performance during 21 days L-DOPA treatment*

There was a significant effect of 21 days L-DOPA treatment on rotorod performance in unilateral 6-OHDA-lesioned rats (F₃,₂₅=10.40; p<0.0001, Figure 37). Rotorod performance was significantly increased on day 2 of L-DOPA priming compared to vehicle treatment (24%, p<0.05). Rotorod performance was significantly reduced on treatment day 8 (21%, p<0.05), day 22 (35%, p<0.001) and post-cannulation (32%, p<0.001), compared to day 2 of L-DOPA priming. Rotorod performance was significantly reduced on day 22 of L-DOPA priming (28%, p<0.01) and post-cannulation (24%, p<0.05), compared to day 15 of L-DOPA priming. There was no significant difference in rotorod performance on any other treatment days (p>0.05).
Figure 36. Abnormal involuntary movement (AIMs) scores over 21 days, once daily L-DOPA/ benzerazide (6/15 mg/kg; i.p.) treatment in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. (A) Axial, limb and orolingual (ALO) and (B) locomotor AIMs scores displayed. Points represent data from individual animals (n=7) and bars represent median value of treatment group. *p<0.05 cf. L-DOPA treatment day 1 (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Figure 37. Rotorod performance over 22 days, once daily L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=7). *p<0.05 cf. vehicle treatment; #p<0.05, ###p<0.001 cf. L-DOPA treatment day 2; @p<0.05, @@p<0.01 cf. L-DOPA treatment day 15 (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
3.3.2 Microinjection of RGS4 protein inhibitors into the lateral striatum

**CCG-2046**

*L-DOPA-induced AIMs*

Following intrastriatal administration of CCG-2046 (0.02, 0.2, 2 mM) or vehicle, there was a significant effect of treatment group on the sum of axial, limb and orolingual (ALO) AIMs induced by L-DOPA treatment over 3 h post administration (Fr=12.57; p=0.0057, Figure 38A and B). Sum of ALO AIMs were reduced by CCG-2046 0.2 mM (82%, p<0.05) and 2 mM (76%, p<0.05) compared to vehicle.

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=10.71; p=0.0134, Figure 38C), which were reduced by CCG-2046 2 mM (67%, p<0.05) compared to vehicle.

At 1-2 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMs (Fr=7.588; p=0.0553, Figure 38D).

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=13.69; p=0.0034, Figure 38E), which were reduced by CCG-2046 2 mM (100%, p<0.05) compared to vehicle.

Following intrastriatal administration of CCG-2046 (0.02, 0.2, 2 mM) or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=4.327; p=0.2283, Figure 38F).

When administered alone into the striatum, CCG-2046 (0.02, 0.2, 2 mM) did not induce significant AIMs compared to vehicle (data not shown).
Figure 38. The effects of CCG-2046 (0.02, 0.2, 2 mM; intrastriatal administration) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05 cf. vehicle (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Rotorod performance
Following intrastriatal administration of CCG-2046 (0.02, 0.2, 2 mM) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration (F3,15=12.39; p=0.0002, Figure 39A). Time on the rotorod was increased by CCG-2046 0.02 mM (66%, p<0.01), 0.2 mM (95%, p<0.001) and 2 mM (73%, p<0.01), compared to vehicle.

Following intrastriatal administration of CCG-2046 (0.02, 0.2, 2 mM) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration (F3,18=2.940; p=0.0611, Figure 39B).

Following intrastriatal administration of CCG-2046 (0.02, 0.2, 2 mM) or vehicle, in combination with vehicle administration, there was a significant effect of treatment group on average rotorod performance (F3,15=3.707; p=0.0355, Figure 40A). There was, however, no group to group difference found following post hoc analysis (p>0.05).

Following intrastriatal administration of CCG-2046 (0.02, 0.2, 2 mM) or vehicle, in combination with vehicle administration, there was no significant effect on peak rotorod performance (F3,18=1.997; p=0.1506, Figure 40B).
Figure 39. The effects of CCG-2046 (0.02, 0.2, 2 mM; intrastriatal administration) on rotorod performance in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=7). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. **p<0.01, ***p<0.001 cf. vehicle (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
Figure 40. The effects of CCG-2046 (0.02, 0.2, 2 mM; intrastriatal administration) on rotorod performance in combination with vehicle (i.p.) administration in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=7). (A) Rotorod performance from 0-3 hours post vehicle administration; (B) Peak rotorod performance at 1 h post vehicle administration (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**CCG-4986**

*L-DOPA-induced AIMs*

Following intrastriatal administration of CCG-4986 (0.01, 0.02, 0.2 mM) or vehicle, there was a significant effect of treatment group on sum of axial, limb and orolingual (ALO) AIMs induced by L-DOPA treatment over 3 h post administration (Fr=17.15; p=0.0007, Figure 41A and B). Sum of ALO AIMs were reduced by CCG-4986 0.02 mM (76%, p<0.05) and 0.2 mM (76%, p<0.01) compared to vehicle.

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=14.77; p=0.0020, Figure 41C), which were reduced by CCG-4986 0.02 mM (66%, p<0.05) and 0.2 mM (78%, p<0.05) compared to vehicle.

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=14.13; p=0.0027, Figure 41D), which were reduced by CCG-4986 0.2 mM (86%, p<0.01) compared to vehicle.

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=15.13; p=0.0017, Figure 41E), which were reduced by CCG-4986 0.02 mM (100%, p<0.05) compared to vehicle.

Following intrastriatal administration of CCG-4986 (0.01, 0.02, 0.2 mM) or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=6.500; p=0.0897, Figure 41F).

When administered alone into the striatum, CCG-4986 (0.01, 0.02, 0.2 mM) did not induce significant AIMs compared to vehicle (data not shown).
Figure 41. The effects of CCG-4986 (0.01, 0.02, 0.2 mM; intrastriatal administration) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Rotorod performance

Following intrastriatal administration of CCG-4986 (0.01, 0.02, 0.2 mM) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration (F_{3,20}=9.361; p=0.0005, Figure 42A). Time on the rotorod was increased by CCG-4986 0.02 mM (75%, p<0.05) and 0.2 mM (129%, p<0.001) compared to vehicle. Time on the rotorod was increased by CCG-4986 0.2 mM (65%, p<0.05) compared to CCG-4986 0.01 mM.

Following intrastriatal administration of CCG-4986 (0.01, 0.02, 0.2 mM) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration (F_{3,23}=1.807; p=0.1741, Figure 42B).

Following intrastriatal administration of CCG-4986 (0.01, 0.02, 0.2 mM) or vehicle, in combination with vehicle (i.p.), there was no significant effect of treatment group on average rotorod performance (F_{3,15}=3.241; p=0.0520, Figure 43A) or peak rotorod performance (F_{3,18}=0.8837; p=0.4682, Figure 43B).
Figure 42. The effects of CCG-4986 (0.01, 0.02, 0.2 mM; intrastriatal administration) on rotord performance in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=6-7). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. *p<0.05, ***p<0.001 cf. vehicle; #p<0.05 cf. CCG-4986 0.01 mM (one-way ANOVA, Tukey’s post hoc analysis).
Figure 43. The effects of CCG-4986 (0.01, 0.02, 0.2 mM; intrastriatal administration) on rotorod performance in combination with vehicle (i.p.) administration in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=7). (A) Rotorod performance from 0-3 hours post vehicle administration; (B) Peak rotorod performance at 1 h post vehicle administration (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**RGS4 antisense oligonucleotide**

*L-DOPA-induced AIMs*

Following 8 days intrastriatal administration of RGS4 antisense oligonucleotide or vehicle, there was a significant effect of treatment on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=39.23; p<0.0001, Figure 44A). Percentage of ALO AIMs were reduced on treatment day 9 (71%, p<0.05), 11 (81%, p<0.01), 13 (68%, p<0.05) and 15 (68%, p<0.05), compared to baseline. Sum of ALO AIMs were reduced on treatment day 11 (62%, p<0.01) compared to day 5. There was no significant difference in ALO AIMs between any other treatment days (p>0.05).

Following 8 days intrastriatal administration of RGS4 antisense oligonucleotide or vehicle, there was no significant effect of treatment on locomotor AIMs (Fr=10.69; p=0.2974, Figure 44B).
Figure 44. The effects of 8 days RGS4 antisense oligonucleotide treatment, via intrastriatal administration, on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as percentage of L-DOPA induced AIMs ± SEM (n=6). (A) Percentage of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) Percentage of locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. baseline; ##p<0.01 cf. sub-chronic treatment day 5 (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Rotorod performance

Following 8 days intrastriatal administration of RGS4 antisense oligonucleotide or vehicle, there was a significant effect of treatment on average rotorod performance over 3 h post L-DOPA administration ($F_{9,45}=4.309; p=0.0004$, Figure 45A). Time on the rod was increased on treatment day 12 compared to baseline (33%, $p<0.05$), day 6 (54%, $p<0.001$), 8 (43%, $p<0.01$), 18 (31%, $p<0.05$) and 35 (32%, $p<0.05$). Time on the rod was increased on treatment day 14 (41%, $p<0.05$) compared to day 6. There was no significant difference in rotorod performance on any other treatment day ($p>0.05$).

Following 8 days intrastriatal administration of RGS4 antisense oligonucleotide or vehicle, there was no significant effect of treatment on peak rotorod performance at 1 h post L-DOPA administration ($F_{9,45}=1.274; p=0.2770$, Figure 45B).

Validation of cannula injection site

For each animal, cresyl violet stained sections showed a cannula tract entering into the rostral striatum (Figure 46).
Figure 45. The effects of 8 days RGS4 antisense oligonucleotide treatment, via intrastriatal administration, on rotorod performance in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=6). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. *p<0.05 cf. baseline; #p<0.05, ###p<0.001 cf. sub-chronic treatment day 6; $$p<0.01 cf. sub-chronic treatment day 8; @p<0.05 cf. sub-chronic treatment day 12 (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**Figure 46.** Coronal sections through the rat striatum showing cannula injection sites in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease and L-DOPA-induced dyskinesia. (A) Schematic diagram of the rostral striatum (+1.0 mm, with respect to bregma) adapted from Paxinos and Watson (1986); ● denotes terminal target sites. (B) Grey scale image of cresyl violet stained coronal section showing the cannula tract in the rostral striatum ipsilateral to the lesioned side. (C) Microscope image (1.6x magnification) of the cresyl violet stained coronal section.
3.3.3 Systemic administration of RGS4 protein inhibitors

**Induction of AIMs**
Following repeated once daily L-DOPA treatment for 21 days, unilateral 6-OHDA-lesioned rats developed AIMs (Fr=15.14; p=0.0017, Figure 47A). Sum of ALO AIMs scores over 3 h post L-DOPA treatment were significantly increased in 6-OHDA-lesioned rats on day 21 (125%, p<0.01) compared to day 1 of L-DOPA priming. There was no significant difference in ALO AIMs scores on days 1, 7 or 14 of L-DOPA priming (p>0.05). There was no significant difference in locomotor AIMs scores on days 1, 7, 14 or 21 of L-DOPA priming (Fr=5.231; p=0.1557, Figure 47B).

**Rotorod performance during L-DOPA priming**
There was a significant effect of 21 days L-DOPA treatment on rotorod performance in unilateral 6-OHDA-lesioned rats (F4,20=66.43; p<0.0001, Figure 48). Time on the rod was significantly decreased on day 8 of L-DOPA priming, compared to vehicle treatment (27%, p<0.001) and day 2 of L-DOPA priming (30%, p<0.001). Time on the rod was significantly decreased on day 15 of L-DOPA priming, compared to vehicle treatment (44%, p<0.001), L-DOPA treatment day 2 (46%, p<0.001) and day 8 (23%, p<0.01). Time on the rotorod was significantly decreased on day 22 of L-DOPA treatment compared to vehicle treatment (52%, p<0.001), L-DOPA treatment day 2 (54%, p<0.001) and day 8 (34%, p<0.001).
Figure 47. Abnormal involuntary movement (AIMs) scores over 21 days, once daily L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. (A) Axial, limb and orolingual (ALO) AIMs scores and (B) locomotor AIMs scores displayed. Points represent data from individual animals (n=7) and bars represent median value of treatment group. **p<0.01 cf. L-DOPA treatment day 1 (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Figure 48. Rotorod performance over 22 days, once daily L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=7). ***p<0.001 cf. vehicle treatment; ###p<0.001 cf. L-DOPA treatment day 2; $$p<0.01, $$$p<0.001 cf. L-DOPA treatment day 8 (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**CCG-2046**

*L-DOPA-induced AIMS*

Following administration of CCG-2046 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMS induced by L-DOPA treatment over 3 h post administration (Fr=21.25; p=0.0003, Figure 49A and B). Sum of ALO AIMS were reduced by CCG-2046 0.03 mg/kg (61%, p<0.05) and 0.1 mg/kg (65%, p<0.05) compared to vehicle. Sum of ALO AIMS were reduced by CCG-2046 0.03 mg/kg (63%, p<0.05) and 0.1 mg/kg (66%, p<0.01) compared to CCG-2046 0.003 mg/kg.

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=17.14; p=0.0018, Figure 49C), which were reduced by CCG-2046 0.1 mg/kg compared to vehicle (67%, p<0.01) and CCG-2046 0.003 mg/kg (67%, p<0.05).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=13.94; p=0.0075, Figure 49D). There was, however, no group to group difference found following post hoc analysis (p>0.05).

At 2-3 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMS (Fr=7.250; p=0.1233, Figure 49E).

Following administration of CCG-2046 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was no significant effect of treatment group on locomotor AIMS induced by L-DOPA over 3 h post administration (Fr=3.540; p=0.4718, Figure 49F).

When administered alone, CCG-2046 (0.003, 0.01, 0.03, 0.1 mg/kg) did not induce significant AIMS compared to vehicle (data not shown).
Figure 49. The effects of CCG-2046 (0.003, 0.01, 0.03, 0.1 mg/kg; i.p.) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle; #p<0.05, ##p<0.01 cf. CCG-2046 0.003 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Rotorod performance
Following administration of CCG-2046 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration (F_{4,20}=4.749; p=0.0074, Figure 50A). Time on the rod was increased by CCG-2046 0.01 (42%, p<0.05), 0.03 (38%, p<0.05) and 0.1 mg/kg (40%, p<0.05), compared to CCG-2046 0.003 mg/kg. There was no significant difference in rotorod performance between any other treatment group (p>0.05).

Following administration of CCG-2046 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration (F_{4,24}=1.333; p=0.2864, Figure 50B).
Figure 50. The effects of CCG-2046 (0.003, 0.01, 0.03, 0.1 mg/kg; i.p.) on rotorod performance in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=7). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. #p<0.05 cf. CCG-2046 0.003 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**CCG-4986**

*L-DOPA-induced AIMS*

Following administration of CCG-4986 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMS induced by L-DOPA treatment over 3 h post administration (Fr=19.97; p=0.0005, Figure 51A and B). Sum of ALO AIMS were reduced by CCG-4986 0.03 mg/kg (55%, p<0.05) and 0.1 mg/kg (58%, p<0.01) compared to vehicle. Sum of ALO AIMS were reduced by CCG-4986 0.03 mg/kg (52%, p<0.05) and 0.1 mg/kg (55%, p<0.05) compared to CCG-4986 0.003 mg/kg.

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=18.26; p=0.0011, Figure 51C), which were reduced by CCG-4986 0.03 mg/kg (44%, p<0.01) and 0.1 mg/kg (56%, p<0.01) compared to vehicle.

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=19.27; p=0.0007, Figure 51D), which were reduced by CCG-4986 0.1 mg/kg compared to vehicle (83%, p<0.01) and CCG-4986 0.003 mg/kg (85%, p<0.01).

At 2-3 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMS (Fr=4.278; p=0.3697, Figure 51E).

Following administration of CCG-4986 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was a significant effect of treatment group on locomotor AIMS induced by L-DOPA over 3 h post administration (Fr=9.522; p=0.0493, Figure 51F). There was, however, no group to group difference found following post hoc analysis (p>0.05).

When administered alone, CCG-4986 (0.003, 0.01, 0.03, 0.1 mg/kg) did not induce significant AIMS compared to vehicle (data not shown).
Figure 51. The effects of CCG-4986 (0.003, 0.01, 0.03, 0.1 mg/kg; i.p.) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle; #p<0.05, ##p<0.01 cf. CCG-4986 0.003 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
**Rotorod performance**

Following administration of CCG-4986 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was no significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{4,20}=0.5592; p=0.6948$, Figure 52A), or peak rotorod performance at 1 h post L-DOPA administration ($F_{4,24}=1.205; p=0.3345$, Figure 52B).
Figure 52. The effects of CCG-4986 (0.003, 0.01, 0.03, 0.1 mg/kg; i.p.) on rotorod performance in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=7). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**CCG-63802**

*L-DOPA-induced AIMs*

Following administration of CCG-63802 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=23.66; p<0.0001, Figure 53A and B). Sum of ALO AIMs were reduced by CCG-63802 0.03 mg/kg (43%, p<0.05) and 0.1 mg/kg (59%, p<0.001) compared to vehicle. Sum of ALO AIMs were reduced by CCG-63802 0.1 mg/kg (52%, p<0.01) compared to CCG-63802 0.003 mg/kg.

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=20.67; p=0.0004, Figure 53C), which were reduced by CCG-63802 0.1 mg/kg compared to vehicle (61%, p<0.01) and CCG-63802 0.003 mg/kg (56%, p<0.01).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=18.32; p=0.0011, Figure 53D), which were reduced by CCG-63802 0.03 mg/kg (56%, p<0.05) and 0.1 mg/kg (69%, p<0.01) compared to vehicle.

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=13.38; p=0.0096, Figure 53E). There was, however, no group to group difference found following post hoc analysis (p>0.05).

Following administration of CCG-63802 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was a significant effect of treatment group on locomotor AIMs induced by L-DOPA over 3 h post administration (Fr=11.73; p=0.0195, Figure 53F). There was, however, no group to group difference found following post hoc analysis (p>0.05).

When administered alone, CCG-63802 (0.003, 0.01, 0.03, 0.1 mg/kg) did not induce significant AIMs compared to vehicle (data not shown).
Figure 53. The effects of CCG-63802 (0.003, 0.01, 0.03, 0.1 mg/kg; i.p.) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01, ***p<0.001 cf. vehicle; ##p<0.01 cf. CCG-63802 0.003 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Rotorod performance

Following administration of CCG-63802 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration (F_{4,20}=15.67; p<0.0001, Figure 54A). Time on the rod was increased by CCG-63802 0.01 (40%, p<0.05), 0.03 (49%, p<0.01) and 0.1 mg/kg (91%, p<0.001), compared to vehicle. Time on the rotorod was increased by CCG-63802 0.1 mg/kg compared to CCG-63802 0.003 (51%, p<0.001), 0.01 (37%, p<0.01) and 0.03 mg/kg (28%, p<0.05). There was no significant difference in rotorod performance between any other treatment groups (p>0.05).

Following administration of CCG-63802 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration (F_{4,24}=1.675; p=0.1886, Figure 54B).
Figure 54. The effects of CCG-63802 (0.003, 0.01, 0.03, 0.1 mg/kg; i.p.) on rotorod performance in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=7). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration.

* p<0.05, ** p<0.01, *** p<0.001 cf. vehicle; ### p<0.001 cf. CCG-63802 0.003 mg/kg; $$ p<0.01 cf. CCG-63802 0.01 mg/kg; @ p<0.05 cf. CCG-63802 0.03 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**CCG-63808**

*L-DOPA-induced AIMs*

Following administration of CCG-63808 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=24.20; p<0.0001, Figure 55A and B). Sum of ALO AIMs were reduced by CCG-63808 0.03 mg/kg (44%, p<0.05) and 0.1 mg/kg (79%, p<0.001) compared to vehicle. Sum of ALO AIMs were reduced by CCG-63808 0.1 mg/kg (75%, p<0.01) compared to CCG-63808 0.003 mg/kg.

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=24.36; p<0.0001, Figure 55C), which were reduced by CCG-63808 0.1 mg/kg compared to vehicle (63%, p<0.001) and CCG-63808 0.003 mg/kg (56%, p<0.01).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=22.52; p=0.0002, Figure 55D), which were reduced by CCG-63808 0.03 mg/kg (53%, p<0.05) and 0.1 mg/kg (82%, p<0.001) compared to vehicle. ALO AIMs were reduced by CCG-63808 0.1 mg/kg (79%, p<0.05) compared to CCG-63808 0.003 mg/kg.

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=19.96; p=0.0005, Figure 55E), which were reduced by CCG-63808 0.1 mg/kg (100%, p<0.05) compared to CCG-63808 0.003 mg/kg.

Following administration of CCG-63808 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=3.415; p=0.4909, Figure 55F).

When administered alone, CCG-63808 (0.003, 0.01, 0.03, 0.1 mg/kg) did not induce significant AIMs compared to vehicle (data not shown).
Figure 55. The effects of CCG-63808 (0.003, 0.01, 0.03, 0.1 mg/kg; i.p.) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, ***p<0.001 cf. vehicle; #p<0.05, ##p<0.01 cf. CCG-63808 0.003 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Rotorod performance

Following administration of CCG-63808 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{4,20}=35.14; p<0.0001$, Figure 56A). Time on the rotorod was increased by CCG-63808 0.01 (44%, $p<0.05$), 0.03 (69%, $p<0.001$) and 0.1 mg/kg (131%, $p<0.001$) compared to vehicle. Time on the rotorod was increased by CCG-63808 0.03 mg/kg (34%, $p<0.05$) and 0.1 mg/kg (83%, $p<0.001$), compared to CCG-63808 0.003 mg/kg. Time on the rotorod was increased by CCG-63808 0.1 mg/kg compared to CCG-63808 0.01 mg/kg (61%, $p<0.001$) and 0.03 mg/kg (36%, $p<0.001$).

Following administration of CCG-63808 (0.003, 0.01, 0.03, 0.1 mg/kg) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration ($F_{4,24}=2.138; p=0.1071$, Figure 56B).
Figure 56. The effects of CCG-63808 (0.003, 0.01, 0.03, 0.1 mg/kg; i.p.) on rotorod performance in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=7). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. *p<0.05, ***p<0.001 cf. vehicle; #p<0.05, ###p<0.001 cf. CCG-63808 0.003 mg/kg; $$$p<0.001 cf. CCG-63808 0.01 mg/kg; @@ @p<0.001 cf. CCG-63808 0.03 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
3.4 Discussion
The major finding from this study was that acute inhibition of RGS4 proteins attenuated AIMs and improved motor ability in the unilateral 6-OHDA-lesioned rat model of LID.

3.4.1 Methodological considerations

**Behavioural assessments of the unilateral 6-OHDA-lesioned rat model**
In the current study, the behavioural effects of potential pharmacological treatments for dyskinesia were tested in the unilateral 6-OHDA-lesioned rat model of LID (Lundblad et al., 2002; Dekundy et al., 2007). This model is based on the unilateral 6-OHDA-lesioned rat model of PD (Ungerstedt, 1971), which models motor deficits on the side contralateral to the lesion. These motor deficits are quantifiable as discussed in Chapter 2.

The dose of L-DOPA (6 mg/kg) used in this study falls within the dose range (0.71-17.14 mg/kg) for PD patients (Rascol et al., 2000). Repeated L-DOPA treatment for 21 days leads to gradual induction of AIMs in unilateral 6-OHDA-lesioned rats (Dekundy et al., 2007). These AIMs are defined as purposeless, repetitive movements that affect different topographical areas such as forelimb, trunk and orolingual musculature (Cenci et al., 2002), and are characteristically different to other dopamine-dependent behaviours (sniffing, licking and gnawing) (Andersson et al., 2001). The severity of AIMs is quantified by amplitude and duration present, similar to the criteria used to assess dyskinesia in PD patients (Hagell and Widner, 1999; Cenci et al., 2002). In parallel with the induction of AIMs, molecular correlates associated with dyskinesia (opioid precursor PPE-B and ΔfosB proteins) are up-regulated in the lesioned striatum of unilateral 6-OHDA-lesioned rats (Cenci et al., 1998; Andersson et al., 1999; Henry et al., 1999; Konradi et al., 2004), which are consistent with findings in dyskinetic MPTP-lesioned NHPs (Henry et al., 2003; Berton et al., 2009).

The time course of AIMs expression is used to model peak-dose dyskinesia in PD patients (Lee et al., 2000). Peak AIMs expression occurs approximately 1 h after L-DOPA treatment, when extracellular dopamine levels are highest in the striatum (Zhang et al., 2003; Carta et al., 2006; Lindgren et al., 2010), similar to peak-dose dyskinesia in PD patients (de la Fuente-Fernandez et al., 2004; Pavese et al., 2006). Clinically used antidyskinetic treatments, such as amantadine, effectively reduce AIMs in the unilateral 6-OHDA-lesioned rat model of LID (Dekundy et al., 2007; Kobylecki et al., 2011). Thus, the
AIMs model is widely used to assess for novel anti-dyskinetic treatments (Lundblad et al., 2002; Dekundy et al., 2007; Mela et al., 2007; Rylander et al., 2009).

In this study, the rotorod test was used to evaluate general motor ability in unilateral 6-OHDA-lesioned rats (Rozas et al., 1997; Rozas and Labandeira, 1997). The accelerating rotorod trial was used to measure the effects on the L-DOPA-induced anti-akinetic response (Lundblad et al., 2003; Dekundy et al., 2007; Kobylecki et al., 2010). We also extended the rotorod testing period to 0-3 hours post L-DOPA administration, which is the time course dopamine levels are elevated in the basal ganglia following L-DOPA treatment in unilateral 6-OHDA-lesioned rats (Lindgren et al., 2010). This testing schedule was employed to thoroughly assess the overall motor activity following L-DOPA treatment.

**Mechanisms of action of RGS4 protein inhibitors**

*RGS4 antisense oligonucleotides*

RGS4 antisense oligonucleotides were used to inhibit RGS4 protein activity (Garzon et al., 2001). The RGS4 antisense oligonucleotide sequence was complementary to rat RGS4 mRNA (GenBank accession No. NW_047339) and was synthesised as previously described (Garzon et al., 2001). Phosphorothioate bonds were inserted at the 5'- and 3'-ends to reduce degradation by intracellular endo- and exo- nucleases (Wickstrom, 1986; Campbell et al., 1990; Akhtar et al., 1991; Eder et al., 1991). Antisense oligonucleotides mediate protein knockdown by binding to specific mRNA sequences via Watson-Crick base pair hybridisation (Dias and Stein, 2002). Upon duplex formation, protein translational machinery is blocked, while endogenous RNAase H enzyme mediates hydrolysis of bound RNA (Dias and Stein, 2002). The concentration of RGS4 antisense oligonucleotide used in this study has been shown to change opioid-mediated behavioural responses in mice following intracerebroventricular administration (Garzon et al., 2001).

*RGS4 protein inhibitors*

The RGS domain of the RGS4 protein can interact with Gα subunits through two defined interaction sites, ‘A’ and ‘B’ (Zhong and Neubig, 2001; Neubig and Siderovski, 2002). Firstly, site ‘A’ of the RGS domain mediates interaction through five specific amino acid residues that interact with amino acid Thr182 of the Gα subunit (Tesmer et al., 1997a; de Alba et al., 1999; Moy et al., 2000). This interaction occurs at the focal point of the RGS domain, where the two helical bundles form a ‘V’-like structure (Neubig and Siderovski,
The ‘B’ site on the RGS domain represents an allosteric modulatory site. Endogenous signalling factors, such as phosphatidylinositol-3,4,5-trisphosphate, calcium and calmodulin, bind to the ‘B’ site and regulate RGS protein function (Popov et al., 2000; Ishii et al., 2005).

RGS4-\(G_{\alpha_0}\) interaction inhibitors, CCG-2046 and CCG-4986, have IC\(_{50}\) values 4.3 ± 0.1 \(\mu\)M and 4.2 ± 0.1 \(\mu\)M, respectively (Roman et al., 2007). CCG-4986 inhibits RGS4-\(G_{\alpha_0}\) interaction by covalent modification of cysteine residues at site ‘B’ of RGS4 proteins (Kimple et al., 2007; Roman et al., 2010), but not RGS8, a closely related RGS protein (Roman et al., 2007; Roman et al., 2010). CCG-4986 also inhibits RGS4 GTPase activating activity (10 \(\mu\)M induces 68% inhibition) (Roman et al., 2007). The effects of CCG-4986 are reduced when tested in the presence of excess (2 mM) glutathione (GSH) (Blazer et al., 2010). However, these levels of GSH exceed physiological concentrations found in the striatum (Raps et al., 1989; Sian et al., 1994; Langeveld et al., 1996), blood and plasma (Michelet et al., 1995). Novel reversible RGS inhibitors, CCG-63802 and CCG-63808, block RGS4-\(G_{\alpha_0}\) interaction with IC\(_{50}\) values of 1.9 and 1.4 \(\mu\)M, respectively. These compounds bind to three specific cysteine residues (Cys95, Cys148 and Cys132) at site ‘B’ of the RGS4 protein (Blazer et al., 2010). CCG-63802 and CCG-63808 block RGS4 protein function but also exhibit weaker activity on other RGS proteins in the following order of efficacy: RGS4 > RGS19 = RGS16 > RGS8 >> RGS7 (Blazer et al., 2010).

### 3.4.2 Behavioural effects of RGS4 protein inhibitors in PD and LID

The acute behavioural effects of RGS4 protein inhibitors are summarised in Table 7. There were no significant differences compared to vehicle treatment when compounds were administered alone.
<table>
<thead>
<tr>
<th>Intrastrial administration</th>
<th>ALO AIMS</th>
<th>Lo AIMS</th>
<th>Overall rotorod performance</th>
<th>Peak rotorod performance</th>
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<td>CCG-2046</td>
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<td>CCG-4986</td>
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<td>RGS4 antisense</td>
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<tr>
<th>Systemic administration</th>
<th>ALO AIMS</th>
<th>Lo AIMS</th>
<th>Overall rotorod performance</th>
<th>Peak rotorod performance</th>
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<tr>
<td>CCG-2046</td>
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<td>CCG-4986</td>
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<td>CCG-63808</td>
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Table 7. The effects of RGS4 protein inhibitors on abnormal involuntary movements (AIMs) and rotorod performance when given in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Behavioural effects are compared to vehicle treatment in combination with L-DOPA/benserazide. Arrows represent increased or decreased magnitude of change; ↔, no change cf. vehicle treatment; ALO, sum of axial/limb/oroligual; Lo, locomotor.
**RGS4 protein inhibitors did not alleviate experimental parkinsonism**

Systemic administration of RGS4 inhibitors did not change the anti-parkinsonian effects of L-DOPA, either by improved peak rotorod performance or locomotor AIMs. Moreover, there were no differences in rotorod performance, or locomotor AIMs, compared to vehicle when compounds were administered alone.

Previous studies have suggested that inhibition of RGS4 proteins may alleviate parkinsonism (Ding et al., 2006; Lerner and Kreitzer, 2012). However, we found that when given alone, RGS4 inhibitors did not significantly improve rotorod performance or induce contralateral rotations, both of which are measures of anti-parkinsonian action (Schneider et al., 1984; Schwarting and Huston, 1996; Lundblad et al., 2003; Dekundy et al., 2007). A previous study suggested that blockade of striatal RGS4 proteins could potentiate M₄-cholinergic autoreceptor signalling (Ding et al., 2006), thereby restoring the loss of acetylcholinergic tone and decreasing elevated cholinergic transmission in PD (Barbeau, 1962). In contrast to this hypothesis, behavioural studies have shown blockade of M₄ cholinergic autoreceptors alleviates experimental parkinsonism (Gomeza et al., 1999; Mayorga et al., 1999; Karasawa et al., 2003).

Locomotor AIMs, or contraversive rotations, are induced following stimulation of supersensitive postsynaptic dopamine receptors in the striatum (Ungerstedt, 1971). Contraversive rotations are unlikely to be induced following RGS4 protein inhibition, as RGS4 proteins do not directly regulate dopamine receptor signalling (Ghavami et al., 2004). Indeed, unilateral overexpression of RGS4 in the rat striatum is unable to induce rotational bias following apomorphine or quinpirole treatment (Rahman et al., 2003). Although some studies have utilised contralateral rotation as an anti-parkinsonian response (Hudson et al., 1993), others have excluded this behavioural measure, since it is not representative of motor improvement (Lundblad et al., 2002). We must also consider that the compounds were tested in the ‘off state’ after induction of dyskinesia, where repeated L-DOPA treatment has caused long-term changes in RGS4 protein activity (see Chapter 2). Thus, RGS4 protein activity following dopamine denervation (Geurts et al., 2003; Taymans et al., 2004; Ding et al., 2006) is likely to differ from that following L-DOPA priming. We have explored the effects of RGS4 protein inhibition in PD in more detail in Chapter 4.
**Anti-dyskinetic properties of RGS4 protein inhibitors**

The RGS4 protein inhibitors tested in this study showed anti-dyskinetic effects in the unilateral 6-OHDA-lesioned rat model of LID. Intrastriatal administration of CCG-2046, CCG-4986 or RGS4 antisense oligonucleotides reduced ALO AIMs, while systemic administration of CCG-2046, CCG-4986, CCG-63802 or CCG-63808, dose-dependently reduced ALO AIMs.

The behavioural data presented in this study supports the role of RGS4 proteins in LID. Previous studies have shown RGS4 proteins are needed for general motor function (Grillet et al., 2005) and are implicated in hypokinetic (PD) and hyperkinetic (Huntington’s disease) movement disorders (Ding et al., 2006; Runne et al., 2008). RGS4 proteins are expressed in proximal and distal dendritic spines, and along the axon (Paspalas et al., 2009). These proteins are able to regulate different GPCRs at both pre- and postsynaptic regions (Ghavami et al., 2004; Ding et al., 2006; Ho et al., 2007; Leontiadis et al., 2009). More specifically, the N-terminal domain of the RGS4 protein mediates interaction with GPCRs (Zeng et al., 1998), which confers selectivity to G\(_{\alpha}\) subunit subtypes (Leontiadis et al., 2009). In many cases, RGS4 proteins regulate the G\(_{\alpha}\) subunits, which are the most highly expressed G\(_{\alpha}\) subunit subtype in the brain (Milligan et al., 1987). Blockade of RGS4-G\(_{\alpha}\) interaction is likely to disrupt effector antagonism (Hepler et al., 1997; Tesmer et al., 1997a), protein scaffold formation (Hepler, 1999; Siderovski et al., 1999) and second messenger signals (Roman et al., 2007; Blazer et al., 2010). In the dyskinetic state, RGS4 proteins may have several potential sites of action, as a wide range of GPCRs mediate abnormal neurotransmission in LID (Iravani and Jenner, 2011).

**Muscarinic acetylcholine receptors**

RGS4 proteins negatively modulate the G\(_{\alpha}\) subunits of M\(_4\) cholinergic receptors in the striatum (Ding et al., 2006). M\(_4\)-cholinergic receptors are expressed on striatal cholinergic interneurons and MSNs (Weiner et al., 1990; Bernard et al., 1992; Yan et al., 2001) and modulate local extracellular dopamine levels (Smolders et al., 1997). Given that fluctuations of striatal dopamine levels correlate to the expression of dyskinesia (de la Feunte-Fernandez et al., 2004; Pavese et al., 2006; Lindgren et al., 2010), blockade of RGS4-G\(_{\alpha}\) interactions at M\(_4\)-cholinergic receptors may reduce dyskinesia by controlling unregulated dopamine efflux. In striatal MSNs, M\(_4\)-cholinergic receptors are primarily localised on striatonigral neurons (Santiago and Potter, 2001) and specifically inhibit
dopamine D₁-induced locomotion (Gomeza et al., 1999). These data suggest M₄-cholinergic receptor activation inhibits the direct pathway of the basal ganglia. It is possible that blockade of RGS4 proteins could alleviate the inhibition of M₄-cholinergic receptors, leading to a dampened hyperactive striatonigral pathway in LID (Obeso et al., 2008). However, further studies would be needed to confirm this hypothesis.

µ-opioid receptors

RGS4 proteins regulate activated Gαi/o subunits of µ-opioid receptors in living cells (Georgoussi et al., 2006; Leontiadis et al., 2009; Talbot et al., 2010). In the striatum, µ-opioid receptors modulate GABAergic neurons that form the striatonigral and striatopallidal pathways (Olive et al., 1997; Kalyuzhnny and Wessendorf, 1998; Lindskog et al., 1999). Recent evidence has shown that striatal RGS4 proteins can positively modulate µ-opioid receptor function in vivo (Han et al., 2010). It is possible that RGS4 proteins contribute to increased µ-opioid receptor signalling in the striatum in the dyskinetic state (Chen et al., 2005). While selective antagonists of µ-opioid receptors effectively reduce dyskinesia in animal models of LID (Henry et al., 2001; Koprich et al., 2011), blockade of RGS4 proteins may present a novel mechanism for inhibition of µ-opioid receptor signalling.

Noradrenergic α₂ receptors

RGS4 proteins act as GTPase activating proteins on activated Gαi/o subunits of noradrenergic α₂ receptors (Cavalli et al., 2000). These receptors are highly expressed in the striatum (Unnerstall et al., 1984; Nicholas et al., 1993; Scheinin et al., 1994; Rosin et al., 1996) and modulate GABA release from MSNs (Zhang and Ordway, 2003). Noradrenergic α₂ receptor agonist, clonidine reduces dyskinesia in animal models of LID (Gomez-Mancilla and Bedard, 1993; Dekundy et al., 2007) and in dyskinetic PD patients (Nishikawa et al., 1984). The anti-dyskinetic effects following noradrenergic α₂ receptor activation is not yet fully understood. Noradrenergic α₂ receptors are expressed on over 90% of MSNs (Holmberg et al., 1999) and are likely to modulate the activity of the basal ganglia pathways (Wilbur et al., 1988; Zhang and Ordway, 2003). Interestingly, a novel mechanism has shown that noradrenergic α₂ receptors mediate crosstalk with NMDA receptor activity via RGS4 proteins in cultured neuronal cells (Liu et al., 2006). More specifically, combined RGS4 protein inhibition and clonidine treatment potentiated noradrenergic α₂ receptor-mediated reduction of NMDA receptor signalling (Liu et al.,
2006). Speculatively, this could represent an innovative way of reducing increased NMDA receptor signalling in LID. Indeed, increased NMDA receptor signalling is a well characterised pathological feature of LID (Oh et al., 1998; Hallet et al., 2005; Hurley et al., 2005; Gardoni et al., 2006; Calabresi et al., 2008), with non-selective NMDA receptor antagonist, amantadine commonly used as a clinical treatment for dyskinesia (Metman et al., 1999).

5-HT\textsubscript{1A} receptors

5-HT\textsubscript{1A} receptor agonists effectively reduce dyskinesia in animal models of LID (Carta et al., 2007; Dupre et al., 2007; Eskow et al., 2007) and dyskinetic PD patients (Bonifati et al., 1994; Olanow et al., 2004; Bara-Jimenez et al., 2005). Experimental evidence suggests that increased 5-HT\textsubscript{1A} signalling can reduce dyskinesia by attenuating unregulated dopamine efflux (Carta et al., 2007; Carta et al., 2008). These effects are thought to be attributed to activation of presynaptic somatodendritic autoreceptors on neurons in the dorsal and median raphe nuclei (Chalmers and Watson, 1991; Riad et al., 2000; Carta et al., 2008). Moreover, recent data suggested that activation of 5-HT\textsubscript{1A} receptors within the striatum are also responsible for the anti-dyskinetic effects of 5-HT\textsubscript{1A} receptor agonists (Bishop et al., 2009; Dupre et al., 2011). In vivo microdialysis studies have shown that RGS4 proteins inhibit 5-HT\textsubscript{1A} receptor-mediated neurotransmitter release in the striatum (Beyer et al., 2004). It is possible that blockade of RGS4 proteins could potentiate 5-HT\textsubscript{1A} receptor signalling, leading to reduced dopamine efflux and, subsequent reduction of AIMS in unilateral 6-OHDA-lesioned rats.

5-HT\textsubscript{1A} receptors can be found located on postsynaptic regions and can activate G\textsubscript{\alpha0} subunits in the cortex (Aoki et al., 1992; Dupuis et al., 1999; Mannoury la Cour et al., 2006). These 5-HT\textsubscript{1A} receptors are found on pyramidal neurons in the middle layers of cerebral cortex that form corticostriatal afferent projections (DeFelipe et al., 2001; Cruz et al., 2004), where RGS4 proteins are also densely expressed (Gold et al., 1997; Paspalas et al., 2009). Postsynaptic 5-HT\textsubscript{1A} receptor activation is thought to reduce glutamatergic transmission from corticostriatal afferents by hyperpolarising pyramidal neurons (Ceci et al., 1994; Casanovas et al., 1999; Hajos et al., 1999; Antonelli et al., 2005; Mignon and Wolf, 2005). These effects are suggested to contribute to the anti-dyskinetic effects of 5-HT\textsubscript{1A} agonists following systemic administration (Carta et al., 2007; Dupre et al., 2007; Eskow et al., 2007; Iravani et al., 2006b), which would reduce increased corticostriatal
glutamatergic transmission in LID (Calabresi et al., 2008). Indeed, systemic and intrastriatal administration of 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT reduced glutamate levels in the striatum of L-DOPA-treated unilateral 6-OHDA-lesioned rats (Dupre et al., 2011). It is conceivable that potentiating 5-HT$_{1A}$ receptor signalling through inhibition of RGS4 protein activity could also mediate reduced corticostriatal glutamatergic transmission. Indeed, electrophysiological recordings from prefrontal cortical neurons have shown that RGS4 protein blockade increased 5-HT$_{1A}$ agonist-induced inhibition on NMDA excitatory post-synaptic currents (EPSCs) (Gu et al., 2007). The effects of RGS4 protein inhibition on neurotransmission are explored in Chapter 5.

**RGS4 protein inhibitors improve overall motor ability when administered with L-DOPA**

RGS4 protein inhibitor treatment in combination with L-DOPA had variable effects on overall rotorod performance. Intrastriatal administration of CCG-2046, CCG-4986 or RGS4 antisense oligonucleotides improved rotorod performance when administered with L-DOPA. Systemic administration of CCG-63802 and CCG-63808 increased rotorod performance when combined with L-DOPA treatment, while CCG-2046 and CCG4986 did not.

These data suggest that inhibition of RGS4 proteins in the striatum can improve motor function in LID. Following treatment with RGS4 protein inhibitors, improved physiological motor activity is likely to be attributed to reduced expression of AIMs (Lundblad et al., 2002; Winkler et al., 2002). In the striatum, RGS4 proteins are expressed at higher levels in the lateral regions (Geurts et al., 2002), which are innervated by sensorimotor neuronal projections from the cortex (McGeorge and Faull, 1969). RGS4 protein mRNA becomes elevated following induction of LID (see Chapter 2), which may inhibit the outflow of information from the direct and indirect pathways of the basal ganglia (Herrera-Marschitz et al., 2010). It is possible that blockade of RGS4 proteins helps mediate physiological activity of basal ganglia output, facilitating intended motor action sequences, while dampening dyskinetic motor behaviours. The improved overall rotorod performance, following RGS4 protein inhibitor treatment in combination with L-DOPA, suggests the effects of RGS4 protein inhibition mediates specific reduction in AIMs, rather than suppression of general motor behaviours (Grillet et al., 2005).
In support of this hypothesis, we found that 8 days treatment with RGS4 antisense oligonucleotides, gradually increased rotorod performance in parallel with reduced AIMs. However, following cessation of RGS4 antisense oligonucleotide treatment, AIMs remained reduced and then reappeared after one week of continued L-DOPA treatment. These behavioural data indicate that repeated RGS4 protein inhibitor treatment could maintain reduced expression of LID. The effects of repeated RGS4 protein inhibition on L-DOPA priming i.e. induction of dyskinesia are explored in Chapter 4.

Although at lower levels than in the brain, RGS4 proteins are also present in other major organs of the mammalian system such as the heart, spleen and lungs (Nomoto et al., 1997). Thus, inhibition of RGS4 proteins could potentially induce adverse effects in these peripheral sensory systems. However, we report no noticeable adverse effects following systemic administration of RGS4 protein inhibitors in unilateral 6-OHDA-lesioned rats.

**Conclusion**

RGS4 protein inhibition reduced AIMs and improved overall rotorod performance in the unilateral 6-OHDA-lesioned rat model of LID. These behavioural data support the involvement of RGS4 proteins in the pathophysiology of LID. Thus, RGS4 proteins may represent novel therapeutic targets for future treatments in LID. In the next chapter, we investigate the effects of 28 days and 7 days RGS4 protein inhibitor treatment on L-DOPA priming in LID.
Chapter 4

The effects of RGS4 protein inhibition on L-DOPA priming for L-DOPA-induced dyskinesia in the unilateral 6-OHDA-lesioned rat model of PD
4.1 Introduction

Long-term L-DOPA treatment in PD patients can lead to severe motor complications such as LID (Cotzias et al., 1969). The incidence of these side-effects increases with the time course of L-DOPA treatment. Approximately 40% of PD patients develop LID after 4-6 years of L-DOPA treatment and this rises to 69% after 9-15 years (Ahlskog and Muenter, 2001). A major contributing factor in the development of LID is the unregulated dopamine efflux in the striatum following intermittent L-DOPA administration. Indeed, extracellular dopamine levels in the striatum show patterns of non-physiological fluctuations between cycles of L-DOPA treatment (de la Fuente-Fernandez et al., 2004; Carta et al., 2006; Pavese et al., 2006; Troiano et al., 2009), which stimulate dopamine receptors in a pulsatile manner (Olanow and Obeso, 2000; Stocchi et al., 2005; Olanow et al., 2006). These effects contribute to the sensitisation of dopamine receptors, which ultimately prime for dyskinesia (Blanchet et al., 1995; Goulet et al., 1996; Morisette et al., 1997; Grondin et al., 1999).

In L-DOPA priming, maladaptive changes occur in the striatum that mediate the expression of dyskinesia (Cenci and Konradi, 2010). Behavioural and molecular data have revealed increased signalling, downstream of certain GPCRs in the striatum. For example, the expression of dyskinesia is closely related to the activation of dopamine D<sub>1</sub> receptors (D<sub>1</sub>R) (Grondin et al., 1999a; Grondin et al., 1999b; Rascol et al., 2001b). In animal models of LID, molecular studies have revealed increased dopamine D<sub>1</sub>R binding at the plasma membrane and in the cytoplasm (Aubert et al., 2005; Guigoni et al., 2007). Moreover, the striatal G<sub>a</sub> subunit that couples to dopamine D<sub>1</sub>R, G<sub>olf</sub>, is also up-regulated in LID (Corvol et al., 2004), which is likely to contribute to dopamine D<sub>1</sub>R sensitisation (Aubert et al., 2005), and up-regulation of second messenger signals, such as DARPP-32 and cdk5 (Oh and Chase, 2002; Picconi et al., 2003, Guan et al., 2007).

Numerous molecular markers have been used to uncover pathophysiological mechanisms of dyskinesia (Andersson et al., 1999; Andersson et al., 2001; Konradi et al., 2004; Santini et al., 2007; Westin et al., 2007; Berton et al., 2009). Opioid precursors; PPE-A and PPE-B, are found up-regulated in the striatum following repeated L-DOPA treatment in PD (Cenci et al., 1998; Henry et al., 1999; Henry et al., 2003; Ravenscroft et al., 2004). PPE-B is specifically increased in LID and is used as a molecular marker for L-DOPA priming (Mela et al., 2007; Rylander et al., 2009; Kobylecki et al., 2010). This opioid precursor is cleaved into opioid peptides (prodynorphin, leu-enkephalin and α-neoendorphin) (Dhawan
et al., 1996), which stimulate certain GPCRs, such as μ-opioid receptors (You et al., 1996). These GPCRs have functional roles in the expression of LID. For example, an *in vitro* functional binding study in coronal brain sections of MPTP-lesioned NHPs showed that striatal μ-opioid receptors are sensitised in the dyskinetic state (Chen et al., 2005), while in behavioural studies, acute administration of μ-opioid receptor antagonists reduced dyskinesia in L-DOPA-treated MPTP-lesioned NHPs (Henry et al., 2001; Koprich et al., 2011). Other GPCRs have also been linked to the pathophysiology of LID. Previous behavioural studies have shown that repeated modulation of specific GPCR signalling pathways can reduce L-DOPA priming and subsequent expression of dyskinesia. For example, *de novo* treatment with mGluR5 receptor antagonists (Mela et al., 2007; Rylander et al., 2009) or 5-HT1A receptor agonists (Bishop et al., 2009), in combination with L-DOPA, reduced the associated behavioural and molecular correlates of dyskinesia in animal models of PD.

GPCRs in the striatum are modulated by certain endogenous proteins (RGS proteins) (Gold et al., 2007; Han et al., 2010; Schwendt et al., 2011; Lerner and Kreitzer, 2012). Although the primary roles of RGS proteins are inhibitory (Hepler, 1999; Ross and Wilkie, 2000), recent data suggests that RGS proteins can also positively modulate certain GPCR signalling pathways (Zhong et al., 2003; Smith et al., 2009; Han et al., 2010). *In vivo* studies have found that RGS4 proteins can negatively modulate 5-HT1A receptor signalling in the dorsal raphe nucleus (Beyer et al., 2004), while these proteins also positively modulate specific μ-opioid receptor signalling pathways in the striatum (Han et al., 2010). The roles of RGS4 proteins in L-DOPA priming for LID remain unknown. In the parkinsonian state, RGS4 protein mRNA is up-regulated following initial treatment with dopaminergic agonists (Taymans et al., 2004). These immediate changes may have pathophysiological roles in L-DOPA priming, possibly by mediating neurotransmitter receptor sensitisation.

### 4.1.1 Aims and objectives

We hypothesize that blocking RGS4 proteins may prevent L-DOPA priming in LID. Using the unilateral 6-OHDA-lesioned rat model of PD and LID, we aim to:

1) Characterise the effects of intrastriatal administration of RGS4 antisense treatment on AIMs and rotorod performance, prior to and during L-DOPA priming.
2) Characterise the effects of *de novo* RGS4 protein inhibitor treatment on the expression of AIMs and rotorod performance over 28 days of L-DOPA priming.

3) Characterise the effects of *de novo* RGS4 protein inhibitor treatment on expression of molecular markers, PPE-A and PPE-B, following 28 days of L-DOPA priming.

4) Characterise the behavioural and molecular effects of 7 day RGS4 protein inhibitor treatment in L-DOPA-primed animals.
4.2 Materials and Methods
All animal work was carried out under the regulations of the Animals (Scientific Procedures) Act, 1986.

4.2.1 Animals
Animals were housed in husbandry conditions as described in Chapter 2 (2.2.1).

Unilateral 6-hydroxydopamine lesion of the right MFB
Male Sprague-Dawley rats received unilateral 6-OHDA-lesion of the right MFB as described in Chapter 2 (2.2.1).

4.2.2 Drug treatment
Drugs were administered systemically into the i.p. cavity at a volume of 1 ml/kg, unless otherwise stated.

L-DOPA/benserazide
L-DOPA methyl ester hydrochloride (D1507, Sigma-Aldrich, UK) and benserazide hydrochloride (B7283, Sigma-Aldrich, UK) were dissolved in sterile saline (0.9% w/v; Braun AG, Germany).

RGS4 protein inhibitors
CCG-2046 (Tocris Bioscience, Bristol, UK) and CCG-63802 (Sequoia Research Products Ltd, UK) were dissolved in 10% (v/v) DMSO-sterile saline (0.9%, w/v) at 0.1 mg/ml and administered systemically (i.p.) 30 min prior to L-DOPA/benserazide injection.

4.2.3 Behavioural analysis
Cylinder test
The cylinder test was conducted at three weeks post-lesion as described in Chapter 2 (2.2.3). Animals with <30% left paw contacts were not included in this study (Mela et al., 2007).

Assessment of AIMS
AIMs were scored as described in Chapter 2 (2.2.3). The sum of ALO score was calculated over the three hour testing period for each assessment session. To further assess the effects
of repeated drug treatment on different topographical areas; axial, limb, orolingual and locomotor AIMS were also analysed separately.

**Rotorod training and testing**

At two weeks post-lesion, animals were rotorod trained and tested, as fully described in Chapter 3 (3.2.3). The effect of L-DOPA/benserazide + drug treatment on motor performance was evaluated using the acceleration trial (4 to 40 r.p.m), every 30 min for 3 h post-drug administration. Each trial run lasted a maximum of 5 min. Rotorod performance following vehicle only was used as a baseline score. Rotorod tests were performed on selected days during drug treatment plans.

Average group rotorod performance over three hours was used for statistical analyses. ‘Peak’ rotorod performance, the rotorod score at 1 h post L-DOPA treatment, was analysed separately to assess the effect of drug on the L-DOPA-induced anti-akinetic response (Dekundy et al., 2007; Kobylecki et al., 2011). All rotorod assessments were carried out between 8:00 am and 5:00 pm.

### 4.2.4 Experiment 1: RGS4 mRNA knockdown

The experimental timeline for investigating RGS4 mRNA knockdown is shown in Figure 57. Initial baseline rotorod and cylinder test scores of treatment naïve animals were used to match two groups of equal motor ability prior to RGS4 mRNA knockdown. At four weeks post-lesion, an osmotic mini-pump (Alzet® pump model 2004; maximum capacity 200 µl; infusion rate 0.25 µl/h; Charles River Ltd, UK) was implanted in unilateral 6-OHDA-lesioned rats, ipsilateral to the lesioned side, targeting the lateral rostral striatum. On the day prior to surgery, osmotic mini-pumps were filled with an RGS4 antisense oligonucleotide sequence (5’-G*G*ACTTAGTTTAGAA*G*G-3’,*inserted phosphorothioate bonds; Integrated DNA technologies, Belgium) or control sequence (5’-A*G*ATATGCGTAGATG*T*G-3’; Integrated DNA technologies, Belgium) diluted in molecular grade water (W4502, Sigma-Aldrich, UK) at a concentration of 0.2 nM/µl. Mini-pumps were constructed to manufacturer’s instructions and attached to an Alzet® brain infusion kit (product code: 0008663, Charles River Ltd, UK) via portex tubing (0.71 mm outer diameter). Oligonucleotide sequences were released via a protruding steel cannula (28G, 0.36 mm outer diameter) at a cumulative dose of 1.2 nM/day.
On the day of mini-pump implantation, unilateral 6-OHDA-lesioned rats were anaesthetised with gaseous flow of 2% isoflurane mixed with oxygen and nitrous oxide. Hair above the incision site was shaved and cleaned with 70% ethanol-distilled water solution. Rats were fixed into a stereotactic frame (David Kopf instruments, California, USA) using ear bars. An incision was made on the skin along the midline and a burr hole was made into the skull. The dura matter was penetrated and the protruding steel cannula (28G, 0.36 mm outer diameter) was inserted into the lateral striatum, ipsilateral to the lesioned hemisphere, at the following stereotactic coordinates, in mm relative to bregma: A = +1.0, L = -3.5, V = -5.5 (Paxinos and Watson, 1986). An additional burr hole was made into the skull where a small dental screw was fixed to hold the cannula in position. Dental cement (MPAIR1KG & L, Metrorodent, UK) was used to fix the cannula in an upright position, perpendicular to the skull. The osmotic mini-pump was inserted subcutaneously into the scruff. The wound was closed over the cannula using standard sutures (3-0 Mersilk®), cleaned with Betadine® for antisepsis, and EMLA® cream was applied for analgesia. Animals were placed in an incubator (29°C) until consciousness was regained.

One day post mini-pump implantation, control (n=6) and RGS4 antisense oligonucleotide (n=6) treatment groups received, once daily, vehicle (sterile saline, 0.9% w/v; Braun AG, Germany; i.p.) treatment for 8 days to test the effects of treatment on motor ability as measured from rotorod tests, which were performed on days 1, 3, 5, 6 and 8. Thereafter, L-DOPA/benserazide (6/15 mg/kg; i.p.) was administered once daily over 21 days for L-DOPA priming. During this period, rotorod tests were performed on L-DOPA treatment days 2, 5, 8, 11, 15, 18 and 20, while AIMs were assessed on days 1, 4, 7, 10, 14, 17 and 21 to assess the effects of RGS4 mRNA knockdown during L-DOPA priming. On day 22, unilateral 6-OHDA-lesioned rats were anaesthetised with a gaseous mixture of 2% isoflurane, oxygen and nitrous oxide, and mini-pumps were removed. Hair over the rats’ scruff was shaved and cleaned with 70% ethanol-distilled water solution. A small incision was made on the scruff and the mini-pump was detached and removed. The wound was closed with standard sutures (3-0 Mersilk®) and cleaned with Betadine®, before EMLA® cream was applied. Animals were placed in an incubator (29°C) until consciousness was regained. On day 23, all animals received an L-DOPA/benserazide (6/15 mg/kg; i.p.) only challenge to assess for the overall effects of RGS4 mRNA knockdown on L-DOPA priming i.e. the effect on induction of AIMs. On day 24, rats were killed by exposure to a
rising concentration of carbon dioxide. Rat brains were removed and immediately frozen in -45°C isopentane and stored at -80°C.

4.2.5 Experiment 2: 28 days RGS4 protein inhibitor treatment

The experimental timeline for investigating 28 days RGS4 inhibitor treatment is shown in Figure 58. Initial baseline rotorod and cylinder test scores of treatment naïve animals were used to match three groups of equal motor ability for RGS4 protein inhibitor treatment. Animals were then allocated to the following treatment groups (i) L-DOPA/benserazide (6/15 mg/kg; i.p.) + vehicle (10% (v/v) DMSO-sterile saline) (n=7), (ii) L-DOPA/benserazide (6/15 mg/kg; i.p.) + CCG-2046 (0.1 mg/kg; i.p.) (n=8), (iii) L-DOPA/benserazide (6/15 mg/kg; i.p.) + CCG-63802 (0.1 mg/kg; i.p.) (n=7) and received once daily treatment for 28 days. During this period, rotorod tests were performed on treatment days 2, 5, 8, 11, 15, 18, 20, 23, 25 and 27, while AIMs were assessed on days 1, 4, 7, 10, 14, 17, 21, 22, 24, 26 and 28 to assess the effects of RGS4 protein inhibition on L-DOPA priming. On day 29, all animals received an L-DOPA/benserazide (6/15 mg/kg; i.p.) only challenge to assess the overall effects of 28 days treatment with RGS4 protein inhibitors on L-DOPA priming i.e. the effect on induction of AIMs. On day 30, rats were killed by exposure to a rising concentration of carbon dioxide. Rat brains were removed and immediately frozen in -45°C isopentane and stored at -80°C.

4.2.6 Experiment 3: 7 days RGS4 protein inhibitor treatment

The experimental timeline for investigating 7 days RGS4 protein inhibitor treatment in L-DOPA primed unilateral 6-OHDA-lesioned rats is shown in Figure 59. Unilateral 6-OHDA-lesioned rats treated with L-DOPA/benserazide (6/15 mg/kg; i.p.) + vehicle (10% (v/v) DMSO-sterile saline) for 21 days were matched into three equal groups through rotorod (L-DOPA treatment day 20) and AIMs (L-DOPA treatment day 21) scores. Immediately after 21 day L-DOPA priming, animals were allocated to the following treatment groups: (i) L-DOPA/benserazide (6/15 mg/kg; i.p.) + vehicle (10% (v/v) DMSO-sterile saline) (n=7), (ii) L-DOPA/benserazide (6/15 mg/kg; i.p.) + CCG-2046 (0.1 mg/kg; i.p.) (n=8), (iii) L-DOPA/benserazide (6/15 mg/kg; i.p.) + CCG-63802 (0.1 mg/kg; i.p.) (n=7) and received once daily treatment for 7 days (treatment days 22-28). During this period, rotorod tests were performed on treatment days 20, 23 and 27, while AIMs were assessed on days 22, 24, 26 and 28, to evaluate the effects of 7 days RGS4 protein inhibitor treatment on L-DOPA primed animals. On day 29, all animals received an L-
DOPA/benserazide (6/15 mg/kg; i.p.) only challenge to assess for the long-standing effect of 7 day treatment with RGS4 protein inhibitors i.e. the potential reversal of L-DOPA priming. On day 30, rats were killed by exposure to a rising concentration of carbon dioxide. Rat brains were removed and immediately frozen in -45°C isopentane and stored at -80°C.
**Figure 57.** A timeline for investigating the behavioural effects of RGS4 mRNA knockdown with an RGS4 antisense oligonucleotide delivered by intrastriatal infusion in the unilateral 6-hydroxydopamine (6-OHDA)-lesioned rat model of Parkinson’s disease. AIMs, abnormal involuntary movements.
**Figure 58.** A timeline for investigating the behavioural effects of RGS4 protein inhibition in the unilateral 6-hydroxydopamine (6-OHDA)-lesioned rat model of Parkinson’s disease. AIMs, abnormal involuntary movements.
Figure 59. A timeline for investigating the behavioural effects of RGS4 protein inhibition in the unilateral 6-hydroxydopamine (6-OHDA)-lesioned rat model of L-DOPA-induced dyskinesia. AIMS, abnormal involuntary movements.
4.2.7 Tissue preparation
Tissue processing was performed as described in Chapter 2 (2.2.4). With respect to bregma (Paxinos and Watson, 1986), four series of three equivalent sections were cut rostral-caudal through the striatum of the rat brain:

- Series one (12 slides collected) – rostral striatum, +1.70 mm
- Series two (12 slides collected) – intermediate striatum, +1.0 mm
- Series three (12 slides collected) – caudal striatum, -0.26 mm
- Series four (12 slides collected) – caudal striatum/ globus pallidus, -0.92 mm

4.2.8 DAT binding
$[^{125}]$-RTI-121 binding was carried out as described in Chapter 2 (2.2.5).

4.2.9 In situ hybridisation

Tissue fixation
Tissue sections were fixed as described in Chapter 2 (2.2.6).

Radiolabelling of synthetic oligonucleotide probes
Oligonucleotide probe sequences (Invitrogen, UK) complementary to rat mRNA for: PPE-A (5’-CTT CAT GAA GCC TCC ATA CCG TTT GAT GAA CCC CCT ATA CTT-3’; accession number: M28263) (Henry et al., 1999) were labelled at the 3’-end as described in Chapter 2 (2.2.6). Oligonucleotide probes targeting PPE-B and RGS4 mRNA were the same as described in Chapter 2 (Table 3).

Oligonucleotide/mRNA hybridisation
Radioactive oligonucleotides were hybridised to mRNA sequences as described in Chapter 2 (2.2.6).

Experimental control
For control experiments, competitive in situ hybridisation and RNAase pre-treatment were carried out as described in Chapter 2 (2.2.7).
**Image analysis**

Autoradiograph images of the rostral striatum were analysed as described in Chapter 2 (2.2.8).

4.2.10 Validation of cannula injection site

To validate the cannula target site in rats implanted with mini-pumps, cresyl violet staining of coronal brain sections was carried out as described in Chapter 3 (3.2.4). Sections were observed under a light microscope (Leica DMRB) at x1.6 magnification for confirmation of the cannula tract entering the striatum. Animals with cannula sites outside the striatum were removed from all analyses.

4.2.11 $^{35}$S-GTP$\gamma$S binding autoradiography

*In vitro* $^{35}$S-GTP$\gamma$S binding autoradiography was used to assess the effects of repeated drug treatment on agonist-stimulated G-protein activation, as previously described (Sim et al., 1995; He et al., 2000; Newman-Tancredi et al., 2001). For each animal, slides containing three equivalent coronal sections of the rostral striatum were removed from the -80°C freezer and left for 20 min to equilibrate to room temperature. Slides were pre-incubated in assay buffer (50 mM Tris-HCL, 3 mM MgCl$_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) for 10 min at 25°C. Slides were then incubated in assay buffer containing 2 mM guanosine 5’-diphosphate (GDP; G7127, Sigma-Aldrich UK) for 15 min at room temperature. One slide from each animal was then incubated for 2 h at room temperature in the following conditions:

(i) Basal - assay buffer + 2 mM GDP + 0.01 nM $^{35}$S-GTP$\gamma$S (Perkin-Elmer, UK).

(ii) Agonist stimulated - assay buffer + 2 mM GDP + 0.01 nM $^{35}$S-GTP$\gamma$S + 100 µM dopamine hydrochloride (H8502, Sigma-Aldrich UK).

(iii) Non-specific (control) - assay buffer + 2 mM GDP + 0.01 nM $^{35}$S-GTP$\gamma$S + GTP$\gamma$S (Roche, UK).

Following incubation, slides were rinsed twice, for 2 min, in ice cold Tris-HCL (pH 7.0) and once, for 30 seconds, in distilled water. Slides were air dried overnight at room temperature and exposed to autoradiographic film (Kodak Biomax MR-1, Sigma-Aldrich, UK) for 48 h. A $^{14}$C microscale standard (radioactivity range: 39.3-1117.5 nCi/g;
Amersham) was included in each cassette to determine the radioactivity range. Films were developed using an automatic film developer.

\[^{35}\text{S}\]GTP\(_{\gamma}\text{S}\) binding autoradiographs were analysed with Image-Pro PLUS™ software (©1993-2004 Media Cybernetics Version 5.1.0.20) connected to a digital camera. The optical density (OD) was found for each tissue section and converted to radioactivity (nCi/g). The whole rostral striatum and the four separate sub-regions (dorsolateral, dorsomedial, ventrolateral and ventromedial) were analysed (Figure 7). Percentage change to basal activity induced by agonist stimulation was calculated for each treatment group and used for statistical analyses.

### 4.2.12 Statistical analysis

Statistical analyses were carried out using Prism 5.0 (GraphPad Software, San Diego, CA). Cylinder test performances and DAT binding results were analysed using two-way ANOVA followed by Bonferroni multiple comparisons. The effect of repeated drug treatment was analysed using two-way ANOVA followed by Bonferroni multiple comparisons, with ‘time’ and ‘treatment group’ as given factors. Rotorod performance from single test sessions was compared using an unpaired t-test when there were only two treatment groups. For >2 treatment groups, one-way ANOVA followed by Tukey’s post hoc analysis was used. The effect of repeated drug treatment on AIMs was analysed using two-way ANOVA followed by Bonferroni multiple comparisons, with ‘time’ and ‘treatment group’ as given factors. Since AIMs scores represent non-parametric data, individual testing sessions were compared using the Mann-Whitney test when there were only two treatment groups. For >2 treatment groups, Kruskal-Wallis test followed by Dunn’s multiple comparison test was used. Since all animals used were 6-OHDA-lesioned, in situ hybridisation results were analysed using two-way ANOVA followed by Bonferroni multiple comparisons, with ‘lesion’ and ‘treatment group’ as given factors. Correlations between molecular markers were carried out using Pearson’s linear correlation, while molecular vs. behavioural markers were carried out using Spearman’s linear correlation. \[^{35}\text{S}\]GTP\(_{\gamma}\text{S}\) autoradiography results were compared by using an unpaired t-test when there were only two treatment groups. For >2 treatment groups, one-way ANOVA followed by Tukey’s post hoc analysis was used. A significance of p<0.05 was used for all analyses.
4.3 Results

4.3.1 RGS4 mRNA knockdown

DAT binding
There was a significant effect of lesion (F_{1,20}=292.31; p<0.0001; Figure 60A) but not treatment group (F_{1,20}=0.46; p=0.5050) on [^{125}I]-RTI-121 binding levels. [^{125}I]-RTI-121 binding was reduced in the operated compared to the unoperated side in control (81%, p<0.001) and RGS4 antisense oligonucleotide (82%, p<0.001) treatment groups.

Autoradiographic images from DAT binding in the striatum are shown in Figure 61.

Cylinder test
There was a significant effect of lesion (F_{1,20}=516.42; p<0.0001; Figure 60B) but not treatment group (F_{1,20}=0.00; p=1.0000) on cylinder test performance. Left paw contacts were reduced compared to right paw contacts in control (78%, p<0.001) and RGS4 antisense oligonucleotide (78%, p<0.001) treatment groups.
Figure 60. The effect of unilateral 6-OHDA-lesion on (A) dopamine active transporter binding and (B) cylinder test performance in control or RGS4 antisense oligonucleotide-treated rats. (A) $^{[125]}$I-RTI-121 binding in the unoperated (□) and operated (■) striatum. ***p<0.001 cf. unoperated side (B) % of total paw contacts made with right (□) or left (■) paw. ***p<0.001 cf. right paw same treatment group. Bars represent mean ± SEM (n=6) (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 61. Pseudocolour transformations of autoradiographic images from dopamine active transporter (DAT) binding using $[^{125}\text{I}]$-RTI-121 autoradiography. Representative coronal sections of the (U) unoperated and (O) operated striatum in unilateral 6-OHDA-lesioned rats following continuous intrastriatal infusion of control or RGS4 antisense oligonucleotide (1.2 nM/day), in combination with, once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment.
**Rotorod performance**

There was a significant effect of time ($F_{12,117}=3.27; p=0.0004$, Figure 62A) and treatment group ($F_{1,117}=19.77; p<0.0001$), but not time x treatment interaction ($F_{12,117}=0.58; p=0.8530$) on mean rotorod performance during RGS4 mRNA knockdown. Rotorod performance was reduced in RGS4 antisense oligonucleotide-treated rats on treatment day - 8 (31%; $p=0.0004$, Figure 63A) and day 2 (50%; $p<0.0001$, Figure 63B), compared to control-treated rats.

There was a significant effect of time ($F_{12,117}=3.35; p=0.0003$, Figure 62B) and treatment group ($F_{1,117}=8.80; p=0.0036$), but not time x treatment interaction ($F_{12,117}=0.36; p=0.9753$) on peak rotorod performance during RGS4 mRNA knockdown. There were no significant differences in peak rotorod performance between control and RGS4 antisense oligonucleotide treatment groups on any of the treatment days.
Figure 62. The effect of continuous intrastriatal infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) on rotorod performance over 8 days vehicle (i.p.) and 21 days L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent mean ± SEM (n=5-6); some error bars have been omitted for clarity. (A) Mean rotorod performance. (B) Peak rotorod performance at 1 hour post vehicle or L-DOPA/benserazide treatment (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 63. The effect of continuous intrastriatal infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Bars represent mean ± SEM (n=5-6). (A) Rotorod performance on day 8 of vehicle treatment (i.p.). (B) Rotorod performance on day 2 of L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. ***p<0.001 cf. control (unpaired t-test).
Aims

There was a significant effect of time ($F_{6,60}=13.13; p<0.0001$, Figure 64A) and treatment group ($F_{1,10}=49.69; p<0.001$), but not time x treatment interaction ($F_{6,60}=0.64; p=0.6981$) on sum of ALO AIMS during 21 days L-DOPA treatment. Individual analysis of L-DOPA treatment days showed sum of ALO AIMS were reduced in the L-DOPA + RGS4 antisense oligonucleotide-treated group on days 1 (68%; $U=0.000; p=0.0049$), 4 (50%; $U=0.000; p=0.0050$), 7 (65%; $U=1.000; p=0.0081$), 10 (55%; $U=0.000; p=0.0050$), 14 (37%; $U=0.000; p=0.0022$), 17 (33%; $U=0.000; p=0.0050$) and 21 (48%; $U=2.500; p=0.0161$), compared to L-DOPA + control-treated group.

There was a significant effect of time ($F_{6,60}=11.07; p<0.0001$, Figure 64B) and treatment group ($F_{1,10}=13.72; p=0.0041$), but not time x treatment interaction ($F_{6,60}=0.18; p=0.9817$) on axial AIMS during 21 days L-DOPA treatment. Individual analysis of L-DOPA treatment days showed axial AIMS were reduced in the L-DOPA + RGS4 antisense oligonucleotide-treated group on days 4 (47%; $U=3.000; p=0.0189$), 14 (30%; $U=5.000; p=0.0435$), 17 (25%; $U=4.500; p=0.0357$) and 21 (33%; $U=4.000; p=0.0292$), but not on days 1 ($U=7.500; p=0.1056$), 7 ($U=5.500; p=0.0526$) or 10 ($U=7.500; p=0.1081$), compared to L-DOPA + control-treated group.

There was a significant effect of time ($F_{6,60}=13.57; p<0.0001$, Figure 64C) and treatment group ($F_{1,10}=69.57; p<0.0001$), but not time x treatment interaction ($F_{6,60}=0.86; p=0.5262$) on limb AIMS during 21 days L-DOPA treatment. Individual analysis of L-DOPA treatment days showed limb AIMS were reduced in the L-DOPA + RGS4 antisense oligonucleotide-treated group on days 1 (79%; $U=0.000; p=0.0050$), 4 (52%; $U=1.000; p=0.0074$), 7 (65%; $U=1.500; p=0.0097$), 10 (46%; $U=1.000; p=0.0080$), 14 (45%; $U=0.000; p=0.0049$), 17 (36%; $U=0.500; p=0.0060$) and 21 (43%; $U=2.000; p=0.0126$), compared to L-DOPA + control-treated group.

There was a significant effect of time ($F_{6,60}=2.99; p=0.0127$, Figure 64D) and treatment group ($F_{1,10}=67.79; p<0.0001$), but not time x treatment interaction ($F_{6,60}=0.74; p=0.6192$) on orolingual AIMS during 21 days L-DOPA treatment. Individual analysis of L-DOPA treatment days showed orolingual AIMS were reduced in the L-DOPA + RGS4 antisense oligonucleotide-treated group on days 1 (55%; $U=0.000; p=0.0050$), 4 (58%; $U=0.000; p=0.0048$), 7 (70%; $U=1.500; p=0.0101$), 10 (65%; $U=0.000; p=0.0047$), 14 (54%;
U=0.500; p=0.0064), 17 (48%; U=1.500; p=0.0101) and 21 (58%; U=3.000; p=0.0196), compared to L-DOPA + control-treated group.

There was a significant effect of time (F_{6,60}=4.80; p=0.0005, Figure 64E) but not treatment (F_{1,10}=2.27; p=0.1630) on locomotor AIMS during 21 days L-DOPA treatment. Individual analysis of L-DOPA treatment days showed locomotor AIMS were not significantly different between the treatment groups on any of the treatment days (p>0.05).
Figure 64. The effect of continuous intrastratal infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) on abnormal involuntary movements (AIMs) over 21 day, once daily, L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent mean ± SEM (n=6); some error bars have been omitted for clarity. (A) Sum of axial, limb, orolingual (ALO); (B) axial; (C) limb; (D) orolingual; (E) locomotor AIMs over 3 hours post L-DOPA administration. **p<0.01, *p<0.05, cf. control-treated (Mann-Whitney test).
**Overall effect on L-DOPA priming**

Following mini-pump removal, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (U=0.000; p=0.0050, Figure 65A). Sum of ALO AIMs were reduced in animals pre-treated with L-DOPA + RGS4 antisense oligonucleotide (54%; p<0.01), compared to L-DOPA + control-treated group.

Following mini-pump removal, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (U=6.000; p=0.0599, Figure 65B).

**Validation of cannula injection site**

Cresyl violet stained sections from each animal showed the cannula tract entering the rostral striatum (Figure 66).
Figure 65. The effect of L-DOPA/benserazide (6/15 mg/kg; i.p.) challenge on abnormal involuntary movements (AIMs) in unilateral 6-OHDA-lesioned rats following pre-treatment with an RGS4 antisense or control oligonucleotide (continuous intrastriatal infusion, 1.2 nM/day) in combination with once daily, L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment for 21 days. Points represent data from individual animals (n=6) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs. (B) Locomotor AIMs from 0-3 hours post L-DOPA administration. **p<0.01 cf. control-treated (Mann-Whitney test).
Figure 66. Coronal sections through the rat striatum showing the mini-pump administration site for continuous intrastriatal infusion of control or RGS4 antisense oligonucleotide in the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. (A) Schematic diagram of the rostral striatum (+1.70 mm, with respect to bregma) adapted from Paxinos and Watson (1986); ↓ denotes terminal target site. (B) Grey scale image of cresyl violet stained coronal section showing the cannula tract in the rostral striatum ipsilateral to the lesioned side.
**Expression of molecular markers of LID following RGS4 mRNA knockdown**

**PPE-A**

**Rostral striatum**

In the whole rostral striatum, there was a significant effect of lesion \((F_{1,20}=344.96; \ p<0.0001, \ \text{Figure 67})\) but not treatment group \((F_{1,20}=0.10; \ p=0.7592)\) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + control-treated \((101\%, \ p<0.001)\) and L-DOPA + RGS4 antisense oligonucleotide-treated \((106\%, \ p<0.001)\) groups.

In the dorsolateral region of the rostral striatum, there was a significant effect of lesion \((F_{1,20}=140.83; \ p<0.0001, \ \text{Figure 68A})\) but not treatment group \((F_{1,20}=0.78; \ p=0.3871)\) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + control-treated \((90\%, \ p<0.001)\) and L-DOPA + RGS4 antisense oligonucleotide-treated \((109\%, \ p<0.001)\) groups.

In the dorsomedial region of the rostral striatum, there was a significant effect of lesion \((F_{1,20}=269.13; \ p<0.0001, \ \text{Figure 68B})\) but not treatment group \((F_{1,20}=0.65; \ p=0.4301)\) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + control-treated \((115\%, \ p<0.001)\) and L-DOPA + RGS4 antisense oligonucleotide-treated \((116\%, \ p<0.001)\) groups.

In the ventrolateral region of the rostral striatum, there was a significant effect of lesion \((F_{1,20}=255.43; \ p<0.0001, \ \text{Figure 68C})\) but not treatment group \((F_{1,20}=0.01; \ p=0.9374)\) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + control-treated \((98\%, \ p<0.001)\) and L-DOPA + RGS4 antisense oligonucleotide-treated \((100\%, \ p<0.001)\) groups.

In the ventromedial region of the rostral striatum, there was a significant effect of lesion \((F_{1,20}=139.88; \ p<0.0001, \ \text{Figure 68D})\) but not treatment group \((F_{1,20}=0.28; \ p=0.6050)\) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + control-treated \((87\%, \ p<0.001)\) and L-DOPA + RGS4 antisense oligonucleotide-treated \((84\%, \ p<0.001)\) groups.
In the nucleus accumbens core, there was a significant effect of lesion ($F_{1,20}=39.02; p<0.0001$; Figure 68E) but not treatment group ($F_{1,20}=0.09; p=0.7727$) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + control-treated (49%, $p<0.001$) and L-DOPA + RGS4 antisense oligonucleotide-treated (50%, $p<0.001$) groups.

In the nucleus accumbens shell, there was a significant effect of lesion ($F_{1,20}=15.64; p=0.0008$, Figure 68F) but not treatment group ($F_{1,20}=0.04; p=0.8369$) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + control-treated (56%, $p<0.01$) and L-DOPA + RGS4 antisense oligonucleotide-treated (40%, $p<0.05$) groups.

Autoradiographic images of PPE-A mRNA expression in the rostral striatum are shown in Figure 69.
Figure 67. PPE-A mRNA expression in the rostral striatum of unilateral 6-OHDA-lesioned rats following continuous intrastriatal infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) in combination with, once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6). ***p<0.001 cf. unoperated side (two-way ANOVA, Bonferroni multiple comparisons test).
**Figure 68.** PPE-A mRNA expression in the sub-regions of the rostral striatum of unilateral 6-OHDA-lesioned rats following continuous intrastratal infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) in combination with, once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6). ***p<0.001, **p<0.01, *p<0.05 cf. unoperated side (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 69. Pseudocolour image transformations of autoradiographs from *in situ* hybridisation targeting preproenkephalin-A (PPE-A) mRNA in the (U) unoperated and (O) operated sides of the rostral striatum following continuous intrastriatal infusion of control or RGS4 antisense oligonucleotide (1.2 nM/day), in combination with once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment, in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease.
PPE-B

Rostral striatum

In the whole rostral striatum, there was a significant effect of lesion (F_{1,20}=22.23; \ p=0.0001; \ Figure \ 70) and treatment group (F_{1,20}=10.74; \ p=0.0038), but not lesion x treatment interaction (F_{1,20}=1.61; \ p=0.2194), on PPE-B mRNA expression. PPE-B mRNA was increased in the operated compared to the unoperated side in the L-DOPA + control-treated (50\%, \ p<0.001), but not in the L-DOPA + RGS4 antisense oligonucleotide-treated group (p>0.05). PPE-B mRNA expression in the operated side was increased in L-DOPA + control-treated (25\%, \ p<0.05) compared to the L-DOPA + RGS4 antisense oligonucleotide-treated group.

In the dorsolateral region of the rostral striatum, there was a significant effect of lesion (F_{1,19}=39.05; \ p<0.0001, \ Figure \ 71A) and treatment group (F_{1,19}=11.22; \ p=0.0034), but not lesion x treatment interaction (F_{1,19}=2.41; \ p=0.1371), on PPE-B mRNA expression. PPE-B mRNA expression was increased in the operated compared to the unoperated side in the L-DOPA + control-treated (74\%, \ p<0.001) and L-DOPA + RGS4 antisense oligonucleotide-treated groups (53\%, \ p<0.05). PPE-B mRNA expression in the operated side was increased in L-DOPA + control-treated (36\%, \ p<0.01) compared to the L-DOPA + RGS4 antisense oligonucleotide-treated group.

In the dorsomedial region of the rostral striatum, there was a significant effect of lesion (F_{1,19}=21.66; \ p=0.0002, \ Figure \ 71B), treatment group (F_{1,19}=40.20; \ p<0.0001) and lesion x treatment interaction (F_{1,19}=9.33; \ p=0.0065) on PPE-B mRNA expression. PPE-B mRNA was increased in the operated compared to the unoperated side in the L-DOPA + control-treated (38\%, \ p<0.001), but not in the L-DOPA + RGS4 antisense oligonucleotide-treated group (p>0.05). PPE-B mRNA expression in the operated side was increased in L-DOPA + control-treated (50\%, \ p<0.001) compared to the L-DOPA + RGS4 antisense oligonucleotide-treated group.

In the ventrolateral region of the rostral striatum, there was a significant effect of lesion (F_{1,19}=33.09; \ p<0.0001, \ Figure \ 71C), treatment group (F_{1,19}=13.96; \ p=0.0014) and lesion x treatment interaction (F_{1,19}=5.26; \ p=0.0335) on PPE-B mRNA expression. PPE-B mRNA was increased in the operated compared to the unoperated side in the L-DOPA + control-treated (86\%, \ p<0.001), but not in the L-DOPA + RGS4 antisense oligonucleotide-treated
group (p>0.05). PPE-B mRNA expression in the operated side was increased in L-DOPA + control-treated (53%, p<0.01) compared to the L-DOPA + RGS4 antisense oligonucleotide-treated group.

In the ventromedial region of the rostral striatum, there was a significant effect of treatment group (F_{1,19}=12.22; p=0.0024, Figure 71D) but not lesion (F_{1,19}=1.49; p=0.2366) on PPE-B mRNA expression. PPE-B mRNA expression in the operated side was increased in L-DOPA + control-treated (44%, p<0.05) compared to the L-DOPA + RGS4 antisense oligonucleotide-treated group.

In the nucleus accumbens core, there was no significant effect of lesion (F_{1,20}=0.03; p=0.8746, Figure 71E) or treatment group (F_{1,20}=3.98; p=0.0598) on PPE-B mRNA expression.

In the nucleus accumbens shell, there was no significant effect of lesion (F_{1,18}=0.84; p=0.3728, Figure 71F) or treatment group (F_{1,18}=3.97; p=0.0618) on PPE-B mRNA expression.

Autoradiographic images of PPE-B mRNA expression in the rostral striatum are shown in Figure 72.
Figure 70. PPE-B mRNA expression in the rostral striatum of unilateral 6-OHDA-lesioned rats following continuous intrastriatal infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) in combination with, once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6). **p<0.001 cf. unoperated side; #p<0.05 cf. ipsilateral control-treated (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 71. PPE-B mRNA expression in the sub-regions of the rostral striatum of unilateral 6-OHDA-lesioned rats following continuous intrastrital infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) in combination with, once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=5-6). ***p<0.001, *p<0.05 cf. unoperated side; ###p<0.001, ##p<0.01, #p<0.05 cf. ipsilateral control-treated (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 72. Pseudocolour image transformations of autoradiographs from in situ hybridisation targeting preproenkephalin-B (PPE-B) mRNA in the (U) unoperated and (O) operated sides of the rostral striatum following continuous intrastriatal infusion of control or RGS4 antisense oligonucleotide (1.2 nM/day), in combination with once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment, in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease.
**RGS4**

### Rostral striatum

In the whole rostral striatum, there was a significant effect of lesion ($F_{1,17}=5.05; p=0.0383$, Figure 73) and treatment group ($F_{1,17}=10.75; p=0.0044$) on RGS4 mRNA expression. RGS4 mRNA was increased in the operated compared to the unoperated side in the L-DOPA + control-treated (30%, $p<0.05$), but not in the L-DOPA + RGS4 antisense oligonucleotide-treated group ($p>0.05$). RGS4 mRNA expression in the operated side was increased in L-DOPA + control-treated (42%, $p<0.05$) compared to the L-DOPA + RGS4 antisense oligonucleotide-treated group.

In the dorsolateral region of the rostral striatum, there was a significant effect of treatment group ($F_{1,16}=9.46; p=0.0073$; Figure 74A) but not lesion ($F_{1,16}=1.11; p=0.3081$) on RGS4 mRNA expression. RGS4 mRNA expression in the operated side was increased in L-DOPA + control-treated (61%, $p<0.05$) compared to the L-DOPA + RGS4 antisense oligonucleotide-treated group.

In the dorsomedial region of the rostral striatum, there was no effect of lesion ($F_{1,20}=0.22; p=0.6420$, Figure 74B) or treatment group ($F_{1,20}=1.10; p=0.3068$) on RGS4 mRNA expression.

In the ventrolateral region of the rostral striatum, there was a significant effect of treatment group ($F_{1,17}=6.77; p=0.0186$, Figure 74C) but not lesion ($F_{1,17}=3.30; p=0.0867$) on RGS4 mRNA expression. RGS4 mRNA expression in the operated side was increased in L-DOPA + control-treated (58%, $p<0.05$) compared to the L-DOPA + RGS4 antisense oligonucleotide-treated group.

In the ventromedial region of the rostral striatum, there was no significant effect of lesion ($F_{1,18}=0.38; p=0.5459$, Figure 74D) or treatment group ($F_{1,18}=0.40; p=0.5360$) on RGS4 mRNA expression.

In the nucleus accumbens core, there was no significant effect of lesion ($F_{1,20}=0.43; p=0.5184$, Figure 74E) or treatment group ($F_{1,20}=0.52; p=0.4800$) on RGS4 mRNA expression.
In the nucleus accumbens shell, there was no significant effect of lesion ($F_{1,20}=0.08$; $p=0.7829$; Figure 74F) or treatment group ($F_{1,20}=0.34$; $p=0.5643$) on RGS4 mRNA expression.

Autoradiographic images of RGS4 mRNA expression in the rostral striatum are shown in Figure 75.
Figure 73. RGS4 mRNA expression in the rostral striatum of unilateral 6-OHDA-lesioned rats following continuous intrastral infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) in combination with, once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=5-6). *p<0.05 cf. unoperated side; #p<0.05 cf. ipsilateral control-treated (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 74. RGS4 mRNA expression in the sub-regions of the rostral striatum of unilateral 6-OHDA-lesioned rats following continuous intrastriatal infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) in combination with, once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=5-6). #p<0.05 cf. ipsilateral control-treated (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 75. Pseudocolour image transformations of autoradiographs from in situ hybridisation targeting RGS4 mRNA in the (U) unoperated and (O) operated sides of the rostral striatum following continuous intrastralatal infusion of control or RGS4 antisense oligonucleotide (1.2 nM/day), in combination with once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment, in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease.
**Correlation analysis of behavioural and molecular markers of LID**

In the whole rostral striatum of the operated side, there was a significant linear correlation between PPE-B mRNA expression and sum of ALO AIMs (Spearman’s $r=0.7356$; 95% CI 0.2617 to 0.9237; $p=0.0087$, Figure 76A).

In the whole rostral striatum of the operated side, there was a significant linear correlation between RGS4 mRNA expression and sum of ALO AIMs (Spearman’s $r=0.7244$; 95% CI 0.2004 to 0.9261; $p=0.0144$, Figure 76B).

In the whole rostral striatum of the operated side, there was a significant linear correlation between RGS4 mRNA and PPE-B mRNA expression (Pearson’s $r=0.7791$; 95% CI 0.3363 to 0.9398; $p=0.0047$, Figure 76C).
Figure 76. Correlation between molecular and behavioural markers of L-DOPA-induced dyskinesia in the whole rostral striatum of the operated side in unilateral 6-OHDA-lesioned rats following continuous intrastriatal infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) in combination with, once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. (A) PPE-B mRNA vs. sum of axial, limb and orolingual abnormal involuntary movements (AIMs) following L-DOPA challenge on day 23. (B) RGS4 mRNA vs. sum of ALO AIMs following L-DOPA challenge (**p<0.01, *p<0.05; Spearman’s rank correlation). (C) RGS4 mRNA vs. PPE-B mRNA (**p<0.01; Pearson’s linear correlation).
In the whole rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}S]$GTP$_\gamma$S binding ($p=0.0080$, Figure 77A), which was reduced in RGS4 antisense oligonucleotide-treated (23%, $p<0.01$) compared to control-treated.

In the dorsolateral rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}S]$GTP$_\gamma$S binding ($p=0.0256$, Figure 77B), which was reduced in RGS4 antisense oligonucleotide-treated (19%, $p<0.05$) compared to control-treated.

In the dorsomedial rostral striatum of the operated side, there was no significant effect of treatment group on dopamine stimulated $[^{35}S]$GTP$_\gamma$S binding ($p=0.1482$, Figure 77C).

In the ventrolateral rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}S]$GTP$_\gamma$S binding ($p=0.0186$, Figure 77D), which was reduced in RGS4 antisense oligonucleotide-treated (29%, $p<0.05$) compared to control-treated.

In the ventromedial rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}S]$GTP$_\gamma$S binding ($p=0.0077$, Figure 77E), which was reduced in RGS4 antisense oligonucleotide-treated (26%, $p<0.01$) compared to control-treated.

Autoradiographic images of $[^{35}S]$GTP$_\gamma$S binding in the rostral striatum are shown in Figure 78.
Figure 77. Dopamine stimulated $^{35}$S GTPγS binding autoradiography in the operated side of the rostral striatum of unilateral 6-OHDA-lesioned rats following continuous intrastriatal infusion of an RGS4 antisense or control oligonucleotide (1.2 nM/day) in combination with, once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. (A) whole striatum, (B) dorsolateral, (C) dorsomedial, (D) ventrolateral (E) ventromedial striatum. Bars represent mean ± SEM (n=5-6). **p<0.01, *p<0.05 cf. control-treated (unpaired t-test).
Figure 78. Pseudocolour image transformations of autoradiographs from dopamine (100 µM) stimulated $[^{35}S]$GTPγS binding in the operated side of the rostral striatum following continuous intrastratal infusion of control or RGS4 antisense oligonucleotide (1.2 nM/day), in combination with once daily, 21 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment, in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease.
4.3.2  28 days RGS4 protein inhibitor treatment

**DAT binding**
There was a significant effect of lesion (F\(_{1,42}=3659.58;\) p<0.0001, Figure 79A) but not treatment group (F\(_{2,42}=0.28;\) p=0.7561) on [\(^{125}\)I]-RTI-121 binding levels. [\(^{125}\)I]-RTI-121 binding was reduced in the operated compared to the unoperated side in L-DOPA + vehicle (85%, p<0.001), L-DOPA + CCG-2046 (84%, p<0.001) and L-DOPA + CCG-63802 (84%, p<0.001) treatment groups.

Autoradiographic images from DAT binding in the striatum are shown in Figure 80.

**Cylinder test**
There was a significant effect of lesion (F\(_{1,42}=214.57;\) p<0.0001, Figure 79B) but not treatment group (F\(_{2,42}=0.00;\) p=1.000) on cylinder test performance. Left paw contacts were reduced compared to right paw contacts in L-DOPA + vehicle (85%, p<0.001), L-DOPA + CCG-2046 (79%, p<0.001) and L-DOPA + CCG-63802 (75%, p<0.001) treatment groups.
Figure 79. The effect of unilateral 6-OHDA-lesion on (A) dopamine transporter binding and (B) cylinder test performance in rats treated once daily with L-DOPA + vehicle, L-DOPA + CCG-2046 or L-DOPA + CCG-63802 for 28 days. (A) $[^{125}]$-RTI-121 binding in the unoperated (□) and operated (■) striatum. ***p<0.001 cf. unoperated side (B) % of total paw contacts made with right (□) or left (■) paw. ***p<0.001 cf. right paw same treatment group. Bars represent mean ± SEM (n=8) (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 80. Pseudocolour transformations of autoradiographic images from dopamine active transporter (DAT) binding using $^{125}$I-RTI-121 autoradiography. Representative coronal sections of the (U) unoperated and (O) operated striatum in unilateral 6-OHDA-lesioned rats following once daily treatment with vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.), in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) for 28 days.
Rotorod performance

There was a significant effect of time ($F_{10,200}=3.83; p<0.0001$, Figure 81A), treatment group ($F_{2,200}=3.76; p=0.0412$) and time x treatment interaction ($F_{20,200}=2.74; p=0.0002$) on overall rotorod performance during 28 days drug treatment. Rotorod performance was increased in L-DOPA + CCG-63802 compared to L-DOPA + vehicle on day 15 (156%, $p<0.05$), day 20 (275%, $p<0.01$), day 23 (407%, $p<0.01$) and day 25 (250%, $p<0.05$). There were no significant differences in rotorod performance between any of the treatment groups on any other treatment day.

There was a significant effect of time ($F_{10,200}=2.56; p=0.0063$, Figure 81B), treatment group ($F_{2,200}=4.48; p=0.0246$) and time x treatment interaction ($F_{20,200}=2.37; p=0.0014$) on peak rotorod performance during 28 days drug treatment. Peak rotorod performance was increased in L-DOPA + CCG-63802 compared to L-DOPA + vehicle on day 15 (287%, $p<0.01$), day 20 (431%, $p<0.01$), day 23 (649%, $p<0.01$), day 25 (519%, $p<0.05$) and day 27 (301%, $p<0.05$). There were no significant differences in rotorod performance between any of the treatment groups on any other treatment day.
Figure 81. The effect of 28 days vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent mean ± SEM (n=7-8); some error bars have been omitted for clarity. (A) Mean rotorod performance. (B) Peak rotorod performance at 1 hour post vehicle or L-DOPA/benserazide treatment. **p<0.01, *p<0.05 cf. vehicle (two-way ANOVA, Bonferroni multiple comparisons test).
AIMs

There was a significant effect of time ($F_{10,215}=4.34; p<0.0001$, Figure 82A) and treatment group ($F_{2,215}=144.89; p<0.0001$), but not time $\times$ treatment interaction ($F_{20,215}=0.58; p=0.9243$) on sum of ALO AIMs during 28 days drug treatment. Individual analysis of treatment days showed ALO AIMs were reduced in L-DOPA + CCG-2046 and L-DOPA + CCG-63802 treatment groups on day 1 (94% and 97%, respectively; $H=11.85; p=0.0027$), day 4 ($H=6.701; p=0.0351$), day 7 (71% and 71%, respectively; $H=8.717; p=0.0128$), day 10 (84% and 81%, respectively; $H=11.61; p=0.0030$), day 14 (82% and 69%, respectively; $H=11.55; p=0.0031$), day 17 (75% and 75%, respectively; $H=12.39; p=0.0020$), day 21 (65% and 63%, respectively; $H=11.20; p=0.0037$), day 22 (66% and 55%, respectively; $H=12.11; p=0.0023$), day 24 (70% and 58%, respectively; $H=11.46; p=0.0032$), day 26 (78% and 74%, respectively; $H=14.07; p=0.0009$) and day 28 (69% and 80%, respectively; $H=13.85; p=0.0010$), compared to L-DOPA + vehicle.

There was a significant effect of time ($F_{10,215}=2.68; p=0.0042$, Figure 82B) and treatment group ($F_{2,215}=126.52; p<0.0001$), but not time $\times$ treatment interaction ($F_{20,215}=0.59; p=0.9161$) on axial AIMs during 28 days drug treatment. Individual analysis of treatment days showed axial AIMs were reduced in L-DOPA + CCG-2046 and L-DOPA + CCG-63802 treatment groups on day 1 (100% and 100%, respectively; $H=12.61; p=0.0018$), day 7 (80% and 100%, respectively; $H=9.012; p=0.0110$), day 10 (96% and 92%, respectively; $H=10.73; p=0.0047$), day 14 (92% and 80%, respectively; $H=12.24; p=0.0022$), day 17 (90% and 90%, respectively; $H=10.02; p=0.0067$), day 21 (91% and 86%, respectively; $H=10.64; p=0.0049$), day 22 (77% and 68%, respectively; $H=11.83; p=0.0027$), day 24 (86% and 82%, respectively; $H=11.69; p=0.0029$), day 26 (86% and 100%, respectively; $H=15.11; p=0.0005$) and day 28 (77% and 91%, respectively; $H=14.09; p=0.0009$), compared to L-DOPA + vehicle. On treatment day 4, ALO AIMs were reduced in L-DOPA + CCG-63802 (88%; $H=7.327; p=0.0256$), but not L-DOPA + CCG-2046 ($p>0.05$), compared to L-DOPA + vehicle.

There was a significant effect of time ($F_{10,215}=4.82; p<0.0001$, Figure 82C) and treatment group ($F_{2,215}=117.41; p<0.0001$), but not time $\times$ treatment interaction ($F_{20,215}=0.56; p=0.9358$) on limb AIMs during 28 days drug treatment. Individual analysis of treatment days showed limb AIMs were reduced in L-DOPA + CCG-2046 and L-DOPA + CCG-63802 treatment groups on day 1 (100% and 100%, respectively; $H=10.44; p=0.0054$), day
7 (65% and 55%, respectively; H=8.189; p=0.0167), day 10 (78% and 78%, respectively; H=12.41; p=0.0020), day 14 (74% and 70%, respectively; H=12.23; p=0.0022), day 17 (65% and 65%, respectively; H=12.03; p=0.0024), day 21 (70% and 55%, respectively; H=9.136; p=0.0104), day 22 (67% and 54%, respectively; H=11.06; p=0.0040), day 24 (67% and 54%, respectively; H=10.87; p=0.0044), day 26 (63% and 54%, respectively; H=13.14; p=0.0014) and day 28 (58% and 75%, respectively; H=14.22; p=0.0008), compared to L-DOPA + vehicle. There were no significant differences between treatment groups on any other treatment day (p>0.05).

There was a significant effect of time (F_{10,215}=4.67; p<0.0001, Figure 82D) and treatment group (F_{2,215}=136.17; p<0.0001), but not time x treatment interaction (F_{20,215}=0.53; p=0.9506) on orolingual AIMs during 28 days drug treatment. Individual analysis of treatment days showed orolingual AIMs were reduced in L-DOPA + CCG-2046 and L-DOPA + CCG-63802 treatment groups on day 1 (85% and 92%, respectively; H=12.53; p=0.0019), day 7 (67% and 62%, respectively; H=9.551; p=0.0084), day 10 (73% and 65%, respectively; H=12.48; p=0.0020), day 14 (81% and 58%, respectively; H=11.71; p=0.0029), day 17 (70% and 65%, respectively; H=13.78; p=0.0010), day 21 (60% and 55%, respectively; H=10.24; p=0.0060), day 22 (65% and 55%, respectively; H=12.25; p=0.0022), day 24 (64% and 45%, respectively; H=10.75; p=0.0046), day 26 (77% and 64%, respectively; H=12.64; p=0.0018) and day 28 (59% and 64%, respectively; H=13.18; p=0.0014), compared to L-DOPA + vehicle. There were no significant differences between treatment groups on any other treatment day (p>0.05).

There was no significant effect of time (F_{10,215}=1.80; p=0.0629, Figure 82E) or treatment group (F_{2,215}=2.20; p=0.1130) on locomotor AIMs during 28 days drug treatment. Individual analysis of treatment days showed no significant difference in locomotor AIMs between treatment groups (days 1-28, p>0.05).
**Figure 82.** The effect of 28 days, once daily, vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment on abnormal involuntary movements (AIMs) in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent mean ± SEM (n=6–8); some error bars have been omitted for clarity. (A) Sum of axial, limb, orolingual (ALO); (B) axial; (C) limb; (D) orolingual; (E) locomotor AIMs over 3 hours post L-DOPA administration. ***p<0.001, **p<0.01, *p<0.05 cf. vehicle (Kruskal-Wallis test, Dunn’s multiple comparison test).
**Overall effect on L-DOPA priming**

Following L-DOPA challenge after 28 days drug treatment period, there was a significant effect of treatment group on sum of ALO AIMs (H=12.44; p=0.0020, Figure 83A), which was reduced in groups pre-treated with L-DOPA + CCG-2046 (55%, p<0.05) and L-DOPA + CGG-63802 (52%, p<0.01), compared to L-DOPA + vehicle.

Following L-DOPA challenge after 28 day drug treatment period, there was no significant effect of treatment group on locomotor AIMs (H=0.2590; p=0.8785, Figure 83B).
Figure 83. The effect of L-DOPA/benserazide (6/15 mg/kg; i.p.) challenge on abnormal involuntary movements (AIMs) in unilateral 6-OHDA-lesioned rats following once daily pre-treatment with vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.), in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.), for 28 days. Points represent data from individual animals (n=7-8) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs. (B) Locomotor AIMs from 0-3 hours post L-DOPA administration. **p<0.01, *p<0.05 cf. vehicle (Kruskal-Wallis test, Dunn’s multiple comparison test).
Expression of molecular markers of LID following 28 days RGS4 protein inhibitor treatment

PPE-A

Rostral striatum

In the whole rostral striatum, there was a significant effect of lesion (F_{1,40}=81.68; p<0.0001, Figure 84) but not treatment group (F_{2,40}=0.24; p=0.7894) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (100%, p<0.001), L-DOPA + CCG-2046 (59%, p<0.001) and L-DOPA + CCG-63802 (81%, p<0.001) treatment groups.

In the dorsolateral region of the rostral striatum, there was a significant effect of lesion (F_{1,40}=106.31; p<0.0001, Figure 85A) but not treatment group (F_{2,40}=0.35; p=0.7086) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (109%, p<0.001), L-DOPA + CCG-2046 (94%, p<0.001) and L-DOPA + CCG-63802 (99%, p<0.001) treatment groups.

In the dorsomedial region of the rostral striatum, there was a significant effect of lesion (F_{1,40}=57.54; p<0.0001, Figure 85B) but not treatment group (F_{2,40}=0.32; p=0.7297) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (117%, p<0.001), L-DOPA + CCG-2046 (72%, p<0.01) and L-DOPA + CCG-63802 (91%, p<0.001) treatment groups.

In the ventrolateral region of the rostral striatum, there was a significant effect of lesion (F_{1,40}=73.13; p<0.0001, Figure 85C) but not treatment group (F_{2,40}=0.03; p=0.9696) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (88%, p<0.001), L-DOPA + CCG-2046 (52%, p<0.001) and L-DOPA + CCG-63802 (72%, p<0.001) treatment groups.

In the ventromedial region of the rostral striatum, there was a significant effect of lesion (F_{1,38}=39.95; p<0.0001, Figure 85D) but not treatment group (F_{2,38}=0.72; p=0.4927) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (81%, p<0.001), L-DOPA + CCG-2046 (32%, p<0.05) and L-DOPA + CCG-63802 (62%, p<0.001) treatment groups.
In the nucleus accumbens core, there was a significant effect of lesion (F_{1,38}=12.43; p=0.0011, Figure 85E) but not treatment group (F_{2,38}=0.95; p=0.3955) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (32%, p<0.05) and L-DOPA + CCG-63802 (37%, p<0.05), but not L-DOPA + CCG-2046 (p>0.05) treatment group.

In the nucleus accumbens shell, there was a significant effect of lesion (F_{1,39}=12.47; p=0.0011, Figure 85F) but not treatment group (F_{2,39}=1.19; p=0.3138) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (53%, p<0.01) and L-DOPA + CCG-63802 (50%, p<0.05), but not L-DOPA + CCG-2046 (p>0.05) treatment group.

 Autoradiographic images of PPE-A mRNA expression in the rostral striatum are shown in Figure 86.
Figure 84. PPE-A mRNA expression in the rostral striatum of unilateral 6-OHDA-lesioned rats following once daily, 28 day vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=7-8). ***p<0.001 cf. unoperated side (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 85. PPE-A mRNA expression in the sub-regions of the rostral striatum of unilateral 6-OHDA-lesioned rats following once daily, 28 day, vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment in combination with 28 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=7-8). ***p<0.001, **p<0.01, *p<0.05 cf. unoperated side (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 86. Pseudocolour image transformations of autoradiographs from *in situ* hybridisation targeting preproenkephalin-A (PPE-A) mRNA in the (U) unoperated and (O) operated sides of the rostral striatum of unilateral 6-OHDA-lesioned rats following once daily 28 day vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment.
**PPE-B**

**Rostral striatum**

In the whole rostral striatum, there was a significant effect of lesion (F<sub>1,33</sub>=17.18; p=0.0002, Figure 87), treatment group (F<sub>2,33</sub>=6.75; p=0.0035) and lesion x treatment interaction (F<sub>2,33</sub>=8.52; p=0.0010) on PPE-B mRNA expression. PPE-B mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (54%, p<0.001), but not L-DOPA + CCG-2046 (p>0.05) or L-DOPA + CCG-63802 (p>0.05) treatment groups. PPE-B mRNA expression in the operated side was increased in L-DOPA + vehicle compared to L-DOPA + CCG-2046 (40%, p<0.05) and L-DOPA + CCG-63802 (42%, p<0.05) treatment groups. There was no significant difference between the operated sides of L-DOPA + CCG-2046 and L-DOPA + CCG-63802 (p>0.05) treatment groups.

In the dorsolateral region of the rostral striatum, there was a significant effect of lesion (F<sub>1,34</sub>=42.12; p<0.0001, Figure 88A), treatment group (F<sub>2,34</sub>=9.00; p=0.0007) and lesion x treatment interaction (F<sub>2,34</sub>=6.38; p=0.0044) on PPE-B mRNA expression. PPE-B mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (109%, p<0.001) and L-DOPA + CCG-63802 (36%, p<0.01) treatment groups. PPE-B mRNA expression in the operated side was increased in L-DOPA + vehicle compared to L-DOPA + CCG-2046 (64%, p<0.05) and L-DOPA + CCG-63802 (54%, p<0.01) treatment groups. There was no significant difference between the operated sides of L-DOPA + CCG-2046 and L-DOPA + CCG-63802 (p>0.05) treatment groups.

In the dorsomedial region of the rostral striatum, there was a significant effect of lesion (F<sub>1,34</sub>=22.10; p<0.0001, Figure 88B), treatment group (F<sub>2,34</sub>=6.21; p=0.0050) and lesion x treatment interaction (F<sub>2,34</sub>=5.41; p=0.0092) on PPE-B mRNA expression. PPE-B mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (67%, p<0.001), but not L-DOPA + CCG-2046 (p>0.05) or L-DOPA + CCG-63802 (p>0.05) treatment groups. PPE-B mRNA expression in the operated side was increased in L-DOPA + vehicle compared to L-DOPA + CCG-2046 (48%, p<0.05) and L-DOPA + CCG-63802 (42%, p<0.05) treatment groups. There was no significant difference between the operated sides of L-DOPA + CCG-2046 and L-DOPA + CCG-63802 (p>0.05) treatment groups.
In the ventrolateral region of the rostral striatum, there was a significant effect of lesion ($F_{1,34}=19.65; p<0.0001$, Figure 88C), treatment group ($F_{2,34}=11.77; p=0.0001$) and lesion x treatment interaction ($F_{2,34}=10.01; p=0.0004$) on PPE-B mRNA expression. PPE-B mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (66%, $p<0.001$), but not L-DOPA + CCG-2046 ($p>0.05$) or L-DOPA + CCG-63802 ($p>0.05$) treatment groups. PPE-B mRNA expression in the operated side was increased in L-DOPA + vehicle compared to L-DOPA + CCG-2046 (67%, $p<0.01$) and L-DOPA + CCG-63802 (46%, $p<0.05$) treatment groups. There was no significant difference between the operated sides of L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$) treatment groups.

In the ventromedial region of the rostral striatum, there was no significant effect of lesion ($F_{1,35}=1.35; p=0.2530$, Figure 88D) or treatment group ($F_{2,35}=0.65; p=0.5269$) on PPE-B mRNA expression.

In the nucleus accumbens core, there was no significant effect of lesion ($F_{1,37}=3.13; p=0.0853$, Figure 88E) or treatment group ($F_{2,37}=0.22; p=0.8022$) on PPE-B mRNA expression.

In the nucleus accumbens shell, there was no significant effect of lesion ($F_{1,35}=2.19; p=0.1418$, Figure 88F) or treatment group ($F_{2,35}=0.87; p=0.4294$) on PPE-B mRNA expression.

Autoradiographic images of PPE-B mRNA expression in the rostral striatum are shown in Figure 89.
Figure 87. PPE-B mRNA expression in the rostral striatum of unilateral 6-OHDA-lesioned rats following once daily, 28 day vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6-8). ***p<0.001 cf. unoperated side; #p<0.05 cf. ipsilateral vehicle-treated (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 88. PPE-B mRNA expression in the sub-regions of the rostral striatum of unilateral 6-OHDA-lesioned rats following once daily, 28 day vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6-8). ***p<0.001, **p<0.01 cf. unoperated side; #p<0.01, #p<0.05 cf. ipsilateral vehicle-treated (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 89. Pseudocolour image transformations of autoradiographs from *in situ* hybridisation targeting preproenkephalin-B (PPE-B) mRNA in the (U) unoperated and (O) operated sides of the rostral striatum of unilateral 6-OHDA-lesioned rats following once daily, 28 day, vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment.
In the whole rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding ($F_{2,15}=7.428$; $p=0.0057$, Figure 90A), which was reduced in L-DOPA + CCG-2046 (16%, $p<0.01$) and L-DOPA + CCG-63802 (14%, $p<0.05$), compared to L-DOPA + vehicle. There was no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$).

In the dorsolateral rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding ($F_{2,14}=6.294$; $p=0.0112$, Figure 90B), which was reduced in L-DOPA + CCG-2046 (14%, $p<0.05$) and L-DOPA + CCG-63802 (17%, $p<0.05$), compared to L-DOPA + vehicle. There was no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$).

In the dorsomedial rostral striatum of the operated side, there was no significant effect of treatment group on dopamine stimulated $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding ($F_{2,15}=2.059$; $p=0.1622$, Figure 90C).

In the ventrolateral rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding ($F_{2,15}=5.811$; $p=0.0135$, Figure 90D), which was reduced in L-DOPA + CCG-2046 (19%, $p<0.05$) and L-DOPA + CCG-63802 (19%, $p<0.05$), compared to L-DOPA + vehicle. There was no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$).

In the ventromedial rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding ($F_{2,15}=3.756$; $p=0.0476$, Figure 90E), which was reduced in L-DOPA + CCG-63802 (9%, $p<0.05$), but not L-DOPA + CCG-2046 ($p>0.05$), compared to L-DOPA + vehicle. There was no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$).

Autoradiographic images of $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding in the rostral striatum are shown in Figure 91.
Figure 90. Dopamine stimulated $[^{35}]$GTP$_{\gamma}$S binding autoradiography in the operated side of the rostral striatum of unilateral 6-OHDA-lesioned rats following once daily, 28 day, vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment in combination with 28 day L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. (A) Whole striatum, (B) dorsolateral, (C) dorsomedial, (D) ventrolateral (E) ventromedial striatum. Bars represent mean ± SEM (n=5-6). **p<0.01, *p<0.05 cf. vehicle-treated (one-way ANOVA, Tukey’s post hoc analysis).
Figure 91. Pseudocolour image transformations of autoradiographs from dopamine (100 µM) stimulated [³⁵S]GTPγS binding in the operated side of the rostral striatum of unilateral 6-OHDA-lesioned rats following once daily, 28 day, vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment.
4.3.3  7 days RGS4 protein inhibitor treatment

**DAT binding**
There was a significant effect of lesion (F\(_{1,40}=2578.80; \ p<0.0001\), Figure 92A) but not treatment group (F\(_{2,40}=0.27; \ p=0.7678\)) on \[^{125}\text{I}]-\text{RTI-121}\) binding levels. \[^{125}\text{I}]-\text{RTI-121}\) binding was reduced in the operated compared to the unoperated side in L-DOPA + vehicle (85%, \(p<0.001\)), L-DOPA + CCG-2046 (86%, \(p<0.001\)) and L-DOPA + CCG-63802 (83%, \(p<0.001\)) treatment groups.

Autoradiographic images from DAT binding in the striatum are shown in Figure 93.

**Cylinder test**
There was a significant effect of lesion (F\(_{1,42}=557.39; \ p<0.0001\), Figure 92B) but not treatment group (F\(_{2,42}=0.00; \ p=1.000\)) on cylinder test performance. Left paw contacts were reduced compared to right paw contacts in L-DOPA + vehicle (85%, \(p<0.001\)), L-DOPA + CCG-2046 (88%, \(p<0.001\)) and L-DOPA + CCG-63802 (87%, \(p<0.001\)) treatment groups.
Figure 92. The effect of unilateral 6-OHDA-lesion on (A) dopamine transporter binding and (B) cylinder test performance in rats after 7 days treatment with L-DOPA + vehicle, L-DOPA + CCG-2046 or L-DOPA + CCG-63802 following L-DOPA priming. (A) $[^{125}I]$-RTI-121 binding in the unoperated (□) and operated (■) striatum. ***p<0.001 cf. unoperated side. (B) % of total paw contacts made with right (□) or left (■) paw. ***p<0.001 cf. right paw same treatment group. Bars represent mean ± SEM (n=7-8) (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 93. Pseudocolour transformations of autoradiographic images from dopamine active transporter (DAT) binding using $[^{125}]$RTI-121 autoradiography. Representative coronal sections of the (U) unoperated and (O) operated striatum in the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia following 7 day vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.).
Rotorod performance

On day 20 of L-DOPA treatment, there was no significant difference between overall rotorod performance in L-DOPA-treated groups (F\(_{2,15}=3.407; \ p=0.0603\), Figure 94A).

On day 23 of L-DOPA treatment, there was a significant effect of treatment group (F\(_{2,15}=39.37; \ p<0.0001\), Figure 94B) on overall rotorod performance, which was increased in L-DOPA + CCG-2046 (122%; \ p<0.001) and L-DOPA + CCG-63802 (136%, \ p<0.001), compared to L-DOPA + vehicle. There were no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 (p>0.05).

On day 27 of L-DOPA treatment, there was a significant effect of treatment group (F\(_{2,15}=10.92; \ p=0.0012\), Figure 94C) on overall rotorod performance, which was increased in L-DOPA + CCG-63802 compared to L-DOPA + vehicle (86%, \ p<0.01) and L-DOPA + CCG-2046 (133%, \ p<0.01). There was no significant difference between L-DOPA + vehicle and L-DOPA + CCG-2046 (p>0.05).

There was no significant effect of time (F\(_{2,30}=0.83; \ p=0.4475\), Figure 94D) or treatment group (F\(_{2,30}=3.58; \ p=0.0535\) on peak rotorod performance during 7 days RGS4 protein inhibitor treatment.
Figure 94. The effect of 7 day vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on rotorod performance in unilateral 6-OHDA-lesioned rats primed with L-DOPA/benserazide for 21 days. Rotorod performance on (A) day 20, (B) day 23 and (C) day 27 of L-DOPA/benserazide treatment. Bars represent mean ± SEM (n=5-7). ***p<0.001, **p<0.01 cf. vehicle; ##p<0.01 cf. L-DOPA + CCG-2046 (one-way ANOVA, Tukey’s post hoc analysis). (D) peak rotorod performance at 1 hour post L-DOPA/benserazide treatment. Points represent mean ± SEM (n=5-7); some error bars have been omitted for clarity (two-way ANOVA, Bonferroni multiple comparisons test).
AIMs

There was a significant effect of time (F_{4,85}=6.98; p<0.0001, Figure 95A), treatment group (F_{2,85}=24.90; p<0.0001) and time x treatment interaction (F_{8,85}=3.55; p=0.0014) on sum of ALO AIMS during 7 days treatment. Individual analysis of treatment days showed ALO AIMS were reduced in L-DOPA + CCG-2046 and L-DOPA + CCG-63802 treatment groups on day 24 (40% and 41%, respectively; H=8.511; p=0.0142), day 26 (56% and 46%, respectively; H=13.14; p=0.0014) and day 28 (59% and 46%, respectively; H=12.94; p=0.0015), compared to L-DOPA + vehicle. On treatment day 22, sum of ALO AIMS were reduced in L-DOPA + CCG-2046 (35%; H=8.236; p=0.0163), but not L-DOPA + CCG-63802 (p>0.05), compared to L-DOPA + vehicle.

There was a significant effect of time (F_{4,85}=5.89; p=0.0003, Figure 95B), treatment group (F_{2,85}=20.15; p<0.0001) and time x treatment interaction (F_{8,85}=2.73; p=0.0099) on axial AIMS during 7 days treatment. Individual analysis of treatment days showed axial AIMS were reduced in L-DOPA + CCG-2046 and L-DOPA + CCG-63802 treatment groups on day 26 (55% and 55%, respectively; H=12.89; p=0.0016) and day 28 (64% and 45%, respectively; H=10.36; p=0.0056), compared to L-DOPA + vehicle. On treatment day 24, axial AIMS were reduced by L-DOPA + CCG-63802 (55%; H=11.10; p=0.0039), but not L-DOPA + CCG-2046 (p>0.05), compared to L-DOPA + vehicle. On treatment day 22, there was no significant difference in axial AIMS between any of the treatment groups (H=5.566; p=0.0618).

There was a significant effect of time (F_{4,85}=2.75; p=0.0331, Figure 95C), treatment group (F_{2,85}=18.33; p<0.0001) and time x treatment interaction (F_{8,85}=2.35; p=0.0247) on limb AIMS during 7 days treatment. Individual analysis of treatment days showed limb AIMS were reduced in L-DOPA + CCG-2046 and L-DOPA + CCG-63802 treatment groups on day 24 (42% and 42%, respectively; H=9.232; p=0.0099) and day 26 (50% and 46%, respectively; H=12.41; p=0.0020), compared to L-DOPA + vehicle. On treatment days 22 and 28, limb AIMS were reduced by L-DOPA + CCG-2046 (42%; H=7.251; p=0.0266 and 58%; H=10.83; p=0.0045, respectively), but not L-DOPA + CCG-63802 (p>0.05), compared to L-DOPA + vehicle.

There was a significant effect of time (F_{4,85}=12.56; p<0.0001, Figure 95D), treatment group (F_{2,85}=28.42; p<0.0001) and time x treatment interaction (F_{8,85}=4.87; p<0.0001) on
orolingual AIMs during 7 days treatment. Individual analysis of treatment days showed orolingual AIMs were reduced in L-DOPA + CCG-2046 and L-DOPA + CCG-63802 treatment groups on day 22 (30% and 30%, respectively; \( H=10.09; p=0.0064 \)), day 26 (55% and 45%, respectively; \( H=13.23; p=0.0013 \)) and day 28 (55% and 41%, respectively; \( H=13.04; p=0.0015 \)), compared to L-DOPA + vehicle. On treatment day 24, orolingual AIMs were reduced by L-DOPA + CCG-2046 (45%; \( H=8.493; p=0.0143 \)), but not L-DOPA + CCG-63802 (\( p>0.05 \)), compared to L-DOPA + vehicle.

There was a significant effect of treatment group (\( F_{2,85}=5.67; p=0.0049 \), Figure 95E) but not time (\( F_{4,85}=0.54; p=0.7096 \)) on locomotor AIMs during 7 days treatment. Individual analysis of treatment days showed locomotor AIMs were increased on day 24 in L-DOPA + CCG-63802 (63%; \( H=7.043; p=0.0296 \)), but not L-DOPA + CCG-2046 (\( p>0.05 \)), compared to L-DOPA + vehicle. There was no significant difference between treatment groups on any other treatment day (\( p>0.05 \)).
Figure 95. The effect of 7 days vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on abnormal involuntary movements (AIMs) in unilateral 6-OHDA-lesioned rats primed with L-DOPA/benserazide for 21 days. Points represent mean ± SEM (n=6-7); some error bars have been omitted for clarity. (A) Sum of axial, limb, orolingual (ALO); (B) axial; (C) limb; (D) orolingual; (E) locomotor AIMs over 3 hours post L-DOPA administration. **p<0.01, *p<0.05 cf. vehicle (Kruskal-Wallis test, Dunn’s multiple comparison test).
Long-standing effect of 7 days RGS4 protein inhibitor treatment
Following L-DOPA challenge after the 7 days treatment period, there was a significant effect of treatment group on sum of ALO AIMs (H=11.11; p=0.0039, Figure 96A), which was reduced in groups pre-treated with L-DOPA + CCG-2046 (31%, p<0.05) and L-DOPA + CGG-63802 (45%, p<0.01), compared to L-DOPA + vehicle.

Following L-DOPA challenge after the 7 days treatment period, there was no significant effect of treatment group on locomotor AIMs (H=3.552; p=0.1693, Figure 96B).
Figure 96. The effect of L-DOPA/benserazide (6/15 mg/kg; i.p.) challenge on abnormal involuntary movements (AIMs) in L-DOPA-primed unilateral 6-OHDA-lesioned rats following once daily pre-treatment with vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.), in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.), for 7 days. Points represent data from individual animals (n=6-7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs. (B) Locomotor AIMs from 0-3 hours post L-DOPA administration. **p<0.01, *p<0.05 cf. vehicle (Kruskal-Wallis test, Dunn’s multiple comparison test).
Expression of molecular markers of LID following 7 days RGS4 protein inhibitor treatment

PPE-A

Rostral striatum

In the whole rostral striatum, there was a significant effect of lesion (F_{1,34}=316.23; p<0.0001, Figure 97) but not treatment group (F_{2,34}=2.20; p=0.1260) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (100%, p<0.001), L-DOPA + CCG-2046 (97%, p<0.001) and L-DOPA + CCG-63802 (105%, p<0.001) treatment groups.

In the dorsolateral region of the rostral striatum, there was a significant effect of lesion (F_{1,34}=343.55; p<0.0001, Figure 98A) but not treatment group (F_{2,34}=3.11; p=0.0575) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (109%, p<0.001), L-DOPA + CCG-2046 (105%, p<0.001) and L-DOPA + CCG-63802 (101%, p<0.001) treatment groups.

In the dorsomedial region of the rostral striatum, there was a significant effect of lesion (F_{1,34}=376.97; p<0.0001, Figure 98B) but not treatment group (F_{2,34}=2.43; p=0.1034) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (117%, p<0.001), L-DOPA + CCG-2046 (118%, p<0.001) and L-DOPA + CCG-63802 (122%, p<0.001) treatment groups.

In the ventrolateral region of the rostral striatum, there was a significant effect of lesion (F_{1,34}=189.34; p<0.0001, Figure 98C) but not treatment group (F_{2,34}=1.43; p=0.2522) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (88%, p<0.001), L-DOPA + CCG-2046 (80%, p<0.001) and L-DOPA + CCG-63802 (85%, p<0.001) treatment groups.

In the ventromedial region of the rostral striatum, there was a significant effect of lesion (F_{1,34}=133.24; p<0.0001, Figure 98D) but not treatment group (F_{2,34}=1.35; p=0.2731) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (81%, p<0.001), L-DOPA + CCG-2046 (68%, p<0.001) and L-DOPA + CCG-63802 (80%, p<0.001) treatment groups.
In the nucleus accumbens core, there was a significant effect of lesion ($F_{1,34}=11.94$; $p=0.0015$, Figure 98E) but not treatment group ($F_{2,34}=1.49; p=0.2396$) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (32%, $p<0.05$) and L-DOPA + CCG-63802 (43%, $p<0.05$), but not in L-DOPA + CCG-2046 ($p>0.05$) treatment group.

In the nucleus accumbens shell, there was a significant effect of lesion ($F_{1,34}=16.90$; $p=0.0002$, Figure 98F) but not treatment group ($F_{2,34}=0.68; p=0.5131$) on PPE-A mRNA expression. PPE-A mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (53%, $p<0.05$) and L-DOPA + CCG-63802 (54%, $p<0.05$), but not in L-DOPA + CCG-2046 ($p>0.05$) treatment group.

 Autoradiographic images of PPE-A mRNA expression in the rostral striatum are shown in Figure 99.
Figure 97. PPE-A mRNA expression in the rostral striatum of L-DOPA-primed unilateral 6-OHDA-lesioned rats following 7 day vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.). Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6-7). ***p<0.001 cf. unoperated side (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 98. PPE-A mRNA expression in the sub-regions of the rostral striatum of L-DOPA-primed unilateral 6-OHDA-lesioned rats following 7 days vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.). (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6-7). ***p<0.001, *p<0.05 cf. unoperated side (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 99. Pseudocolour image transformations of autoradiographs from *in situ* hybridisation targeting preproenkephalin-A (PPE-A) mRNA in the (U) unoperated and (O) operated sides of the rostral striatum of L-DOPA-primed unilateral 6-OHDA-lesioned rats following 7 days vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.).
PPE-B

Rostral striatum

In the whole rostral striatum, there was a significant effect of lesion ($F_{1,34}=30.85; p<0.0001$, Figure 100) but not treatment group ($F_{2,34}=0.23; p=0.7936$) on PPE-B mRNA expression. PPE-B mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (42%, $p<0.01$), L-DOPA + CCG-2046 (52%, $p<0.05$) and L-DOPA + CCG-63802 (79%, $p<0.01$) treatment groups.

In the dorsolateral region of the rostral striatum, there was a significant effect of lesion ($F_{1,34}=87.93; p<0.0001$, Figure 101A) but not treatment group ($F_{2,34}=0.35; p=0.7078$) on PPE-B mRNA expression. PPE-B mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (89%, $p<0.001$), L-DOPA + CCG-2046 (136%, $p<0.001$) and L-DOPA + CCG-63802 (163%, $p<0.01$) treatment groups.

In the dorsomedial region of the rostral striatum, there was a significant effect of lesion ($F_{1,34}=40.47; p<0.0001$, Figure 101B) but not treatment group ($F_{2,34}=0.59; p=0.5586$) on PPE-B mRNA expression. PPE-B mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (54%, $p<0.001$), L-DOPA + CCG-2046 (69%, $p<0.01$) and L-DOPA + CCG-63802 (103%, $p<0.01$) treatment groups.

In the ventrolateral region of the rostral striatum, there was a significant effect of lesion ($F_{1,34}=43.47; p<0.0001$, Figure 101C) but not treatment group ($F_{2,34}=0.28; p=0.7590$) on PPE-B mRNA expression. PPE-B mRNA expression was increased in the operated compared to the unoperated side in L-DOPA + vehicle (58%, $p<0.01$), L-DOPA + CCG-2046 (69%, $p<0.01$) and L-DOPA + CCG-63802 (102%, $p<0.01$) treatment groups.

In the ventromedial region of the rostral striatum, there was no significant effect of lesion ($F_{1,34}=1.98; p=0.1690$, Figure 101D) or treatment group ($F_{2,34}=0.50; p=0.6094$) on PPE-B mRNA expression.

In the nucleus accumbens core, there was no significant effect of lesion ($F_{1,34}=2.00; p=0.1667$, Figure 101E) or treatment group ($F_{2,34}=2.24; p=0.1222$) on PPE-B mRNA expression.
In the nucleus accumbens shell, there was no significant effect of lesion ($F_{1,34}=3.68; p=0.0636$, Figure 101F) or treatment group ($F_{2,34}=1.47; p=0.2441$) on PPE-B mRNA expression.

Autoradiographic images of PPE-B mRNA expression in the rostral striatum are shown in Figure 102.
PPE-B mRNA expression in the rostral striatum of L-DOPA-primed unilateral 6-OHDA-lesioned rats following 7 days vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.). Bars represent mean ± SEM in unoperated (□) or operated (■) sides (n=6-7). **p<0.01, *p<0.05 cf. unoperated side (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 101. PPE-B mRNA expression in the sub-regions of the rostral striatum of L-DOPA-primed unilateral 6-OHDA-lesioned rats following 7 day vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.). (A) dorsolateral, (B) dorsomedial, (C) ventrolateral, (D) ventromedial striatum; nucleus accumbens (E) core and (F) shell regions. Bars represent mean ± SEM in unoperated (●) or operated (■) sides (n=6-7). ***p<0.001, **p<0.01 cf. unoperated side (two-way ANOVA, Bonferroni multiple comparisons test).
Figure 102. Pseudocolour image transformations of autoradiographs from in situ hybridisation targeting preproenkephalin-B (PPE-B) mRNA in the (U) unoperated and (O) operated sides of the rostral striatum of L-DOPA-primed unilateral 6-OHDA-lesioned rats following 7 days vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.).
In the whole rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}S]GTP\gamma S$ binding ($F_{2,17}=13.24; p=0.0003$, Figure 103A), which was reduced in L-DOPA + CCG-2046 (14%, $p<0.001$) and L-DOPA + CCG-63802 (11%, $p<0.01$), compared to L-DOPA + vehicle. There was no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$).

In the dorsolateral rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}S]GTP\gamma S$ binding ($F_{2,16}=6.302; p=0.0096$, Figure 103B), which was reduced in L-DOPA + CCG-2046 (14%, $p<0.05$) and L-DOPA + CCG-63802 (12%, $p<0.05$), compared to L-DOPA + vehicle. There was no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$).

In the dorsomedial rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}S]GTP\gamma S$ binding ($F_{2,16}=6.406; p=0.0090$, Figure 103C), which was reduced in L-DOPA + CCG-2046 (12%, $p<0.01$), but not L-DOPA + CCG-63802 ($p>0.05$), compared to L-DOPA + vehicle. There was no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$).

In the ventrolateral rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}S]GTP\gamma S$ binding ($F_{2,16}=5.878; p=0.0122$, Figure 103D), which was reduced in L-DOPA + CCG-2046 (16%, $p<0.05$) and L-DOPA + CCG-63802 (18%, $p<0.05$), compared to L-DOPA + vehicle. There was no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$).

In the ventromedial rostral striatum of the operated side, there was a significant effect of treatment group on dopamine stimulated $[^{35}S]GTP\gamma S$ binding ($F_{2,16}=4.339; p=0.0312$, Figure 103E), which was reduced in L-DOPA + CCG-2046 (10%, $p<0.05$), but not L-DOPA + CCG-63802 ($p>0.05$), compared to L-DOPA + vehicle. There was no significant difference between L-DOPA + CCG-2046 and L-DOPA + CCG-63802 ($p>0.05$).

Autoradiographic images of $[^{35}S]GTP\gamma S$ binding in the rostral striatum are shown in Figure 104.
Figure 103. Dopamine stimulated [35S]GTPγS binding autoradiography in the operated side of the rostral striatum of L-DOPA primed unilateral 6-OHDA-lesioned rats following 7 days vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment. (A) whole striatum, (B) dorsolateral, (C) dorsomedial, (D) ventrolateral (E) ventromedial striatum. Bars represent mean ± SEM (n=6-7). ***p<0.001, **p<0.01, *p<0.05 cf. vehicle-treated (one-way ANOVA, Tukey’s post hoc analysis).
Figure 104. Pseudocolour image transformations of autoradiographs from dopamine (100 µM) stimulated [35S]GTPγS binding in the operated side of the rostral striatum of L-DOPA-primed unilateral 6-OHDA-lesioned rats following 7 days vehicle, CCG-2046 (0.1 mg/kg; i.p.) or CCG-63802 (0.1 mg/kg; i.p.) treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.).
Experimental control

In situ hybridisation

Autoradiograph signal was absent in the presence of 25-fold excess unlabelled probe and following RNAase pre-treatment (data not shown).

\[^{35}\text{S}]\text{GTP}\gamma\text{S binding autoradiography}\]

Autoradiograph signal was absent in the presence of GTP\gamma\text{S} in comparison to control (Figure 105).
Figure 105. Pseudocolour image transformations of autoradiograph images displaying $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding in the rostral striatum following basal ($[^{35}\text{S}]\text{GTP}_\gamma\text{S}$) and competitive ($[^{35}\text{S}]\text{GTP}_\gamma\text{S} + \text{GTP}_\gamma\text{S}$) incubations.
4.4 Discussion

The main findings from this study were that 28 days RGS4 protein inhibitor treatment, in combination with L-DOPA treatment, reduced L-DOPA priming and subsequent induction of AIMs in the unilateral 6-OHDA-lesioned rat model of PD. These behavioural effects were supported at the molecular level, with reduced striatal expression of preproenkephalin-B, a molecular marker of LID. Moreover, functional binding analysis showed that the striatal dopaminergic response was reduced following 28 days and 7 days RGS4 protein inhibition, suggesting modulated G-protein function.

4.4.1 Methodological considerations

*Behavioural and molecular assessments of the unilateral 6-OHDA-lesioned rat model*

In this study, the unilateral 6-OHDA-lesioned rat model of PD was used to investigate the effects of 28 days RGS4 protein inhibitor treatment on L-DOPA priming in LID. The behavioural motor deficits induced following unilateral 6-OHDA-lesion were measured using the rotord and cylinder tests (Rozas et al., 1997; Tillerson et al., 2001). These motor performance scores were used to match animals into groups of equal motor ability prior to repeated drug treatment (Mela et al., 2007; Kobylecki et al., 2010). A low dose of L-DOPA (6 mg/kg), which was within the dose range (0.71-17.14 mg/kg) for treatment in PD patients (Rascol et al., 2000), was repeatedly administered to induce AIMs in 6-OHDA-lesioned rats (Cenci et al., 1998; Lundblad et al., 2002). As discussed in Chapter 3, repeated intermittent L-DOPA treatment causes induction of stereotypical, purposeless AIMs and up-regulation of associated molecular markers of dyskinesia (Cenci et al., 1998; Winkler et al., 2002). Such behavioural and molecular correlates have been used to measure the anti-dyskinetic (Lundblad et al., 2002; Dekundy et al., 2007) and anti-priming effects of potential novel treatment therapies (Mela et al., 2007; Rylander et al., 2009; Kobylecki et al., 2010). We adopted a de novo treatment plan using RGS4 protein inhibitors, or an RGS4 antisense oligonucleotide, in combination with L-DOPA, to investigate the effects on L-DOPA priming (Mela et al., 2007; Rylander et al., 2009; Kobylecki et al., 2010). As discussed in Chapter 3, we used an antisense oligonucleotide to cause knockdown of RGS4 mRNA, which inhibits RGS4 protein function (Garzon et al., 2001). The control antisense oligonucleotide contained the same bases as the RGS4 antisense sequence but in a randomised order. This sequence did not code for any documented gene, in the rat genome, at the time of this experiment. The doses (0.1 mg/kg) of RGS4 protein inhibitors, CCG-2046 and CCG-63802, were chosen from behavioural
studies in Chapter 3, which were the most effective doses at reducing AIMs. We extended L-DOPA treatment to 28 days, to investigate the effects of 7 days RGS4 protein inhibition on rats primed with L-DOPA. The rotorod test was conducted throughout the 28 days and 7 days drug treatment periods to measure the effect of drug on general motor ability. AIMs were also repeatedly assessed to ensure the anti-dyskinetic effects of repeated drug treatment could be maintained. The behavioural correlate of L-DOPA priming was measured with AIMs assessment, following an L-DOPA challenge, 24 h after the final treatment day (Mela et al., 2007). Animals were killed a further 24 h post final administration, to allow the acute effects of L-DOPA treatment on second messenger signalling to resolve (Westin et al., 2007).

To investigate the effects of 28 days and 7 days RGS4 protein inhibition on L-DOPA priming at the molecular level, we used in situ hybridisation to measure the expression of striatal opioid precursors, PPE-A and PPE-B. These opioid precursors have been characterised as molecular correlates of PD and LID (Cenci et al., 1998; Henry et al., 1998; Henry et al., 2003), and are commonly used to indicate the abnormal activity of the two main striatofugal pathways (Gerfen et al., 1990). Striatal PPE-A mRNA is localised to the indirect pathway of the basal ganglia (Gerfen, 1992b) and becomes elevated following 6-OHDA-lesion (Henry et al., 1999; Ravenscroft et al., 2004). Striatal PPE-A mRNA levels are further up-regulated following induction of dyskinesia in PD patients and animal models (Cenci et al., 1998; Calon et al., 2002). In contrast, striatal PPE-B mRNA, or cleaved opioid product prodynorphin, is localised to the direct pathway (Gerfen et al., 1992b) and is reduced in the parkinsonian state (Gerfen et al., 1991; Cenci et al., 1998; Henry et al., 1999). Following repeated L-DOPA treatment and induction of dyskinesia, striatal PPE-B mRNA levels become overexpressed (Cenci et al., 1998; Henry et al., 1999; Henry et al., 2003) and remain elevated after L-DOPA treatment is discontinued (Andersson et al., 2003). Thus, up-regulation of PPE-B mRNA is used as the primary indicator for L-DOPA priming i.e. the induction of LID (Mela et al., 2007; Rylander et al., 2009; Kobylecki et al., 2010). To further analyse the effects of 28 days and 7 days RGS4 protein inhibition at the molecular level, we measured the functional changes of dopaminergic signalling in the striatum. Since there has been discrepancies in data on striatal dopamine receptor binding in L-DOPA-treated PD patients (Rinne et al., 1991; Turjanksi et al., 1997) and animal models of LID (Gerfen et al., 1990; Konradi et al., 2004;
Visjani et al., 2009), we used $[^{35}\text{S}]$GTPγS autoradiography to analyse dopamine receptor supersensitivity, which is a pathophysiological feature of LID (Aubert et al., 2005).

### 4.4.2 Behavioural and molecular effects of 28 days and 7 days RGS4 protein inhibitor treatment in PD and LID

The effects of RGS4 mRNA knockdown, 28 day and 7 days RGS4 protein inhibition, in combination with L-DOPA treatment, in the unilateral 6-OHDA-lesioned rat model of PD and LID are summarised in Table 8.

<table>
<thead>
<tr>
<th>28 days treatment</th>
<th>ALO AIMS</th>
<th>Lo AIMS</th>
<th>RR</th>
<th>PPE-A</th>
<th>PPE-B</th>
<th>DA stimulated $[^{35}\text{S}]$GTPγS binding</th>
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<tr>
<td>RGS4 antisense (pre-treatment)</td>
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<td>↓↓↓</td>
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<td>CCG-2046 (de novo treatment)</td>
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<td>CCG-63802 (de novo treatment)</td>
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<table>
<thead>
<tr>
<th>7 days treatment</th>
<th>ALO AIMS</th>
<th>Lo AIMS</th>
<th>RR</th>
<th>PPE-A</th>
<th>PPE-B</th>
<th>DA stimulated $[^{35}\text{S}]$GTPγS binding</th>
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<tr>
<td>CCG-2046</td>
<td>↓</td>
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<td>CCG-63802</td>
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**Table 8.** The effects of RGS4 protein inhibitors in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease and L-DOPA-induced dyskinesia. The abnormal involuntary movements (AIMs) scores were recorded following L-DOPA challenge after RGS4 mRNA knockdown, 28 day or 7 days RGS4 protein inhibitor treatment. Behavioural effects were compared to vehicle + L-DOPA/benserazide treated animals. Molecular effects on mRNA levels in the operated side of the whole rostral striatum were compared to vehicle + L-DOPA/benserazide treated animals. Arrows represent increased or decreased magnitude of change; ↔, no change; RR, rotord performance; ALO, sum of axial/limb/orolingual; Lo, locomotor; PPE-A, preproenkephalin-A; PPE-B, preproenkephalin-B.
**RGS4 mRNA knockdown in experimental parkinsonism reduced motor ability**

Continuous infusion of an RGS4 antisense oligonucleotide into the lesioned striatum of unilateral 6-OHDA-lesioned rats caused a gradual reduction in overall rotorod performance. Moreover, when combined with L-DOPA, overall rotorod performance was reduced in RGS4 antisense-treated compared to control-treated rats on day 2 of L-DOPA treatment.

The results from this study are consistent with previous data that demonstrated RGS4 proteins have a direct role in motor behaviours (Grillet et al., 2005; Lerner and Kreitzer, 2012). RGS4 is predominantly expressed in the lateral striatum (Geurts et al., 2002), which corresponds to the sensorimotor regions (McGeorge and Faull, 1969), supporting the role of RGS4 proteins in movement. However, a specific mechanism of action remains to be established. In a recent study, RGS4 proteins were shown to mediate bi-directional control of motor behaviours in amphetamine-treated rats (Schwendt et al., 2011), while an earlier study found that RGS4 knockout mice displayed sensorimotor deficits from tests conducted on the rotorod (Grillet et al., 2005). Our results show that knockdown of striatal RGS4 mRNA reduces motor function and inhibits the initial behavioural response to L-DOPA in unilateral 6-OHDA-lesioned rats. In contrast, previous studies have suggested that RGS4 protein inhibition may improve motor symptoms in PD (Ding et al., 2006; Lerner and Kreitzer, 2012). In one study, it was suggested that RGS4 proteins in striatal intercholinergic neurons may be a therapeutic target in PD (Ding et al., 2006). Inhibition of RGS4 proteins in these neurons could potentially alleviate the inhibition of M₄ cholinergic autoreceptors, leading to reduced acetylcholine and improved motor function (Ding et al., 2006). However, this predicted motor improvement may be masked given the wide range of GPCRs expressed in the striatum that are modulated by RGS4 proteins (Ghavami et al., 2004; Ho et al., 2007; Han et al., 2010; Schwendt et al., 2011). In contrast to our results, a more recent study showed that unilateral 6-OHDA-lesioned RGS4 knockout mice displayed improved balance and increased distance travelled, when compared to unilateral 6-OHDA-lesioned wild-type mice (Lerner and Kreitzer, 2012). These discrepancies in results may be due to the different behavioural tests and models used. In the aforementioned study, foot slips of mice were counted from a balance beam test, while we employed the rotorod test which is commonly used to assess motor performance in rodent models of PD (Rozas et al., 1997; Lundblad et al., 2003; Dekundy et al., 2007; Rylander et al., 2009). Furthermore, RGS4 knockout mice may yield developmental changes of neurons
in different brain areas affecting normal physiological function (Grillet et al., 2003; Ebert et al., 2006).

Previous studies have shown contrasting data into the roles of RGS4 proteins in dopamine receptor signalling (Ghavami et al., 2004; Taymans et al., 2004; Ho et al., 2007). Although not via direct modulation of G_\text{ai/o} subunits (Ghavami et al., 2004), RGS4 proteins can modulate mechanisms downstream of dopamine D_2 receptor signalling (Ho et al., 2007; Lerner and Kreitzer, 2012). A recent study, using RGS4 protein inhibitor, CCG-63802, found that RGS4 proteins regulate LTD in medium spiny neurons of the indirect pathway (Lerner and Kreitzer, 2012). It is possible that chronic RGS4 mRNA knockdown caused functional changes in dopaminergic signalling mechanisms, leading to a reduced anti-parkinsonian response to L-DOPA. However, such potential hindrance on the anti-parkinsonian actions of L-DOPA would be more accurately assessed in MPTP-lesioned NHPs, where these behavioural responses are more clearly displayed.

**RGS4 mRNA knockdown or 28 days treatment with RGS4 protein inhibitors reduced L-DOPA priming and subsequent induction of AIMs**

Continuous infusion of an RGS4 antisense oligonucleotide into the lesioned striatum, or 28 days treatment with RGS4 protein inhibitors, reduced the expression of AIMs from the first day of L-DOPA treatment in unilateral 6-OHDA-lesioned rats. Moreover, induction of AIMs was reduced following repeated treatment with an RGS4 antisense oligonucleotide or RGS4 protein inhibitors, CCG-2046 or CCG-63802. 28 days treatment with CCG-63802, but not CCG-2046, in combination with L-DOPA increased both peak and average rotorod performance, compared to vehicle-treated unilateral 6-OHDA-lesioned rats.

The behavioural data presented in this study, further supports the role of RGS4 proteins in the pathophysiology of LID. These behavioural effects of RGS4 protein inhibition may be caused by modulation of different GPCR signalling pathways in the striatum, which is one of the main neuroanatomical sites where maladaptive changes occur in LID (Cenci and Konradi, 2010). For example, the up-regulation of striatal prodynorphin is a well characterised molecular marker of LID (Cenci et al., 1998; Henry et al., 1999; Henry et al., 2003) and is associated with increased dopamine D_1R signalling in striatonigral neurons (St-Hilaire et al., 2005). In the striatum, prodynorphin is co-expressed with µ-opioid receptors (Guttenberg et al., 1996). These receptors become sensitised in the striatum of
dyskinetic MPTP-lesioned NHPs (Chen et al., 2005), suggesting hyperactivation of opioid transmission in LID (Henry et al., 2001; Aubert et al., 2007; Koprich et al., 2011). In vitro studies have shown that RGS4 proteins can form protein scaffold complexes with μ-opioid receptors (Georgoussi et al., 2006; Leontiadis et al., 2009), which has been suggested to mediate roles in opioid addiction (Bishop et al., 2002; Gold et al., 2003; Han et al., 2010). Interestingly, striatal RGS4 proteins can mediate positive modulatory responses to opioid agonists in vivo, which are dependent on the different Gα subunit complexes formed at the μ-opioid receptor (Han et al., 2010). Thus, increased striatal RGS4 expression (see Chapter 2) may contribute to the sensitisation of striatal μ-opioid receptors in LID (Chen et al., 2005) and by blocking RGS4 protein function, it could attenuate μ-opioid receptor signalling, subsequently reducing dyskinesia (Henry et al., 2001; Koprich et al., 2011). However, further studies would be needed to confirm this hypothesis.

In vivo and in vitro studies have shown that RGS4 proteins negatively modulate 5-HT1A receptors (Beyer et al., 2004; Ghavami et al., 2004). This endogenous role of RGS4 may have functional significance in LID. For example, clinical and preclinical studies have shown that increased 5-HT1A signalling, via administration of 5-HT1A receptor agonists, reduces LID (Bonifati et al., 1994; Pierelli et al., 1998; Bibbani et al., 2001; Carta et al., 2007; Dupre et al., 2007; Eskow et al., 2007). These effects are partly mediated by activation of presynaptic 5-HT1A autoreceptors in the dorsal raphe nucleus, which reduce unregulated dopamine efflux in the striatum (Carta et al., 2008; Lindgren et al., 2010). The RGS4 proteins in the dorsal raphe nucleus can block 5-HT1A-mediated modulation of neurotransmitter release in the striatum (Beyer et al., 2004). Thus, blockade of RGS4 proteins in the dorsal raphe nucleus would increase 5-HT1A signalling, leading to more regulated striatal dopamine efflux. These effects would be expected to reduce the development of dyskinesia (Stocchi et al., 2005; Olanow et al., 2006). However, we showed that specific inhibition of RGS4 proteins in the striatum can also reduce AIMS (Chapter 3). It is possible that RGS4 proteins negatively modulate 5-HT1A receptors that exist in the striatum (Maraziti et al., 1994; Frechilla et al., 2001; Huot et al., 2012). This specific population of 5-HT1A receptors are suggested to act as presynaptic heteroreceptors on glutamatergic corticostriatal terminals (Pasqualetti et al., 1996; Frechilla et al., 2001) and become increased following induction of LID (Huot et al., 2012). Specific activation of striatal 5-HT1A receptors reduced AIMS in the unilateral 6-OHDA-lesioned rat model of LID (Bishop et al., 2009; Dupre et al., 2011), by reducing local glutamate levels and
NMDA glutamate receptor signalling (Dupre et al., 2008b; Dupre et al., 2011). Thus, it is possible that blocking striatal RGS4 proteins leads to increased 5-HT$_{1A}$ signalling and, in turn, reduces the excessive corticostriatal glutamatergic transmission in LID (Robelet et al., 2004; Dupre et al., 2011). Indeed, electrophysiological data in prefrontal cortical neurons has shown that blockade of RGS4 proteins can potentiate 5-HT$_{1A}$-mediated inhibition on NMDA glutamate receptors (Gu et al., 2007). Thus, chronic treatment with RGS4 inhibitors may have reduced the induction of AIMs in L-DOPA-treated unilateral 6-OHDA-lesioned rats by inhibition of excessive glutamatergic signalling during L-DOPA priming.

**Repeated RGS4 protein inhibition reduces the molecular correlates of LID**

Continuous infusion of an RGS4 antisense oligonucleotide into the lesioned striatum, or 28 days treatment with RGS4 protein inhibitors, reduced the up-regulation of PPE-B, but not PPE-A, mRNA in the striatum of L-DOPA-treated unilateral 6-OHDA-lesioned rats. However, there were no significant effects of 7 days treatment with RGS4 protein inhibitors on striatal PPE-A or PPE-B mRNA in unilateral 6-OHDA-lesioned rats primed with L-DOPA.

The data presented in this study support the role of RGS4 proteins in the induction of LID. Consistent with previous studies, repeated L-DOPA treatment in unilateral 6-OHDA-lesioned rats elevated striatal opioid mRNA expression levels (Cenci et al., 1998; Henry et al., 1999; Ravenscroft et al., 2004). Interestingly, the effects on striatal opioid precursor mRNA levels following 28 day RGS4 protein inhibition are similar to those found following repeated de novo treatment with mGluR5 antagonist, MTEP, in combination with L-DOPA, in unilateral 6-OHDA-lesioned rats (reduced up-regulation of striatal PPE-B mRNA but no effect on PPE-A mRNA) (Mela et al., 2007). Striatal mGluR5 receptors have an established pathophysiological role in the expression and development of LID. In dyskinetic PD patients (Ouattara et al., 2011) and dyskinetic MPTP-lesioned NHPs (Samadi et al., 2008), mGluR5 receptor binding is increased in the putamen, while treatment with mGluR5 receptor antagonists reduced dyskinesia in animal models of LID (Mela et al., 2007; Levandis et al., 2008; Rylander et al., 2009; Johnston et al., 2010; Morin et al., 2010; Rylander et al., 2010a). Moreover, inhibition of mGluR5 receptors decreases the expression of molecular correlates of dyskinesia, such as ΔfosB, prodynorphin and phosphorylated ERK1/2 (Mela et al., 2007; Levandis et al., 2008; Rylander et al., 2009).
The mGluR5 receptors signal via $G_{aq}$ subunits, which activate phospholipase C (PLC)-β, and subsequently lead to calcium release from intracellular stores (Ribeiro et al., 2010). This signalling pathway activates ERK1/2 (Mao et al., 2005; Ronesi and Huber, 2008), which regulates striatal gene expression (Choe and Wang, 2001), and plays a fundamental role in the development of LID (Santini et al., 2007; Westin et al., 2007; Calabresi et al., 2008). Striatal RGS4 proteins directly interact with mGluR5 receptors (Schwendt and McGinty, 2007; Schwendt et al., 2011) and antagonise $G_{aq}$-activated subunits that couple to these receptors (Saugstad et al., 1998). Moreover, RGS4 proteins fine tune $G_{aq}$ subunit mediated intracellular calcium signals (Xu et al., 1999; Luo et al., 2001), by binding scaffold proteins such as Homer 2 (Hollinger and Hepler, 2002; Shin et al., 2003) and/or by forming a stable ternary complex with the GPCR (Sierra et al., 2000). Such functions of RGS4 proteins on Ca$^{2+}$ signalling is thought to be regulated by second messenger signalling molecules, such as PIP3 and Ca$^{2+}$/CAMK (Luo et al., 2001; Hollinger and Hepler, 2002). These endogenous signalling factors bind to sites within the RGS domain (Popov et al., 2000; Ishii et al., 2005), while the RGS4 protein inhibitors used in this study compete for these sites (Blazer et al., 2010) and would be expected to disrupt protein scaffold formation, IP3-mediated calcium signalling and ERK1/2 signalling cascade. Thus, it is possible that the anti-priming effects induced by repeated RGS4 protein blockade in L-DOPA-treated unilateral 6-OHDA-lesioned rats are mediated downstream of mGluR5 receptors, by disrupting the activation of ERK1/2. Given that 7 day treatment with RGS4 protein inhibitors had no effect on striatal PPE-A or PPE-B mRNA levels, RGS4 protein inhibition is likely to mediate inhibition of striatal gene expression during development of, rather than following induction of, LID, which is when synaptic plasticity-induced changes occur in the striatum (Cenci and Konradi, 2010).

**Potential effects of repeated RGS4 protein inhibition on abnormal synaptic plasticity in LID**

Repeated RGS4 inhibitor treatment, in combination with L-DOPA, reduced the response to dopamine, with decreased activation of G-protein subunits in the striatum.

The sensitisation of striatal dopamine receptors is recognised as a major factor in the development of LID (Nutt, 1990). *In vitro* GTPγS binding studies have shown that increased G-protein coupling occurs at striatal dopamine $D_1$ and $D_2$ receptors following
dopamine denervation (Geurts et al., 1999; Cai et al., 2002; Aubert et al., 2005). In LID, increased response to dopamine D₁R agonists (Aubert et al., 2005), may be attributed to increased G_{olf} subunits that couple to dopamine D₁Rs. The expression levels of these G_{a} subunits are increased in PD and LID (Herve et al., 1993; Corvol et al., 2004). Thus, following dopamine receptor stimulation, exaggerated activation of downstream signalling pathways, particularly via adenylate cyclase (Pifl et al., 1992a), leads to increased PKA-mediated phosphorylation of protein targets, such as NMDA receptors and DARPP-32 (Konradi et al., 1996; Cepeda and Levine, 1998; Dudman et al., 2003; Picconi et al., 2003; Cepeda and Levine 2006; Santini et al., 2007). Abnormal RGS4 protein function during the development of LID is likely to be mediated through this signalling pathway. In the normal striatum, the opposing effects of dopamine D₁ and D₂ receptor on adenylate cyclase regulate RGS4 mRNA (Geurts et al., 2002; Taymans et al., 2003), while PKA mediates phosphorylation of RGS4 proteins (Huang et al., 2007). Proteins phosphorylated by PKA are important in neuronal plasticity (Schulman, 1995), and many of these proteins mediate major pathophysiological changes in LID (Cenci and Konradi, 2010). Although, PKA-mediated phosphorylated CREB up-regulates prodynorphin mRNA in the intact striatum (Cole et al., 1995), there is transition of gene regulation in LID (Andersson et al., 2001). For example, during L-DOPA priming in PD, ΔfosB-related proteins and JunD bind CRE/AP-1 elements in the prodynorphin promoter region and up-regulate prodynorphin mRNA (Andersson et al., 2001). The RGS4 gene contains CRE/AP-1 elements (Zhang et al., 2005), which could activate its role in the induction of LID. We propose that blockade of RGS4 proteins during L-DOPA priming alleviates feed-back control on GPCR signalling events, which, in turn, dampens certain plasticity-induced changes that occur during the induction of LID.

Abnormal activity-dependent synaptic plasticity is a characterised feature of LID (Picconi et al., 2003; Morgante et al., 2006; Belujon et al., 2010). Electrophysiological data has revealed that there is loss of regulation of LTD in L-DOPA-treated unilateral 6-OHDA-lesioned rats (Belujon et al., 2010). Moreover, a recent study has shown that there is a transition of cortically-induced LTD to LTP, in striatopallidal neurons of unilateral 6-OHDA-lesioned rats chronically treated with L-DOPA (Belujon et al., 2010). Abnormal synaptic plasticity, which is found in dyskinetic PD patients (Morgante et al., 2006), is likely to disrupt motor learning and control (Wiecki and Frank, 2010), leading to the inability to erase irrelevant motor commands (Picconi et al., 2003). The synaptic plasticity
of corticostriatal synapses is dependent on downstream signalling factors, such as DARPP-32 (Calabresi et al., 2000b), that are activated by different GPCRs. These particular GPCRs tend to modulate dopaminergic signalling (Schwarzschild et al., 2006; Cheer et al., 2007). For example, adenosine A2a receptors form heterotrimeric complexes with dopamine D2 receptors in striatopallidal neurons (Morelli et al., 2007) and activate G\textsubscript{olf} subunits (Kull et al., 2000). This signalling pathway is similar to that of dopamine D1R, leading to PKA-mediated activation of DARPP-32 (Svenningsson et al., 2000b; Hakansson et al., 2004), which opposes the dopamine D2R signalling pathway. While endocannabinoids (ECB), which are released following dopamine D2R activation, mediate LTD of corticostriatal synapses through modulation of glutamatergic signals (Gerdeman and Lovinger, 2003; Lerner and Kreitzer, 2012). In a recent study, RGS4 proteins were found to play a fundamental role in LTD in striatopallidal neurons (Lerner and Kreitzer, 2012). RGS4 proteins, which are controlled by PKA downstream of dopamine D2 and adenosine A2a receptors, modulate the calcium signalling induced by mGluR group I (mGluR 1 and 5) receptors in ECB-induced LTD (Lerner and Kreitzer, 2012). Release of intracellular calcium leads to activation of calcium-dependent protein kinases required for striatal LTD (Calabresi et al., 1994; Bonsi et al., 2003). The RGS4 proteins appear to be a key link in converging downstream signalling pathways for LTD. Indeed, the addition of recombinant RGS4 protein blocked LTD in striatopallidal neurons (Lerner and Kreitzer, 2012). Moreover, loss of LTD following unilateral 6-OHDA-lesion in wild-type mice was not seen in RGS4 knockout mice (Lerner and Kreitzer, 2012). These data suggest that RGS4 proteins block LTD, which may have functional relevance in LID. We found that RGS4 is up-regulated in LID (Chapter 2), which could mediate loss of LTD in corticostriatal synapses in dyskinesia (Belujon et al., 2010). It is possible that by blocking RGS4 proteins during L-DOPA priming, reduces the loss of LTD in corticostriatal synapses, allowing removal of redundant motor sequences that underlie the development of LID (Picconi et al., 2003; Pisani et al., 2005). However, future studies using electrophysiological techniques would be needed to confirm this hypothesis.

**Conclusion**

Repeated *de novo* treatment with RGS4 protein inhibitors, in combination with L-DOPA, reduced the behavioural and molecular correlates of LID in the unilateral 6-OHDA-lesioned rat model of PD. These data support the role of RGS4 proteins in L-DOPA priming in LID. Thus, blocking RGS4 proteins in combination with L-DOPA treatment in
PD could reduce the induction of LID. RGS4 proteins may be a therapeutic target for future treatments in LID.
Chapter 5
Mechanisms of action of RGS4 protein inhibitors in the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia
5.1 Introduction

Long-term L-DOPA treatment in PD leads to severe motor complications such as LID (Cotzias et al., 1969). Although the underlying cause of LID remains unknown, certain pathophysiological features have been well characterised. For example, the expression of LID in PD patients has been associated with non-physiological, transient fluctuation of striatal dopamine levels (de la Fuente-Fernandez et al., 2004; Pavese et al., 2006; Troiano et al., 2009). These results have been replicated in the unilateral 6-OHDA-lesioned rat model of LID, where large transient extracellular dopamine effluxes, in both the striatum and SN, show a temporal and quantitative relationship with the expression of AIMs (Lindgren et al., 2010).

The large transient fluctuations of dopamine release in PD are attributed to raphe-striatal serotonergic neurons (Cenci and Lundblad, 2006; Carta et al., 2008; Carta and Bezard, 2011), which can catabolise, store and release dopamine into the striatum (Tison et al., 1991; Arai et al., 1994; Arai et al., 1995; Arai et al., 1996). However, these neurons lack proper autoregulatory function for dopamine transmission, resulting in unregulated efflux (Cenci and Lundblad, 2006; Carta et al., 2008; Carta and Bezard, 2011). In vivo microdialysis studies have shown that striatal dopamine efflux is attenuated, with a concomitant reduction in AIMs, following combined administration of 5-HT\textsubscript{1A/1B} agonists in L-DOPA-treated unilateral 6-OHDA-lesioned rats (Lindgren et al., 2010). These effects are, at least partly, caused by activation of autoreceptors on serotonergic neurons, which, in turn, dampens striatal dopamine release (Cenci and Lundblad, 2006; Carta et al., 2008; Carta and Bezard, 2011).

Another important non-dopaminergic system involved in the pathophysiology of LID is the glutamatergic system (Bezard et al., 2001a; Cenci and Lundblad, 2006; Calabresi et al., 2008; Cenci and Konradi, 2010). In vivo microdialysis studies have shown excessive glutamate release in the striatum following L-DOPA treatment in unilateral 6-OHDA-lesioned rats (Robelet et al., 2004; Dupre et al., 2011). Moreover, following induction of LID there is increased striatal expression of ionotropic glutamate receptor subunits, of both AMPA (Silverdale et al., 2010; Ba et al., 2011) and NMDA receptors (Oh et al., 1998; Hallet et al., 2005; Hurley et al., 2005; Gardoni et al., 2006). Preclinical and clinical studies have shown that attenuation of excessive corticostriatal glutamatergic transmission leads to a reduction of LID. For example, antagonism of NMDA receptors with non-selective
NMDA receptor antagonist amantadine, effectively reduces dyskinesia in animal models of LID (Dekundy et al., 2007; Kobylecki et al., 2011) and PD patients (Metman et al., 1999; Luginger et al., 2000). It is suggested that there is an intimate cross-talk between the non-dopaminergic systems in LID. For example, 5-HT$_{1A}$ receptors are found at low levels in the striatum (Mengod et al., 1996; Pasqualetti et al., 1996), where they are thought to be expressed on presynaptic corticostriatal terminals (Dupre et al., 2011; Huot et al., 2012). Following 5-HT$_{1A}$ agonist treatment, extracellular glutamate release is reduced in the striatum (Antonelli et al., 2005; Mignon and Wolf, 2005; Dupre et al., 2011), which can contribute to an anti-dyskinetic effect (Dupre et al., 2011; Huot and Brotchie, 2011).

The 5-HT$_{1A}$ receptors are GPCRs and are regulated by certain endogenous (RGS) proteins. The RGS4 protein subtype negatively modulates the $G_{\alpha}$ subunit of 5-HT$_{1A}$ receptors (Ghavami et al., 2004). With regard to serotonergic neurons, the functional implications of this cellular regulation are that RGS4 proteins can positively modulate neurotransmitter release, which has been demonstrated in raphe-striatal afferents (Beyer et al., 2004). Moreover, RGS proteins can directly impact specific GPCR signalling mechanisms. For example, in an electrophysiological study conducted in rat prefrontal cortical neurons, specific blockade of RGS4 proteins potentiated 5-HT$_{1A}$ receptor-mediated inhibition of NMDA receptor-induced excitatory postsynaptic currents (Gu et al., 2007). These interdependent mechanisms involving RGS4 protein regulation on serotonergic and glutamatergic signalling may have important implications in the pathophysiology of LID.

In Chapters 3 and 4, we reported that acute and repeated treatment with RGS4 protein inhibitors attenuated expression and induction of AIMs in the unilateral 6-OHDA-lesioned rat model of LID. However, the mechanism of action of RGS4 protein inhibitors on attenuating AIMs remains unknown.

5.1.1 Aims and objectives

We hypothesize that RGS4 proteins mediate roles in LID via a common signalling pathway which involves 5-HT$_{1A}$ and NMDA receptors. Using the unilateral 6-OHDA-lesioned rat model of LID, we aim to:

1) Test whether RGS4 protein inhibitors mediate anti-dyskinetic effects via 5-HT$_{1A}$ receptors.
2) Characterise the acute synergistic effects of subthreshold RGS4 protein inhibitor treatment combined with a 5-HT$_{1A}$ agonist, or NMDA receptor antagonist, on AIMs and rotorod performance.

3) Characterise neurotransmitter release in basal ganglia sub-regions (striatum, GPI and SNr) following acute RGS4 protein inhibitor treatment.
5.2 Materials and Methods
All animal work was carried out under the regulations of the Animals (Scientific Procedures) Act, 1986.

5.2.1 Animals
Animals were housed in husbandry conditions as described in Chapter 2 (2.2.1).

Unilateral 6-hydroxydopamine lesion of the right MFB
Male Sprague-Dawley rats received unilateral 6-OHDA-lesion of the right MFB as described in Chapter 2 (2.2.1).

5.2.2 Drug treatment
Drugs were administered systemically into the i.p. cavity at a volume of 1 ml/kg, unless otherwise stated.

L-DOPA/benserazide
L-DOPA methyl ester hydrochloride (D1507, Sigma-Aldrich, UK) and benserazide hydrochloride (B7283, Sigma-Aldrich, UK) were dissolved in sterile saline (0.9% w/v; Braun AG, Germany).

L-DOPA priming began four to five weeks post-lesion. Animals received one daily injection of L-DOPA/benserazide (6/15 mg/kg; i.p.) between 10:00 - 11:00 am for 21 days. Following L-DOPA priming, animals received two L-DOPA/benserazide injections per week to maintain AIMs scores for behavioural studies (Lee et al., 2000; Dekundy et al., 2007).

RGS4 protein inhibitors
RGS4-G\textsubscript{ao} interaction inhibitors, CCG-2046 (Tocris Bioscience, Bristol, UK) and CCG-4986 (Chembridge, USA), were dissolved in 0.4% (v/v) ethanol-sterile saline (0.9%, w/v) to a final concentration of 2 and 0.2 mM, respectively, for microinjection studies. A total volume of 10 µl was administered through an implanted cannula immediately prior to L-DOPA/benserazide injection as fully in Chapter 3 (3.2.3).
Novel reversible RGS4 inhibitor, CCG-63802 was synthesised by Sequoia Research Products Ltd. RGS4 protein inhibitors were dissolved in 10% (v/v) DMSO-sterile saline (0.9%, w/v) at 0.003, 0.01 and 0.1 mg/ml for systemic (i.p.) administration. RGS4 protein inhibitors were administered 30 min prior to L-DOPA/benserazide injection.

**Amantadine**
Adamantan-1-amine (amantadine) hydrochloride (A1260, Sigma-Aldrich, UK) was dissolved in sterile saline (0.9%, w/v) at 5, 10 and 20 mg/ml and administered (s.c.) 100 min prior to L-DOPA/benserazide injection.

**8-OH-DPAT**
(±)-8-Hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) hydrobromide (H8520, Sigma-Aldrich, UK) was dissolved in sterile saline (0.9%, w/v) at 0.03, 0.1 and 0.3 mg/ml and administered 5 min after L-DOPA/benserazide injection.

**WAY-100635**
[2-(4-(2-methoxyphenyl)-1-piperaziny-1]ethyl]-N-2-pyridinylecyclohexanecarboxamide (WAY-100635) maleate salt (W108, Sigma-Aldrich, UK) was dissolved in sterile saline (0.9%, w/v) at 0.5 mg/ml and administered 5 min prior to L-DOPA/benserazide injection.

5.2.3 **Behavioural analysis**

**Cylinder test**
The cylinder test was conducted at three weeks post-lesion as described in Chapter 2 (2.2.3).

**Assessment of AIMs**
The subtypes of AIMs; axial, limb, orolingual and locomotor, were scored as described in Chapter 2 (2.2.3) and Chapter 3 (3.2.3). The sum of ALO was calculated over the 3 h testing period and used for statistical analyses.

**Rotorod training and testing**
At two weeks post-lesion, animals were rotorod trained and tested, as fully described in Chapter 3 (3.2.3). Briefly, the effect of L-DOPA/benserazide + drug treatment on motor performance was evaluated using the acceleration trial (4 to 40 r.p.m), every 30 min for 3 h.
post L-DOPA/benserazide administration. Each trial run lasted a maximum of 5 min. Rotorod performance following vehicle only was used as a baseline score.

Average group rotorod performance over 3 h was used for statistical analyses. ‘Peak’ rotorod performance, the rotorod score at 1 h post L-DOPA treatment, was analysed separately to assess the effect of drug on the L-DOPA-induced anti-akinetic response (Dekundy et al., 2007; Kobylecki et al., 2011). All rotorod assessments were carried out between 8:00 am and 5:00 pm.

5.2.4 Experiment 1: Behavioural pharmacology of RGS4 protein inhibitors

Systemic administration of RGS4 protein inhibitors

The same cross over design study from Chapter 3 (3.2.4 ‘systemic administration of RGS4 protein inhibitors’) (Figure 35) was used to test the behavioural effects of subthreshold treatment of RGS4 protein inhibitors combined with established anti-dyskinetic compounds, in L-DOPA primed unilateral 6-OHDA-lesioned rats. A subthreshold dose of RGS4 protein inhibitor, in combination with a subthreshold dose of 8-OH-DPAT or amantadine, was administered with L-DOPA/benserazide (6/15 mg/kg; i.p.) to investigate potential synergistic effects. AIMs and rotorod performances were assessed following treatment with the same dose of compound on separate days, with a minimum of two days between each assessment. A minimum of one week was left between testing different compounds. Animals were kept for up to 1 year for completion of all behavioural tests.

Microinjection of RGS4 protein inhibitors into the lateral striatum

The same cross over design study from Chapter 3 (3.2.4 ‘microinjection of RGS4 protein inhibitors into the lateral striatum’) (Figure 35) was used to further investigate the specific effects of RGS4 protein inhibition in the striatum. One group of L-DOPA-primed unilateral 6-OHDA-lesioned rats received cannula implantation in the lateral rostral striatum as fully described in Chapter 3 (3.2.3). Baseline AIMs scores were re-tested two weeks post-cannulation to ensure no significant behavioural effects were caused by surgery.

Acute behavioural effects of CCG-2046 and CCG-4986 via microinjection

Manual injections of CCG-2046 or CCG-4986 were made through a 30 G, 12 mm long, needle inserted into the cannula targeting the right rostral striatum as described in Chapter 3 (3.2.3). To test for potential 5-HT1A receptor dependent effects of RGS4 protein
inhibition, AIMs were assessed following the acute administration of RGS4 protein inhibitor + L-DOPA/benserazide in combination with 5-HT\textsubscript{1A} receptor antagonist, WAY-100635 in the cross over design study described in Chapter 3 (3.2.4) (Figure 35). There was minimum of one week between testing each RGS4 protein inhibitor. Animals were kept for up to 1 year for completion of all behavioural tests.

**Validation of cannula injection site**

Validation of the cannula injection site was carried out as described in Chapter 3 (3.2.4). Animals with cannula sites outside the striatum were removed from all analyses.

**5.2.5 Experiment 2: Triple probe microdialysis**

The timeline for experimental investigation into the acute effects of RGS4 protein inhibitor treatment on neurotransmitter release in basal ganglia sub-regions is shown in Figure 106. Unilateral 6-OHDA-lesioned rats treated with L-DOPA/benserazide (6/15 mg/kg; i.p.) for 21 days were matched into three equal groups by their cylinder test and AIMs (L-DOPA treatment day 21) scores and allocated into the following acute treatment groups (i) L-DOPA/benserazide (6/15 mg/kg; i.p.) + vehicle (10% (v/v) DMSO-sterile saline) (n=4), (ii) L-DOPA/benserazide (6/15 mg/kg; i.p.) + CCG-2046 (0.1 mg/kg; i.p.) (n=4), and (iii) L-DOPA/benserazide (6/15 mg/kg; i.p.) + CCG-63802 (0.1 mg/kg; i.p.) (n=4) for in vivo microdialysis.

On the day of microdialysis, CMA 12 microdialysis probes (8010433, Linton instrumentation, UK) were connected to a microdialysis syringe pump system (CMA100, Sweden) via fluorinated ethylene propylene (FEP) tubing (inner diameter 0.12 mm, Linton instrumentation, UK). Tubing adaptors (3409500, Linton instrumentation, UK) were used to prevent any leakages between connections. The microdialysis system, composed of three 2.5 ml syringes filled with artificial CNS perfusion fluid (NaCl 147 mmol/l, KCl 2.7 mmol/l, CaCl\textsubscript{2} 1.2 mmol/l, MgCl\textsubscript{2} 0.85 mmol/l; product code: P000151, Linton instrumentation, UK), was set at a flow rate of 1 µl/min. CMA 12 microdialysis probes were rinsed with perfusion fluid and free flow was ensured prior to implantation. All microdialysis probes were used once to ensure the probe efficiency was not compromised following repeated use.
Unilateral 6-OHDA-lesioned rats were anaesthetised with gaseous flow of 0.5-1% isoflurane mixed with oxygen and nitrous oxide. Hair above the incision site was shaved and cleaned with 70% ethanol-distilled water solution. Rats were fixed into a stereotactic frame (David Kopf instruments, California, USA) using ear bars. An incision was made on the skin along the midline and a burr hole was made into the skull above each target region. The dura matter was penetrated and one CMA 12 microdialysis probe was inserted into each of the following regions, ipsilateral to the lesioned hemisphere, at the following stereotactic coordinates; in mm relative to bregma: striatum (probe membrane length 3 mm), A = +1.0, L = -3.0, V = -6.0; GPI (probe membrane length 2 mm), A = -1.3, L = -3.3, V = -7.5; SN (probe membrane length 1 mm), A = -5.5, L = -2.2, V = -8.0 (Paxinos and Watson, 1986). Each microdialysis probe was held in position by a CMA 11/12 probe clip (8309013, Linton instrumentation, UK) and fixed into an upright position, perpendicular to the skull, with dental cement (MPAIR1KG & L, Metrorodent, UK).

Following implantation, microdialysis probes were left to stabilise for 2 h before baseline samples were collected for 1 h. Samples were collected in ice-cooled micro-vial inserts (product code: 501304, NLG analytical Ltd, UK) containing 5 µl of 0.1 N perchloric acid to minimise oxidation. Samples were collected in 20 min intervals at a rate of 1 µl/min. L-DOPA/benserazide (6/15 mg/kg; i.p.) was injected at time point 0 min and samples were collected for 180 min thereafter. Samples were then stored at -80°C until analysis using high-performance liquid chromatography (HPLC) with electrochemical detection. Rats were killed following microdialysis by cervical dislocation. Rat brains were removed and immediately frozen in -45°C isopentane and stored at -80°C.

**High-performance liquid chromatography (HPLC) analysis**

The effect of acute drug treatment on extracellular release of monoamines (dopamine, DA; 3,4-dihydroxyphenylacetic acid, DOPAC; homovanillic acid, HVA; serotonin, 5-HT; 5-hydroxyindoleacetic acid, 5-HIAA; noradrenaline, NA) and amino acids (γ-aminobutyric acid, GABA; glutamine) were measured using HPLC with an electrochemical detection system. Microdialysis samples were removed from -80°C storage and placed in a refrigerated autosampler set at 4°C.

For detection of monamines, samples were processed through a system consisting of a MD 150 x 3.2 mm, 3 µm C18 column (ESA), a dual electrode 5014B microdialysis cell (ESA)
with the electrode potentials set at -150 mV and +250 mV. Monoamines were detected at the second electrode with Coulochem II detector (ESA). A 5020 guard cell (ESA) with a potential of +350 mV preceded the autosampler. The mobile phase was set at a flow rate of 0.5 ml/min and contained 90 mM sodium dihydrogen phosphate, 50 mM citric acid, 1.7 mM 1-octane sulphonic acid, 50 µM EGTA, 10% acetonitrile, pH 3.0. A calibration curve was created from a range (6.25 - 24.4 ng/ml) of monoamine calibration standards containing DA (H8502, Sigma-Aldrich, UK), DOPAC (850217, Sigma-Aldrich, UK), HVA (H1252, Sigma-Aldrich, UK), NA (A7257, Sigma-Aldrich, UK), 5-HT (H9523, Sigma-Aldrich, UK) and 5-HIAA (H8896, Sigma-Aldrich, UK).

For detection of amino acids, samples were processed through a system consisting of a Waters Xterra MS 3.0 x 50 mm 2.5 µm column, a dual electrode 5011A high sensitivity analytical cell (ESA) with the electrode potentials set at +150 mV and +550 mV. Amino acids were detected at the second electrode with 5600A CoulArray detector (ESA). The mobile phase was set at a flow rate of 0.6 ml/min and contained 100 mM disodium hydrogen phosphate, 20% methanol, 3.5% acetonitrile, pH 6.7. Amino acids were detected after pre-column derivatisation with o-phthalaldehyde (OPA)/β-mercaptoethanol (β-ME). A calibration curve was created from a range (342.9 – 9259.3 ng/ml) of amino acid calibration standards containing GABA (A2129, Sigma-Aldrich, UK) and glutamine (G3126, Sigma-Aldrich, UK).

Chromatograms of monoamine and amino acid samples were analysed using CoulArray® Data Station Program (Version 3.10). Concentrations were quantified by determining the size of peak areas and converting them to known concentration values from respective standards.

5.2.6 Tissue processing
Tissue processing was performed as described in Chapter 2 (2.2.4). With respect to bregma (Paxinos and Watson, 1986), eight series of three equivalent sections were cut rostral-caudal through the rat brain as fully described in Chapter 2 (2.2.4).

5.2.7 DAT binding
[125I]-RTI-121 binding was carried out as described in Chapter 2 (2.2.5).
5.2.8 Validation of microdialysis probe placement

To validate the microdialysis probe target site, cresyl violet staining of coronal brain sections was carried out as described in Chapter 3 (3.2.4). Sections were observed under a light microscope (Leica DMRB) at x1.6 magnification for confirmation of the probe tract entering the striatum, GPl and SN.

5.2.9 Statistical analysis

Statistical analyses were carried out using Prism 5.0 (GraphPad Software, San Diego, CA). Cylinder test performances and DAT binding results were analysed using two-way ANOVA followed by Bonferroni multiple comparisons test. The AIMs assessment scores following drug treatment were analysed using Friedman’s non-parametric ANOVA followed by Dunn’s multiple comparisons test. Rotorod performances were analysed using one-way ANOVA repeated measures followed by Tukey’s post hoc analysis. The effect of chronic L-DOPA treatment on the induction of AIMs was analysed using two-way ANOVA followed by Bonferroni multiple comparisons, with ‘time’ and ‘treatment group’ as given factors. Extracellular concentrations of monoamines and amino acids were expressed as fmol/µl, pmol/µl or nmol/µl in perfusate samples without correction for recovery across the dialysis membrane. Baseline values were calculated from an average of three initial samples collected. Perfusate concentrations were analysed using two-way ANOVA followed by Bonferroni multiple comparisons, with ‘time’ and ‘treatment group’ as given factors. Individual time points were analysed using one-way ANOVA followed by Tukey’s post hoc analysis. Mean perfusate concentrations over 0-3 h following L-DOPA/benserazide + drug treatment were analysed using one-way ANOVA followed by Tukey’s post hoc analysis. Mean perfusate concentrations were compared using an unpaired t-test when there were only two treatment groups available. In all tests, significance level of p<0.05 was used.
**In vivo** microdialysis following acute RGS4 protein inhibition

**Parameters tested**

Unilateral 6-OHDA lesion  →  Rotorod training  →  Rotorod testing  →  Cylinder test

Two weeks post-lesion  →  Three weeks post-lesion

L-DOPA priming begins  →  AIMs test Day 1, 7, 14 and 21

Once daily, 21 days L-DOPA/benserazide (6/15 mg/kg; i.p) treatment

Acute RGS4 inhibitor (CCG-2046 or CCG-63802; 0.1 mg/kg; i.p), or vehicle, + L-DOPA/benserazide (6/15 mg/kg; i.p) treatment

Microdialysis probe implantation in the striatum, GPI and SN

Sample analysis using HPLC

**Figure 106.** The timeline used for investigating the acute effects of RGS4 protein inhibition on neurotransmitter release in the basal ganglia in the unilateral 6-hydroxydopamine (6-OHDA)-lesioned rat model of L-DOPA-induced dyskinesia. AIMs, abnormal involuntary movements; HPLC, high-performance liquid chromatography.
5.3 Results

5.3.1 Behavioural analysis

5-HT<sub>1A</sub> receptor activation mediates the anti-dyskinetic effects of RGS4 protein inhibitors

CCG-2046
Following intrastriatal administration of CCG-2046 (2 mM) or vehicle, there was a significant effect of treatment group on the sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=12.79; p=0.0051, Figure 107A and B), which was reduced by CCG-2046 compared to vehicle (76%, p<0.01) and WAY-100635 + CCG-2046 (69%, p<0.05). There was no significant difference between WAY-100635 + CCG-2046 and WAY-100635 + vehicle-treated groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=11.24; p=0.0105, Figure 107C), which was reduced by CCG-2046 (70%, p<0.05) compared to WAY-100635 + CCG-2046. There was no significant difference between any of the other treatment groups (p>0.05).

At 1-2 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMs (Fr=4.676; p=0.1971, Figure 107D).

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=14.26; p=0.0026, Figure 107E), which was reduced by CCG-2046 (100%, p<0.05) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

Following intrastriatal administration of CCG-2046 (2 mM) or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=4.620; p=0.2018, Figure 107F).
Figure 107. The effects of CCG-2046 (2 mM; intrastratal administration) + WAY-100635 (0.5 mg/kg; i.p.) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle; #p<0.05 cf. CCG-2046 2 mM + WAY-100635 0.5 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Following intrastriatal administration of CCG-4986 (0.2 mM) or vehicle, there was a significant effect of treatment group on the sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=14.56; p=0.0022, Figure 108A and B), which was reduced by CCG-4986 compared to vehicle (76%, p<0.01) and WAY-100635 + CCG-4986 (78%, p<0.05). There was no significant difference between WAY-100635 + CCG-4986 and WAY-100635 + vehicle-treated groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=8.559; p=0.0358, Figure 108C). There was, however, no significant group to group difference found following post hoc analysis (p>0.05).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=8.603; p=0.0351, Figure 108D). There was, however, no significant group to group difference found following post hoc analysis (p>0.05).

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=11.45; p=0.0095, Figure 108E), which was reduced by CCG-4986 (100%, p<0.05) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

Following intrastriatal administration of CCG-4986 (0.2 mM) or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=1.370; p=0.7127, Figure 108F).
Figure 108. The effects of CCG-4986 (0.2 mM; intrastriatal administration) + WAY-100635 (0.5 mg/kg; i.p.) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle; #p<0.05 cf. CCG-4986 0.2 mM + WAY-100635 0.5 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
**Behavioural pharmacology of selective 5-HT$_{1A}$ agonist, 8-OH-DPAT**

**L-DOPA-induced AIMs**

Following systemic administration of 8-OH-DPAT (0.03, 0.1, 0.3 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration ($F_r=17.52; p=0.0006$, Figure 109A and B), which was reduced by 8-OH-DPAT 0.3 mg/kg (81%, $p<0.001$) compared to vehicle. There was no significant difference between any of the other treatment groups ($p>0.05$).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs ($F_r=13.68; p=0.0034$, Figure 109C), which was reduced by 8-OH-DPAT 0.3 mg/kg, compared to vehicle (85%, $p<0.01$) and 8-OH-DPAT 0.03 mg/kg (86%, $p<0.05$). There was no significant difference between any of the other treatment groups ($p>0.05$).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs ($F_r=14.23; p=0.0026$, Figure 109D), which was reduced by 8-OH-DPAT 0.3 mg/kg (73%, $p<0.01$) compared to vehicle. There was no significant difference between any of the other treatment groups ($p>0.05$).

At 2-3 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMs ($F_r=2.739; p=0.4336$, Figure 109E).

Following administration of 8-OH-DPAT (0.03, 0.1, 0.3 mg/kg) or vehicle, there was a significant effect of treatment group on locomotor AIMs induced by L-DOPA over 3 h post administration ($F_r=14.91; p=0.0019$, Figure 109F), which was reduced by 8-OH-DPAT 0.3 mg/kg (80%, $p<0.01$) and 0.1 mg/kg (60%, $p<0.05$) compared to vehicle. There was no significant difference between any of the other treatment groups ($p>0.05$).
Figure 109. The effects of 8-OH-DPAT (0.03, 0.1, 0.3 mg/kg; i.p.) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01, ***p<0.001 cf. vehicle; #p<0.05 cf. 8-OH-DPAT 0.03 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Rotorod performance

Following systemic administration of 8-OH-DPAT (0.03, 0.1, 0.3 mg/kg) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{3,15}=10.59; p=0.0005$, Figure 110A), which was increased by 8-OH-DPAT 0.3 mg/kg (77%, $p<0.001$) and 0.1 mg/kg (53%, $p<0.01$) compared to vehicle. Time on the rotorod was also increased by 8-OH-DPAT 0.3 mg/kg (33%, $p<0.05$) compared to 8-OH-DPAT 0.03 mg/kg. There was no significant difference in rotorod performance between any other treatment group ($p>0.05$).

Following systemic administration of 8-OH-DPAT (0.03, 0.1, 0.3 mg/kg) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration ($F_{3,21}=1.141; p=0.3554$, Figure 110B).
The effects of 8-OH-DPAT (0.03, 0.1, 0.3 mg/kg; i.p.) in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. **p<0.01, ***p<0.001 cf. vehicle; #p<0.05 cf. 8-OH-DPAT 0.03 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).

Figure 110.
Combined treatment with 8-OH-DPAT and an RGS4 protein inhibitor

8-OH-DPAT and CCG-2046 on L-DOPA-induced AIMs

Following systemic administration of 8-OH-DPAT 0.03 mg/kg + CCG-2046 (0.003, 0.01 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=9.696; p=0.0213, Figure 111A and B), which was reduced by 8-OH-DPAT 0.03 mg/kg + CCG-2046 0.01 mg/kg, compared to vehicle (53%, p<0.05) and 8-OH-DPAT 0.03 mg/kg (44%, p<0.05). There was no significant difference between any of the other treatment groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=12.76; p=0.0052, Figure 111C), which was reduced by 8-OH-DPAT 0.03 mg/kg + CCG-2046 0.01 mg/kg, compared to vehicle (31%, p<0.05) and 8-OH-DPAT 0.03 mg/kg (36%, p<0.05). There was no significant difference between any of the other treatment groups (p>0.05).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=8.864; p=0.0312, Figure 111D), which was reduced by 8-OH-DPAT 0.03 mg/kg + CCG-2046 0.01 mg/kg (60%, p<0.05) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 2-3 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMs (Fr=3.250; p=0.3547, Figure 111E).

Following systemic administration of 8-OH-DPAT 0.03 mg/kg + CCG-2046 (0.003, 0.01 mg/kg) or vehicle, there was a significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=10.55; p=0.0144, Figure 111F), which was reduced by 8-OH-DPAT 0.03 mg/kg + CCG-2046 0.003 mg/kg (70%, p<0.05) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).
Figure 111. The effects of 8-OH-DPAT (0.03 mg/kg; i.p.) + CCG-2046 (0.003, 0.01 mg/kg; i.p.) or vehicle on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05 cf. vehicle; #p<0.05 cf. 8-OH-DPAT 0.03 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Following systemic administration of 8-OH-DPAT 0.1 mg/kg + CCG-2046 0.003 mg/kg or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=12.29; p=0.0003, Figure 112A and B), which was reduced by 8-OH-DPAT 0.1 mg/kg + CCG-2046 0.003 mg/kg, compared to vehicle (72%, p<0.01) and 8-OH-DPAT 0.1 mg/kg (65%, p<0.05). There was no significant difference between vehicle and 8-OH-DPAT 0.1 mg/kg-treated groups (p>0.05).

At 0-1 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMs (Fr=6.00; p=0.0515, Figure 112C).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=11.31; p=0.0003, Figure 112D), which was reduced by 8-OH-DPAT 0.1 mg/kg + CCG-2046 0.003 mg/kg (73%, p<0.01) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 2-3 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMs (Fr=3.176; p=0.2366, Figure 112E).

Following systemic administration of 8-OH-DPAT 0.1 mg/kg + CCG-2046 0.003 mg/kg or vehicle, there was a significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=11.76; p=0.003, Figure 112F), which was reduced by 8-OH-DPAT 0.1 mg/kg + CCG-2046 0.003 mg/kg (70%, p<0.05) and 8-OH-DPAT 0.1 mg/kg-treated (60%, p<0.05) compared to vehicle.
Figure 112. The effects of 8-OH-DPAT (0.1 mg/kg; i.p.) + CCG-2046 (0.003 mg/kg; i.p.) or vehicle on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=7) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle; #p<0.05 cf. 8-OH-DPAT 0.1 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
8-OH-DPAT and CCG-2046 on rotorod performance

Following systemic administration of 8-OH-DPAT 0.03 mg/kg + CCG-2046 (0.003, 0.01 mg/kg) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{3,15}=18.46$; $p<0.0001$, Figure 113A), which was increased by 8-OH-DPAT 0.03 mg/kg + CCG-2046 0.003 mg/kg (104%, $p<0.001$) and 8-OH-DPAT 0.03 mg/kg + CCG-2046 0.01 mg/kg (68%, $p<0.01$), compared to vehicle. Time on the rotorod was also increased by 8-OH-DPAT 0.03 mg/kg + CCG-2046 0.003 mg/kg (54%, $p<0.01$) compared to 8-OH-DPAT 0.03 mg/kg. There was no significant difference in rotorod performance between any other treatment group ($p>0.05$).

Following systemic administration of 8-OH-DPAT 0.03 mg/kg + CCG-2046 (0.003, 0.01 mg/kg) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration ($F_{3,21}=1.358$; $p=0.2828$, Figure 113B).
Figure 113. The effects of 8-OH-DPAT (0.03 mg/kg; i.p.) + CCG-2046 (0.003, 0.01 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. **p<0.01, ***p<0.001 cf. vehicle; ##p<0.01 cf. 8-OH-DPAT 0.03 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
Following systemic administration of 8-OH-DPAT 0.1 mg/kg + CCG-2046 0.003 mg/kg or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{2,10}=27.25; p<0.0001$), Figure 114A), which was increased by 8-OH-DPAT 0.1 mg/kg + CCG-2046 0.003 mg/kg (77%, $p<0.001$) and 8-OH-DPAT 0.1 mg/kg (37%, $p<0.05$), compared to vehicle. Time on the rotorod was also increased by 8-OH-DPAT 0.1 mg/kg + CCG-2046 0.003 mg/kg (29%, $p<0.01$) compared to 8-OH-DPAT 0.1 mg/kg.

Following systemic administration of 8-OH-DPAT 0.1 mg/kg + CCG-2046 0.003 mg/kg or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration ($F_{2,14}=0.4236; p=0.6628$, Figure 114B).
Figure 114. The effects of 8-OH-DPAT (0.1 mg/kg; i.p.) + CCG-2046 (0.003 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle; ##p<0.01 cf. 8-OH-DPAT 0.1 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
8-OH-DPAT and CCG-63802 on L-DOPA-induced AIMS

Following systemic administration of 8-OH-DPAT 0.03 mg/kg + CCG-63802 (0.003, 0.01 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMS induced by L-DOPA treatment over 3 h post administration (Fr=16.06; p=0.001, Figure 115A and B), which was reduced by 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.01 mg/kg compared to vehicle (31%, p<0.001) and 8-OH-DPAT 0.03 mg/kg (27%, p<0.05). There was no significant difference between any of the other treatment groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=13.14; p=0.0043, Figure 115C), which was reduced by 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.01 mg/kg (42%, p<0.01) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=12.21; p=0.0067, Figure 115D), which was reduced by 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.01 mg/kg (30%, p<0.05) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=13.43; p=0.0038, Figure 115E), which was reduced by 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.01 mg/kg (60%, p<0.05) compared to 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.003 mg/kg. There was no significant difference between any of the other treatment groups (p>0.05).

Following systemic administration of 8-OH-DPAT 0.03 mg/kg + CCG-63802 (0.003, 0.01 mg/kg) or vehicle, there was no significant effect of treatment group on locomotor AIMS induced by L-DOPA treatment over 3 h post administration (Fr=1.360; p=0.7149, Figure 115F).
The effects of 8-OH-DPAT (0.03 mg/kg; i.p.) + CCG-63802 (0.003, 0.01 mg/kg; i.p.) or vehicle on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=8) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01, ***p<0.001 cf. vehicle; #p<0.05 cf. 8-OH-DPAT 0.03 mg/kg; $p<0.05 cf. 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.003 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Following systemic administration of 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=16.00; p<0.0001, Figure 116A and B), which was reduced by 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg (50%, p<0.001) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=14.25; p<0.0001, Figure 116C), which was reduced by 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg (64%, p<0.001) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=12.84; p=0.0003, Figure 116D), which was reduced by 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg (56%, p<0.01) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=9.172; p=0.0080, Figure 116E), which was reduced by 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg (67%, p<0.05) compared to 8-OH-DPAT 0.1 mg/kg. There was no significant difference between any of the other treatment groups (p>0.05).

Following systemic administration of 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=3.714; p=0.1495, Figure 116F).
Figure 116. The effects of 8-OH-DPAT (0.1 mg/kg; i.p.) + CCG-63802 (0.003 mg/kg; i.p.) or vehicle on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=8) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. **p<0.01, ***p<0.001 cf. vehicle; #p<0.05 cf. 8-OH-DPAT 0.1 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
8-OH-DPAT and CCG-63802 on rotorod performance

Following systemic administration of 8-OH-DPAT 0.03 mg/kg + CCG-63802 (0.003, 0.01 mg/kg) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration (F_{3,15}=51.24; p<0.0001, Figure 117A), which was increased by 8-OH-DPAT 0.03 mg/kg (33%, p<0.05), 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.003 mg/kg (126%, p<0.001) and 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.01 mg/kg (72%, p<0.001), compared to vehicle. Time on the rotorod was also increased by 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.003 mg/kg (70%, p<0.001) and 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.01 mg/kg (30%, p<0.05), compared to 8-OH-DPAT 0.03 mg/kg. Time on the rotorod was also increased by 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.003 mg/kg (31%, p<0.001) compared to 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.01 mg/kg.

Following systemic administration of 8-OH-DPAT 0.03 mg/kg + CCG-63802 (0.003, 0.01 mg/kg) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration (F_{3,21}=2.286; p=0.1083, Figure 117B).
The effects of 8-OH-DPAT (0.03 mg/kg; i.p.) + CCG-63802 (0.003, 0.01 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. *p<0.05, ***p<0.001 cf. vehicle; #p<0.05, ###p<0.001 cf. 8-OH-DPAT 0.03 mg/kg; $$$p<0.001 cf. 8-OH-DPAT 0.03 mg/kg + CCG-63802 0.003 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
Following systemic administration of 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{2,10}=111.4; p<0.0001$, Figure 118A), which was increased by 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg (128%, $p<0.001$) and 8-OH-DPAT 0.1 mg/kg (37%, $p<0.01$), compared to vehicle. Time on the rotorod was also increased by 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg (67%, $p<0.001$) compared to 8-OH-DPAT 0.1 mg/kg.

Following systemic administration of 8-OH-DPAT 0.1 mg/kg + CCG-63802 0.003 mg/kg or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration ($F_{2,14}=0.9707; p=0.4029$, Figure 118B).
Figure 118. The effects of 8-OH-DPAT (0.1 mg/kg; i.p.) + CCG-63802 (0.003 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. **p<0.01, ***p<0.001 cf. vehicle; ###p<0.001 cf. 8-OH-DPAT 0.1 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**Behavioural pharmacology of Amantadine**

**L-DOPA-induced AIMs**

Following systemic administration of amantadine (5, 10, 20 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=16.90; p=0.0007, Figure 119A and B), which was reduced by amantadine 20 mg/kg compared to vehicle (45%, p<0.01) and amantadine 5 mg/kg (50%, p<0.01). There was no significant difference between any of the other treatment groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=18.51; p=0.0003, Figure 119C), which was reduced by amantadine 20 mg/kg compared to vehicle (58%, p<0.001) and amantadine 5 mg/kg (56%, p<0.01). There was no significant difference between any of the other treatment groups (p>0.05).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=10.32; p=0.0160, Figure 119D), which was reduced by amantadine 20 mg/kg (50%, p<0.05) compared to amantadine 5 mg/kg. There was no significant difference between any of the other treatment groups (p>0.05).

At 2-3 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMs (Fr=1.550; p=0.6708, Figure 119E).

Following systemic administration of amantadine (5, 10, 20 mg/kg) or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA over 3 h post administration (Fr=6.584; p=0.0864, Figure 119F).
The effects of amantadine (5, 10, 20 mg/kg; s.c.) on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=8) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. **p<0.01, ***p<0.001 cf. vehicle; #p<0.05, ##p<0.01 cf. amantadine 5 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
**Rotorod performance**

Following systemic administration of amantadine (5, 10, 20 mg/kg) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{3,15}=25.68; p<0.0001$, Figure 120A), which was increased by amantadine 20 mg/kg compared to vehicle (122%, $p<0.001$), amantadine 5 mg/kg (101%, $p<0.001$) and 10 mg/kg (83%, $p<0.001$). There was no significant difference in rotorod performance between any other treatment group ($p>0.05$).

Following systemic administration of amantadine (5, 10, 20 mg/kg) or vehicle, there was a significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration ($F_{3,21}=5.865; p=0.0045$, Figure 120B), which was increased by amantadine 20 mg/kg compared to vehicle (128%, $p<0.05$), amantadine 5 mg/kg (146%, $p<0.05$) and 10 mg/kg (123%, $p<0.01$). There was no significant difference in peak rotorod performance between any other treatment group ($p>0.05$).
Figure 120. The effects of amantadine (5, 10, 20 mg/kg; s.c.) in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. *p<0.05, **p<0.01, ***p<0.001 cf. vehicle; #p<0.05, ###p<0.001 cf. amantadine 5 mg/kg; $$p<0.01, $$$p<0.001 cf. amantadine 10 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**Combined treatment with Amantadine and an RGS4 protein inhibitor**

**Amantadine and CCG-2046 on L-DOPA-induced AIMs**

Following systemic administration of amantadine 5 mg/kg + CCG-2046 (0.003, 0.01 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=12.49; p=0.0059, Figure 121A and B), which was reduced by amantadine 5 mg/kg + CCG-2046 0.01 mg/kg (60%, p<0.01) compared to amantadine 5 mg/kg. There was no significant difference between any of the other treatment groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=16.82; p=0.0008, Figure 121C), which was reduced by amantadine 5 mg/kg + CCG-2046 0.01 mg/kg compared to vehicle (52%, p<0.01) and amantadine 5 mg/kg (50%, p<0.05). There was no significant difference between any of the other treatment groups (p>0.05).

At 1-2 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMs (Fr=6.908; p=0.0749, Figure 121D).

At 2-3 h post L-DOPA administration, there was no significant effect of treatment group on sum of ALO AIMs (Fr=6.217; p=0.1015, Figure 121E).

Following systemic administration of amantadine 5 mg/kg + CCG-2046 (0.003, 0.01 mg/kg) or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA over 3 h post administration (Fr=2.014; p=0.5696, Figure 121F).
Figure 121. The effects of amantadine (5 mg/kg; s.c.) + CCG-2046 (0.003, 0.01 mg/kg; i.p.) or vehicle on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=8) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. **p<0.01 cf. vehicle; #p<0.05, ##p<0.01 cf. amantadine 5 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Following systemic administration of amantadine 10 mg/kg + CCG-2046 0.003 mg/kg or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=13.00; p=0.0003, Figure 122A and B), which was reduced by amantadine 10 mg/kg + CCG-2046 0.003 mg/kg compared to vehicle (64%, p<0.01) and amantadine 10 mg/kg (53%, p<0.05). There was no significant difference between vehicle and 8-OH-DPAT 0.1 mg/kg-treated groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=13.61; p<0.0001, Figure 122C), which was reduced by amantadine 10 mg/kg + CCG-2046 0.003 mg/kg (55%, p<0.001) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=13.07; p<0.0001, Figure 122D), which was reduced by amantadine 10 mg/kg + CCG-2046 0.003 mg/kg compared to vehicle (65%, p<0.01) and amantadine 10 mg/kg (62%, p<0.05).

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=7.750; p=0.0179, Figure 122E). There was, however, no significant difference between any of the treatment groups following post hoc analysis (p>0.05).

Following systemic administration of amantadine 10 mg/kg + CCG-2046 0.003 mg/kg or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=1.786; p=0.3553, Figure 122F).
Figure 122. The effects of amantadine (10 mg/kg; s.c.) + CCG-2046 (0.003 mg/kg; i.p.) or vehicle on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=8) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. **p<0.01, ***p<0.001 cf. vehicle; #p<0.05 cf. amantadine 10 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Amantadine and CCG-2046 on rotorod performance

Following systemic administration of amantadine 5 mg/kg + CCG-2046 (0.003, 0.01 mg/kg) or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{3,15}$=5.974; $p=0.0069$, Figure 123A), which was increased by amantadine 5 mg/kg + CCG-2046 0.01 mg/kg compared to vehicle (38%, $p<0.01$) and amantadine 5 mg/kg (25%, $p<0.05$). There was no significant difference in rotorod performance between any other treatment group ($p>0.05$).

Following systemic administration of amantadine 5 mg/kg + CCG-2046 (0.003, 0.01 mg/kg) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration ($F_{3,21}=0.2988$; $p=0.8259$, Figure 123B).
Figure 123. The effects of amantadine (5 mg/kg; s.c.) + CCG-2046 (0.003, 0.01 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. **p<0.01 cf. vehicle; #p<0.05 cf. amantadine 5 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
Following systemic administration of amantadine 10 mg/kg + CCG-2046 0.003 mg/kg or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{2,10}=33.32; p<0.0001$, Figure 124A), which was increased by amantadine 10 mg/kg + CCG-2046 0.003 mg/kg compared to vehicle (83%, $p<0.001$) and amantadine 10 mg/kg (51%, $p<0.001$).

Following systemic administration of amantadine 10 mg/kg + CCG-2046 0.003 mg/kg or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration ($F_{2,14}=0.9248; p=0.4195$, Figure 124B).
Figure 124. The effects of amantadine (10 mg/kg; s.c.) + CCG-2046 (0.003 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. ***p<0.001 cf. vehicle; ###p<0.001 cf. amantadine 10 mg/kg (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
**Amantadine and CCG-63802 on L-DOPA-induced AIMS**

Following systemic administration of amantadine 5 mg/kg + CCG-63802 (0.003, 0.01 mg/kg) or vehicle, there was a significant effect of treatment group on sum of ALO AIMS induced by L-DOPA treatment over 3 h post administration (Fr=13.88; p=0.0031, Figure 125A and B), which was reduced by amantadine 5 mg/kg + CCG-63802 0.01 mg/kg compared to vehicle (53%, p<0.05) and amantadine 5 mg/kg (56%, p<0.01). There was no significant difference between any of the other treatment groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=13.66; p=0.0034, Figure 125C), which was reduced by amantadine 5 mg/kg + CCG-63802 0.01 mg/kg compared to vehicle (55%, p<0.01) and amantadine 5 mg/kg (53%, p<0.05). There was no significant difference between any of the other treatment groups (p>0.05).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=10.87; p=0.0124, Figure 125D), which was reduced by amantadine 5 mg/kg + CCG-63802 0.003 mg/kg (53%, p<0.05) compared to amantadine 5 mg/kg. There was no significant difference between any of the other treatment groups (p>0.05).

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMS (Fr=8.016; p=0.0457, Figure 125E). There was, however, no significant difference between any of the treatment groups following post hoc analysis (p>0.05).

Following systemic administration of amantadine 5 mg/kg + CCG-63802 (0.003, 0.01 mg/kg) or vehicle, there was no significant effect of treatment group on locomotor AIMS induced by L-DOPA treatment over 3 h post administration (Fr=6.458; p=0.0913, Figure 125F).
Figure 125. The effects of amantadine (5 mg/kg; s.c.) + CCG-63802 (0.003, 0.01 mg/kg; i.p.) or vehicle on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=8) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle; #p<0.05, ##p<0.01 cf. amantadine 5 mg/kg (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Following systemic administration of amantadine 10 mg/kg + CCG-63802 0.003 mg/kg or vehicle, there was a significant effect of treatment group on sum of ALO AIMs induced by L-DOPA treatment over 3 h post administration (Fr=10.75; p=0.0024, Figure 126A and B), which was reduced by amantadine 10 mg/kg + CCG-63802 0.003 mg/kg (50%, p<0.01) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 0-1 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=11.81; p=0.0011, Figure 126C), which was reduced by amantadine 10 mg/kg + CCG-63802 0.003 mg/kg (58%, p<0.01) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

At 1-2 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=6.690; p=0.0375, Figure 126D). There was, however, no significant difference between any of the treatment groups following post hoc analysis (p>0.05).

At 2-3 h post L-DOPA administration, there was a significant effect of treatment group on sum of ALO AIMs (Fr=6.870; p=0.0303, Figure 126E). There was, however, no significant difference between any of the treatment groups following post hoc analysis (p>0.05).

Following systemic administration of amantadine 10 mg/kg + CCG-63802 0.003 mg/kg or vehicle, there was no significant effect of treatment group on locomotor AIMs induced by L-DOPA treatment over 3 h post administration (Fr=3.379; p=0.1495, Figure 126F).
Figure 126. The effects of amantadine (10 mg/kg; s.c.) + CCG-63802 (0.003 mg/kg; i.p.) or vehicle on abnormal involuntary movements (AIMs) induced by L-DOPA/benserazide (6/15 mg/kg; i.p.) in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent data from individual animals (n=8) and bars represent median value of treatment group. (A) Sum of axial, limb and orolingual (ALO) AIMs from 0-3 hours post L-DOPA administration; (B) % of ALO AIMs from 0-3 hours post L-DOPA administration. Data expressed as mean ± SEM; (C) ALO AIMs from 0-1 hours post L-DOPA administration; (D) ALO AIMs from 1-2 hours post L-DOPA administration; (E) ALO AIMs from 2-3 hours post L-DOPA administration; (F) Locomotor AIMs from 0-3 hours post L-DOPA administration. **p<0.01 cf. vehicle (Friedman’s non-parametric ANOVA, Dunn’s multiple comparison post hoc analysis).
Amantadine and CCG-63802 on rotorod performance

Following systemic administration of amantadine 5 mg/kg + CCG-63802 (0.003, 0.01 mg/kg) or vehicle, there was a significant effect of treatment group on average rotorod performance over a 3 h post L-DOPA administration (F_{3,15}=3.561; p=0.0399, Figure 127A), which was increased by amantadine 5 mg/kg + CCG-63802 0.01 mg/kg (52%, p<0.05) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

Following systemic administration of amantadine 5 mg/kg + CCG-63802 (0.003, 0.01 mg/kg) or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration (F_{3,21}=0.7954; p=0.5101, Figure 127B).
Figure 127. The effects of amantadine (5 mg/kg; s.c.) + CCG-63802 (0.003, 0.01 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. *p<0.05 cf. vehicle (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
Following systemic administration of amantadine 10 mg/kg + CCG-63802 0.003 mg/kg or vehicle, there was a significant effect of treatment group on average rotorod performance over 3 h post L-DOPA administration ($F_{2,10}=6.913; p<0.0130$, Figure 128A), which was increased by amantadine 10 mg/kg + CCG-63802 0.003 mg/kg (45%, $p<0.05$) compared to vehicle. There was no significant difference between any of the other treatment groups ($p>0.05$).

Following systemic administration of amantadine 10 mg/kg + CCG-63802 0.003 mg/kg or vehicle, there was no significant effect of treatment group on peak rotorod performance at 1 h post L-DOPA administration ($F_{2,14}=0.09962; p=0.9058$, Figure 128B).
Figure 128. The effects of amantadine (10 mg/kg; s.c.) + CCG-63802 (0.003 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) administration on rotorod performance in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Data expressed as mean ± SEM (n=8). (A) Rotorod performance from 0-3 hours post L-DOPA administration; (B) Peak rotorod performance at 1 h post L-DOPA administration. *p<0.05 cf. vehicle (one-way ANOVA repeated measures, Tukey’s post hoc analysis).
The acute effects of RGS4 protein inhibitors on neurotransmitter release in the basal ganglia

DAT binding
There was a significant effect of lesion (F_{1,18}=829.80; p<0.0001; Figure 129A) but not treatment group (F_{2,18}=0.94; p=0.4097) on [{\textsuperscript{125}}I]-RTI-121 binding levels. [{\textsuperscript{125}}I]-RTI-121 binding was reduced in the operated compared to the unoperated side in vehicle (87%, p<0.001), CCG-2046 (88%, p<0.001) and CCG-63802 (85%, p<0.001) treatment groups.

Cylinder test
There was a significant effect of lesion (F_{1,18}=353.89; p<0.0001; Figure 129B) but not treatment group (F_{2,18}=0.00; p=1.0000) on cylinder test performance. Left paw contacts were reduced compared to right paw contacts in vehicle (81%, p<0.001), CCG-2046 (77%, p<0.001) and CCG-63802 (86%, p<0.001) treatment groups.
Figure 129. The effect of unilateral 6-OHDA-lesion on (A) dopamine active transporter binding and (B) cylinder test performance in rats treated once daily with L-DOPA/benserazide (6/15 mg/kg; i.p.) for 21 days. (A) $[^{125}]$-RTI-121 binding in the unoperated (□) and operated (■) striatum. ***p<0.001 cf. unoperated side (B) % of total paw contacts made with right (□) or left (■) paw. ***p<0.001 cf. right paw same treatment group. Bars represent mean ± SEM (n=4) (two-way ANOVA, Bonferroni multiple comparisons test).
*Induction of AIMS*

There was a significant effect of time ($F_{3,36}=27.37; \ p<0.0001$, Figure 130A) but not treatment group ($F_{2,36}=0.12; \ p=0.8840$) on sum of ALO AIMS during 21 days L-DOPA treatment. Individual analysis of L-DOPA treatment days 1, 7, 14 and 21, showed sum of ALO AIMS were not significantly different between treatment groups (day 1, $U=2.175, \ p=0.3371$; day 7, $U=1.656, \ p=0.4370$; day 14, $U=0.1846, \ p=0.9118$; day 21, $U=2.936, \ p=0.2304$).

There was no significant effect of time ($F_{3,36}=1.34; \ p=0.2771$, Figure 130B) or treatment group ($F_{2,36}=2.50; \ p=0.0961$) on locomotor AIMS during 21 days L-DOPA treatment. Individual analysis of L-DOPA treatment days 1, 7, 14 and 21, showed locomotor AIMS were not significantly different between the treatment groups (day 1, $U=2.879, \ p=0.2371$; day 7, $U=1.233, \ p=0.5399$; day 14, $U=0.7491, \ p=0.6876$; day 21, $U=1.102, \ p=0.5764$).
Figure 130. The induction of abnormal involuntary movements (AIMs) following, once daily, L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment for 21 days in the unilateral 6-OHDA-lesioned rat model of Parkinson’s disease. Points represent mean ± SEM (n=4); some error bars have been omitted for clarity. (A) Sum of axial, limb, oro-lingual (ALO) AIMs; (B) locomotor AIMs over 3 hours post L-DOPA administration (Kruskal-Wallis test, Dunn’s multiple comparison test).
Triple probe microdialysis - the release of monoamines in basal ganglia sub-regions

The effects of acute RGS4 protein inhibitor treatment on DA efflux in the striatum
There was a significant effect of treatment group (F_{2,93}=12.63; p<0.0001, Figure 131A) but not time (F_{10,93}=0.82; p=0.6071) on DA release following acute drug treatment. Individual analysis of time points showed striatal DA levels were reduced at 160 min (F_{2,11}=9.144; p=0.0068) post L-DOPA, by CCG-2046 (81%, p<0.01) and CCG-63802 (54%, p<0.05), compared to vehicle. There were no significant differences between treatment groups at any other time point (p>0.05).

There was a significant effect of treatment group (F_{2,30}=14.29; p<0.0001, Figure 131B) on mean DA efflux over 0-3 h post L-DOPA, which was reduced by CCG-2046 (65%, p<0.001) and CCG-4986 (54%, p<0.001), compared to vehicle.

The effects of acute RGS4 protein inhibitor treatment on DOPAC efflux in the striatum
There was a significant effect of treatment group (F_{2,88}=3.48; p=0.0352, Figure 131C) and time (F_{10,88}=3.84; p=0.0002), but not treatment x time interaction (F_{20,88}=0.75; p=0.7609), on DOPAC release following acute drug treatment. Individual analysis showed striatal DOPAC levels were not significantly different between treatment groups at any time point (p>0.05).

There was no significant effect of treatment group (F_{2,30}=1.871; p=0.1714, Figure 131D) on mean DOPAC efflux over 0-3 h post L-DOPA.

The effects of acute RGS4 protein inhibitor treatment on HVA efflux in the striatum
There was a significant effect of time (F_{10,88}=4.39; p<0.0001, Figure 131E) but not treatment group (F_{2,88}=1.25; p=0.2907) on HVA release following acute drug treatment. Individual analysis showed striatal HVA levels were not significantly different between treatment groups at any time point (p>0.05).

There was no significant effect of treatment group (F_{2,30}=0.7307; p=0.4899, Figure 131F) on mean HVA efflux over 0-3 h post L-DOPA.
Figure 131. The effects of acute CCG-2046 (0.1 mg/kg; i.p.), CCG-63802 (0.1 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on monoamine release in the striatum of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Points and bars represent mean ± SEM (n=3-4); some error bars have been omitted for clarity. (A) Dopamine (DA); (B) Mean DA; (C) 3,4-dihydroxyphenylacetic acid (DOPAC); (D) Mean DOPAC; (E) homovanillic acid (HVA); (F) Mean HVA efflux over 3 hours post L-DOPA administration. *p<0.05, **p<0.01, ***p<0.001 cf. vehicle (one-way ANOVA, Tukey’s post hoc analysis).
The effects of acute RGS4 protein inhibitor treatment on 5-HT efflux in the striatum

There was no significant effect of treatment group \( (F_{2,77}=2.43; \ p=0.0947, \ Figure \ 132A) \) or time \( (F_{10,77}=0.57; \ p=0.8307) \) on 5-HT release following acute drug treatment. Individual analysis showed striatal 5-HT levels were not significantly different between treatment groups at any time point \( (p>0.05) \).

There was no significant effect of treatment group \( (F_{2,30}=3.098; \ p=0.0598, \ Figure \ 132B) \) on mean 5-HT efflux over 0-3 h post L-DOPA.

The effects of acute RGS4 protein inhibitor treatment on 5-HIAA efflux in the striatum

There was no significant effect of treatment group \( (F_{2,99}=1.69; \ p=0.1902, \ Figure \ 132C) \) or time \( (F_{10,99}=1.19; \ p=0.3090) \) on 5-HIAA release following acute drug treatment. Individual analysis showed striatal 5-HIAA levels were not significantly different between treatment groups at any time point \( (p>0.05) \).

There was a significant effect of treatment group \( (F_{2,30}=3.559; \ p=0.0410, \ Figure \ 132D) \) on mean 5-HIAA efflux over 0-3 h post L-DOPA, which was reduced by CCG-2046 (29\%, \ \ p<0.05) compared to CCG-63802. There was no significant difference between any of the other treatment groups \( (p>0.05) \).

The effects of acute RGS4 protein inhibitor treatment on NA efflux in the striatum

There was no significant effect of treatment group \( (F_{2,99}=2.29; \ p=0.1064, \ Figure \ 132E) \) or time \( (F_{10,99}=0.75; \ p=0.6719) \) on NA release following acute drug treatment. Individual analysis showed striatal NA levels were not significantly different between treatment groups at any time point \( (p>0.05) \).

There was a significant effect of treatment group \( (F_{2,30}=3.473; \ p=0.0440, \ Figure \ 132F) \) on mean NA efflux over 0-3 h post L-DOPA, which was increased by CCG-63802 (37\%, \ \ p<0.05), but not by CCG-2046 \( (p>0.05) \), compared to vehicle.
Figure 132. The effects of acute CCG-2046 (0.1 mg/kg; i.p.), CCG-63802 (0.1 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on monoamine release in the striatum of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Points and bars represent mean ± SEM (n=3-4); some error bars have been omitted for clarity. (A) Serotonin (5-HT); (B) Mean 5-HT; (C) 5-hydroxyindoleacetic acid (5-HIAA); (D) Mean 5-HIAA; (E) noradrenaline (NA); (F) Mean NA efflux over 3 hours post L-DOPA administration. *p<0.05 cf. vehicle; #p<0.05 cf. CCG-2046 0.1 mg/kg (one-way ANOVA, Tukey’s post hoc analysis).
The effects of acute RGS4 protein inhibitor treatment on DA efflux in the Gpi

There was a significant effect of treatment group (F\textsubscript{2,88}=9.81; p=0.0001, Figure 133A) but not time (F\textsubscript{10,88}=0.79; p=0.6429) on DA release following acute drug treatment. Individual analysis showed pallidal dopamine levels were not significantly different between treatment groups at any time point (p>0.05).

There was a significant effect of treatment group (F\textsubscript{2,30}=21.79; p<0.0001, Figure 133B) on mean DA efflux over 0-3 h post L-DOPA, which was reduced by CCG-2046 (79%, p<0.001) and CCG-4986 (56%, p<0.001), compared to vehicle.

The effects of acute RGS4 protein inhibitor treatment on DOPAC efflux in the Gpi

There was a significant effect of time (F\textsubscript{10,88}=2.12; p=0.0312, Figure 133C) but not treatment group (F\textsubscript{2,88}=0.15; p=0.8621) on DOPAC release following acute drug treatment. Individual analysis showed pallidal DOPAC levels were not significantly different between treatment groups at any time point (p>0.05).

There was no significant effect of treatment group (F\textsubscript{2,30}=0.1413; p=0.8688, Figure 133D) on mean DOPAC efflux over 0-3 h post L-DOPA.

The effects of acute RGS4 protein inhibitor treatment on HVA efflux in the Gpi

There was a significant effect of time (F\textsubscript{10,99}=3.65; p=0.0004, Figure 133E) but not treatment group (F\textsubscript{2,99}=2.23; p=0.1125) on HVA release following acute drug treatment. Individual analysis showed pallidal HVA levels were not significantly different between treatment groups at any time point (p>0.05).

There was no significant effect of treatment group (F\textsubscript{2,30}=1.539; p=0.2311, Figure 133F) on mean HVA efflux over 0-3 h post L-DOPA.
Figure 133. The effects of acute CCG-2046 (0.1 mg/kg; i.p.), CCG-63802 (0.1 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on monoamine release in the lateral globus pallidus of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Points and bars represent mean ± SEM (n=3-4); some error bars have been omitted for clarity. (A) Dopamine (DA); (B) Mean DA; (C) 3,4-dihydroxyphenylacetic acid (DOPAC); (D) Mean DOPAC; (E) homovanillic acid (HVA); (F) Mean HVA efflux over 3 hours post L-DOPA administration. ***p<0.001 cf. vehicle (one-way ANOVA, Tukey’s post hoc analysis).
The effects of acute RGS4 protein inhibitor treatment on 5-HT efflux in the GPl

There was a significant effect of treatment group ($F_{2,66}=4.63; p=0.0131$, Figure 134A) but not time ($F_{10,66}=0.52; p=0.8686$) on 5-HT release following acute drug treatment. Individual analysis showed pallidal 5-HT levels were not significantly different between treatment groups at any time point ($p>0.05$).

There was a significant effect of treatment group ($F_{2,30}=6.102; p=0.0060$, Figure 134B) on mean 5-HT efflux over 0-3 h post L-DOPA, which was reduced by CCG-2046 (38%, $p<0.05$) and CCG-63802 (43%, $p<0.01$) compared to vehicle. There was no significant difference between any of the other treatment groups ($p>0.05$).

The effects of acute RGS4 protein inhibitor treatment on 5-HIAA efflux in the GPl

There was no significant effect of treatment group ($F_{2,99}=0.05; p=0.9496$, Figure 134C) or time ($F_{10,99}=0.91; p=0.5282$) on 5-HIAA release following acute drug treatment. Individual analysis showed pallidal 5-HIAA levels were not significantly different between treatment groups at any time point ($p>0.05$).

There was no significant effect of treatment group ($F_{2,30}=0.1176; p=0.8895$, Figure 134D) on mean 5-HIAA efflux over 0-3 h post L-DOPA.

The effects of acute RGS4 protein inhibitor treatment on NA efflux in the GPl

There was a significant effect of treatment group ($F_{2,85}=6.70; p=0.0020$, Figure 134E) and time ($F_{10,85}=2.38; p=0.0152$), but not treatment x time interaction ($F_{20,85}=0.41; p=0.9859$), on NA release following acute drug treatment. Individual analysis showed pallidal NA levels were significantly increased at 60 min by CCG-63802 (72%, $p<0.05$) compared to CCG-2046. There were no significant differences between any of the treatment groups at any other time point ($p>0.05$).

There was a significant effect of treatment group ($F_{2,30}=7.070; p=0.0031$, Figure 134F) on mean NA efflux over 0-3 h post L-DOPA, which was increased by CCG-63802 compared to vehicle (45%, $p<0.05$) and CCG-2046 (54%, $p<0.01$).
Figure 134. The effects of acute CCG-2046 (0.1 mg/kg; i.p.), CCG-63802 (0.1 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on monoamine release in the lateral globus pallidus of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Points and bars represent mean ± SEM (n=3-4); some error bars have been omitted for clarity. (A) Serotonin (5-HT); (B) Mean 5-HT; (C) 5-hydroxyindoleacetic acid (5-HIAA); (D) Mean 5-HIAA; (E) noradrenaline (NA); (F) Mean NA efflux over 3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle; #p<0.05, ##p<0.01 cf. CCG-2046 0.1 mg/kg (one-way ANOVA, Tukey’s post hoc analysis).
The effects of acute RGS4 protein inhibitor treatment on DA efflux in the SN

There was a significant effect of treatment group (F_{2,99}=6.16; p=0.0030, Figure 135A) but not time (F_{10,99}=0.60; p=0.8114) on DA release following acute drug treatment. Individual analysis showed nigral dopamine levels were not significantly different between treatment groups at any time point (p>0.05).

There was a significant effect of treatment group (F_{2,30}=16.27; p<0.0001, Figure 135B) on mean DA efflux over 0-3 h post L-DOPA, which was reduced by CCG-2046 (69%, p<0.001) and CCG-63802 (82%, p<0.001) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

The effects of acute RGS4 protein inhibitor treatment on DOPAC efflux in the SN

There was a significant effect of time (F_{10,99}=4.52; p<0.0001, Figure 135C) but not treatment group (F_{2,99}=2.33; p=0.1029) on DOPAC release following acute drug treatment. Individual analysis showed nigral DOPAC levels were not significantly different between treatment groups at any time point (p>0.05).

There was no significant effect of treatment group (F_{2,30}=1.236; p=0.3050, Figure 135D) on mean DOPAC efflux over 0-3 h post L-DOPA.

The effects of acute RGS4 protein inhibitor treatment on HVA efflux in the SN

There was a significant effect of time (F_{10,99}=7.34; p<0.0001, Figure 135E) but not treatment group (F_{2,99}=0.88; p=0.4196) on HVA release following acute drug treatment. Individual analysis showed nigral HVA levels were not significantly different between treatment groups at any time point (p>0.05).

There was no significant effect of treatment group (F_{2,30}=0.3488; p=0.7083, Figure 135F) on mean HVA efflux over 0-3 h post L-DOPA.
Figure 135. The effects of acute CCG-2046 (0.1 mg/kg; i.p.), CCG-63802 (0.1 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on monoamine release in the substantia nigra of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Points and bars represent mean ± SEM (n=4); some error bars have been omitted for clarity. (A) Dopamine (DA); (B) Mean DA; (C) 3,4-dihydroxyphenylacetic acid (DOPAC); (D) Mean DOPAC; (E) homovanillic acid (HVA); (F) Mean HVA efflux over 3 hours post L-DOPA administration. ***p<0.001 cf. vehicle (one-way ANOVA, Tukey’s post hoc analysis).
The effects of acute RGS4 protein inhibitor treatment on 5-HT efflux in the SN
There was no significant effect of treatment group (F_{2,83}=0.33; p=0.7180, Figure 136A) or time (F_{10,83}=1.07; p=0.3969) on 5-HT release following acute drug treatment. Individual analysis showed nigral 5-HT levels were not significantly different between treatment groups at any time point (p>0.05).

There was no significant effect of treatment group (F_{2,30}=0.4571; p=0.6375, Figure 136B) on mean 5-HT efflux over 0-3 h post L-DOPA.

The effects of acute RGS4 protein inhibitor treatment on 5-HIAA efflux in the SN
There was a significant effect of treatment group (F_{2,88}=7.42; p=0.0011, Figure 136C) but not time (F_{10,88}=1.86; p=0.0612) on 5-HIAA release following acute drug treatment. Individual analysis showed nigral 5-HIAA levels were not significantly different between treatment groups at any time point (p>0.05).

There was a significant effect of treatment group (F_{2,30}=5.557; p=0.0089, Figure 136D) on mean 5-HIAA efflux over 0-3 h post L-DOPA, which was increased by CCG-2046 (58%, p<0.05) and CCG-63802 (49%, p<0.05) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).

The effects of acute RGS4 protein inhibitor treatment on NA efflux in the SN
There was a significant effect of treatment group (F_{2,88}=4.16; p=0.0188, Figure 136E) but not time (F_{10,88}=0.87; p=0.5619) on NA release following acute drug treatment. Individual analysis showed nigral NA levels were not significantly different between treatment groups at any time point (p>0.05).

There was a significant effect of treatment group (F_{2,30}=5.972; p=0.0066, Figure 136F) on mean NA efflux over 0-3 h post L-DOPA, which was increased by CCG-2046 (44%, p<0.05) and CCG-63802 (52%, p<0.001) compared to vehicle. There was no significant difference between any of the other treatment groups (p>0.05).
Figure 136. The effects of acute CCG-2046 (0.1 mg/kg; i.p.), CCG-63802 (0.1 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on monoamine release in the substantia nigra of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Points and bars represent mean ± SEM (n=3-4); some error bars have been omitted for clarity. (A) Serotonin (5-HT); (B) Mean 5-HT; (C) 5-hydroxyindoleacetic acid (5-HIAA); (D) Mean 5-HIAA; (E) noradrenaline (NA); (F) Mean NA efflux over 3 hours post L-DOPA administration. *p<0.05, **p<0.01 cf. vehicle (one-way ANOVA, Tukey’s post hoc analysis).
The effects of acute RGS4 protein inhibitor treatment on GABA efflux in the striatum

There was a significant effect of treatment group ($F_{2,78}=19.71; p<0.0001$, Figure 137A) but not time ($F_{10,78}=0.89; p=0.5424$) on GABA release following acute drug treatment. Individual analysis showed striatal GABA levels were significantly different at 20 ($F_{2,7}=8.526; p=0.0133$) and 40 min ($F_{2,7}=7.948; p=0.0158$), which was increased by CCG-63802 compared to vehicle (361% and 411%, $p<0.05$ and $p<0.05$, respectively) and CCG-2046 (2605% and 2708%, $p<0.05$ and $p<0.05$, respectively). There was no significant difference between the treatment groups at any other time point ($p>0.05$).

There was a significant effect of treatment group ($F_{2,30}=22.46; p<0.0001$, Figure 137B) on mean GABA efflux over 0-3 h post L-DOPA, which was increased by CCG-63802 compared to vehicle (183%, $p<0.001$) and CCG-2046 (413%, $p<0.001$). There was no significant difference between any of the other treatment groups ($p>0.05$).

The effects of acute RGS4 protein inhibitor treatment on glutamine efflux in the striatum

There was no significant effect of treatment group ($F_{2,77}=1.13; p=0.3283$, Figure 137C) or time ($F_{10,77}=0.82; p=0.6126$) on glutamine release following acute drug treatment. Individual analysis showed striatal glutamine levels were not significantly different between treatment groups at any time point ($p>0.05$).

There was no significant effect of treatment group ($F_{2,30}=1.895; p=0.1679$, Figure 137D) on mean glutamine efflux over 0-3 h post L-DOPA.
Figure 137. The effects of acute CCG-2046 (0.1 mg/kg; i.p.), CCG-63802 (0.1 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on amino acid release in the striatum of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Points and bars represent mean ± SEM (n=2-4); some error bars have been omitted for clarity. (A) γ-aminobutyric acid (GABA); (B) Mean GABA; (C) Glutamine; (D) Mean Glutamine efflux over 3 hours post L-DOPA administration. *p<0.05, ***p<0.001 cf. vehicle; #p<0.05, ###p<0.001 cf. CCG-2046 0.1 mg/kg (one-way ANOVA, Tukey’s post hoc analysis).
The effects of acute RGS4 protein inhibitor treatment on GABA efflux in the GPi

There was no significant effect of treatment group (F\(_{1,66}=0.09;\) p=0.7699, Figure 138A) or time (F\(_{10,66}=0.61;\) p=0.7985) on GABA release following acute drug treatment. Individual analysis showed pallidal GABA levels were not significantly different between treatment groups at any time point (p>0.05).

There was no significant effect of treatment group (p=0.6519, Figure 138B) on mean GABA efflux over 0-3 h post L-DOPA.

The effects of acute RGS4 protein inhibitor treatment on glutamine efflux in the GPi

There was no significant effect of treatment group (F\(_{2,88}=2.55;\) p=0.0840, Figure 138C) or time (F\(_{10,66}=1.24;\) p=0.2754) on glutamine release following acute drug treatment. Individual analysis showed pallidal glutamine levels were not significantly different between treatment groups at any time point (p>0.05).

There was a significant effect of treatment group (F\(_{2,30}=4.216;\) p=0.0243, Figure 138D) on mean glutamine efflux over 0-3 h post L-DOPA, which was increased by CCG-63802 (31%, p<0.05) compared to CCG-2046. There was no significant difference between any of the other treatment groups (p>0.05).
Figure 138. The effects of acute CCG-2046 (0.1 mg/kg; i.p.), CCG-63802 (0.1 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on amino acid release in the lateral globus pallidus of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Points and bars represent mean ± SEM (n=3-4); some error bars have been omitted for clarity. (A) γ-aminobutyric acid (GABA); (B) Mean GABA (unpaired t-test); (C) Glutamine; (D) Mean Glutamine efflux over 3 hours post L-DOPA administration. #p<0.05 cf. CCG-2046 0.1 mg/kg (one-way ANOVA, Tukey’s post hoc analysis).
The effects of acute RGS4 protein inhibitor treatment on GABA efflux in the SN
There was a significant effect of treatment group (F_{1,43}=10.98; p=0.0019, Figure 139A) but not time (F_{10,43}=0.45; p=0.9133) on GABA release following acute drug treatment. Individual analysis showed nigral GABA levels were reduced at 20 min post L-DOPA by CCG-63802 compared to vehicle (82%, p=0.0365). There was no significant difference at any other time point (p>0.05).

There was a significant effect of treatment group (p=0.0002; Figure 139B) on mean GABA efflux over 0-3 h post L-DOPA, which was reduced by CCG-63802 compared to vehicle (54%, p<0.001).

The effects of acute RGS4 protein inhibitor treatment on glutamine efflux in the SN
There was a significant effect of treatment group (F_{2,77}=1.97; p=0.1471, Figure 139C) but not time (F_{10,77}=0.85; p=0.5826) on glutamine release following acute drug treatment. Individual analysis showed nigral glutamine levels were not significantly different between treatment groups at any time point (p>0.05).

There was a significant effect of treatment group (F_{2,30}=6.092; p=0.0060, Figure 139D) on mean glutamine efflux over 0-3 h post L-DOPA, which was increased by CCG-63802 compared to CCG-2046 (32%, p<0.01). There was no significant difference between any other treatment group (p>0.05).

Validation of probe placement
Cresyl violet stained sections from each animal showed the probe tract entering the target region (Figure 140).
**Figure 139.** The effects of acute CCG-2046 (0.1 mg/kg; i.p.), CCG-63802 (0.1 mg/kg; i.p.) or vehicle in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.) treatment on amino acid release in the substantia nigra of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Points and bars represent mean ± SEM (n=3-4); some error bars have been omitted for clarity. (A) γ-aminobutyric acid (GABA); (B) Mean GABA (unpaired t-test); (C) Glutamine; (D) Mean Glutamine efflux over 3 hours post L-DOPA administration. ***p<0.001 cf. vehicle; #p<0.01 cf. CCG-2046 0.1 mg/kg (one-way ANOVA, Tukey’s post hoc analysis).
Figure 140. Coronal sections through the rat brain showing microdialysis probe placement sites in the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. (A) Schematic diagram of the rostral striatum (+1.0 mm, with respect to bregma), GPl (-1.3 mm, with respect to bregma) and SN (-5.5 mm, with respect to bregma) adapted from Paxinos and Watson (1986). Grey scale images of cresyl violet stained coronal section showing the probe tract in the (B) striatum, (C) GPl and (D) SN ipsilateral to the lesioned side.
5.4 Discussion

The main findings from this study were that (1) the anti-dyskinetic effects of RGS4 protein inhibitor treatment were blocked following antagonism of 5-HT\textsubscript{1A} receptors; (2) combined treatment with subthreshold doses of an RGS4 protein inhibitor and 8-OH-DPAT, or amantadine, significantly reduced AIMs and improved overall motor ability; and (3) acute RGS4 protein inhibitor treatment, in combination with L-DOPA, caused a reduction of DA efflux in the basal ganglia (striatum, GPl and SN) of the unilateral 6-OHDA-lesioned rat model of LID.

5.4.1 Methodological considerations

*Behavioural and molecular assessments of the unilateral 6-OHDA-lesioned rat model*

In this study, behavioural pharmacology was used in the unilateral 6-OHDA-lesioned rat model of LID to investigate the mechanisms of drug action. As discussed in Chapter 3, AIMs and rotorod scores were used as behavioural markers of LID (Cenci et al., 1998; Lundblad et al., 2002) and motor ability (Rozas et al., 1997; Rozas and Labandeira, 1997), respectively. The doses of 8-OH-DPAT (Dupre et al., 2008a; Dupre et al., 2008b; Bishop et al., 2009) and amantadine (Dekundy et al., 2007; Kobylecki et al., 2011) administered to elicit threshold and subthreshold effects on AIMs and rotorod performances were chosen from previous behavioural studies conducted in L-DOPA-treated unilateral 6-OHDA-lesioned rats. The dose of WAY-100635 was chosen as it had previously been shown to block the anti-dyskinetic effects mediated by 5-HT\textsubscript{1A} receptor agonist treatment (Bishop et al., 2009). Doses of RGS4 protein inhibitors used to induce threshold and subthreshold effects on AIMs and rotorod performances were chosen from behavioural studies conducted in Chapter 3.

To investigate the effects of RGS4 protein inhibitor treatment on neurotransmitter release in the basal ganglia circuitry, we implanted microdialysis probes in the striatum, GPl and SN of L-DOPA-primed unilateral 6-OHDA-lesioned rats. To reduce the disruption on physiological neurotransmission, flow rate of microdialysis probes were kept to a minimum of 1 µl/min (Meissner et al., 2006).
5.4.2 Behavioural effects of RGS4 protein inhibition, in combination with 8-OH-DPAT or amantadine, in LID

The effects of combined treatment with subthreshold doses of an RGS4 protein inhibitor and 8-OH-DPAT, or amantadine, on L-DOPA-induced motor behaviours in the unilateral 6-OHDA-lesioned rat model of LID are summarised in Table 9.

| 8-OH-DPAT (mg/kg) | | |
|-----------------|-----------------|
| **Subthreshold dose** | **0.03** | **0.1** |
| | **ALO AIMs** | **Lo AIMs** | **RR** | **ALO AIMs** | **Lo AIMs** | **RR** |
| RGS4 inhibitor | | | | | | |
| CCG-2046 | ↓ | ↔ | ↑↑ | ↓ | ↔ | ↑↑ |
| CCG-63802 | ↓ | ↔ | ↑↑↑ | ↔ | ↔ | ↑↑↑ |

| Amantadine (mg/kg) | | | | | | |
|-----------------|-----------------|
| **Subthreshold dose** | **5** | **10** |
| | **ALO AIMs** | **Lo AIMs** | **RR** | **ALO AIMs** | **Lo AIMs** | **RR** |
| RGS4 inhibitor | | | | | | |
| CCG-2046 | ↓↓ | ↔ | ↑ | ↓ | ↔ | ↑↑↑ |
| CCG-63802 | ↓↓ | ↔ | ↔ | ↔ | ↔ | ↔ |

| Threshold dose | WAY-100635 (0.5 mg/kg) | | |
|----------------|-------------------------|-----------------|
| **RGS4 inhibitor** | **ALO AIMs** | **Lo AIMs** |
| CCG-2046 | ↓ | ↔ |
| CCG-4986 | ↓ | ↔ |

Table 9. The behavioural effects of RGS4 protein inhibitor treatment at subthreshold, or threshold, doses combined with (±)-8-Hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT), amantadine or WAY-100635 on abnormal involuntary movements (AIMs) and rotorod (RR) performance in L-DOPA/benserazide (6/15 mg/kg; i.p.) treated unilateral 6-OHDA-lesioned rats. Behavioural effects are compared to the same dose of 8-OH-DPAT, or amantadine, combined with L-DOPA. Arrows represent increased or decreased magnitude of change; ↔, no change cf. same dose of treatment; ALO, sum of axial/limb/oroligual; Lo, locomotor.
**RGS4 protein inhibitors act in synergy with 8-OH-DPAT, or amantadine, to reduce the expression of AIMs**

In this study, we found that the anti-dyskinetic effects induced by intrastriatal administration of RGS4 protein inhibitors were blocked following antagonism of 5-HT$_{1A}$ receptors. Moreover, combined subthreshold doses of RGS4 protein inhibitors and 8-OH-DPAT, or amantadine, caused a threshold decrease of AIMs and increased overall rotorod performance in L-DOPA-treated unilateral 6-OHDA-lesioned rats.

Previous studies have shown that RGS4 proteins mediate motor behaviours (Grillet et al., 2005; Lerner et al., 2012). RGS4 protein mRNA is found expressed at higher levels in the lateral striatum (Geurts et al., 2002), which correlate to the sensorimotor regions (McGeorge and Faull, 1989). Although RGS4 proteins have been found to regulate different GPCRs in the striatum including mGluR5 (Schwendt and McGinty, 2007; Schwendt et al., 2011), M$_4$ cholinergic receptors (Ding et al., 2006) and µ-opioid receptors (Georgoussi et al., 2006; Han et al., 2011), their roles in motor behaviour remains poorly understood (Herrera-Marschitz et al., 2010). In this study, we found that blockade of striatal RGS4 proteins can mediate anti-dyskinetic effects via striatal 5-HT$_{1A}$ receptors. Since RGS4 proteins negatively modulate 5-HT$_{1A}$ receptors (Beyer et al., 2004; Ghavami et al., 2004); blockade of these proteins would increase 5-HT$_{1A}$ receptor signalling. Indeed, direct activation of striatal 5-HT$_{1A}$ receptors via intrastrial infusion of selective agonist, 8-OH-DPAT dose-dependently reduces AIMs in L-DOPA-treated unilateral 6-OHDA-lesioned rats (Bishop et al., 2009). Moreover, upon activation of striatal 5-HT$_{1A}$ receptors, AIMs are alleviated as glutamatergic transmission is attenuated (Dupre et al., 2011).

Our behavioural data suggests RGS4 protein inhibition can potentiate 5-HT$_{1A}$ receptor signalling in the striatum to reduce AIMs. In support of this mechanism of action, we found that combined subthreshold doses of an RGS4 protein inhibitor with 8-OH-DPAT, potentiated the reduction of AIMs and increased rotorod performance compared to the same dose when given alone. This synergistic response (Greco et al., 1995) is likely to occur from modulating different components of the same signalling mechanism. Indeed, by blocking the regulatory action of RGS4 proteins on the 5-HT$_{1A}$ receptor activated G$_{a}$ subunit (Ghavami et al., 2004); the effects of a selective agonist would be potentiated (Sjogren et al., 2010). Moreover, combined treatment at subthreshold doses could reduce the side-effects induced by 5-HT$_{1A}$ agonist treatment alone, such as ‘serotonin syndrome’
(Tricklebank et al., 1984; Goodwin et al., 1987; Iravani et al., 2006b; Bishop et al., 2009). Although complete insensitivity of the $G_{\alpha}$ subunit to all RGS proteins can be lethal following administration of serotonergic or cholinergic agonists (Hollinger and Hepler, 2002), we report no visible side-effects or symptoms of serotonin syndrome, such as flattened body posture (Tricklebank et al., 1984), in 6-OHDA-lesioned rats following RGS4 protein inhibitor treatment.

In our final set of behaviour experiments, we showed a synergistic response following combined subthreshold treatment with RGS4 protein inhibitors and amantadine. It has been consistently shown that amantadine produces marked reduction of dyskinesia in unilateral 6-OHDA-lesioned rats (Dekundy et al., 2007; Kobylecki et al., 2011) and MPTP-lesioned NHPs (Blanchet et al., 1998; Hill et al., 2004; Kobylecki et al., 2011). Moreover, amantadine reduces LID by 50-60% in advanced PD patients (Metman et al., 1999; Luginger et al., 2000) but is limited by side-effects such as cognitive decline and psychosis (Luginger et al., 2000; Thomas et al., 2004). The anti-dyskinetic effects of amantadine are attributed to non-competitive antagonism of the phencyclidine site located inside the cation channel of the NMDA receptor, a site where NMDA receptor antagonist, MK-801 also binds (Kornhuber et al., 1991; Parsons et al., 1996).

Treatment with MK-801 effectively reduces dyskinesia in unilateral 6-OHDA-lesioned rats (Dupre et al., 2008b) and MPTP-lesioned NHPs (Gomez-Mancilla and Bedard, 1993). Interestingly, co-administration of MK-801 and 8-OH-DPAT induces a synergistic effect on the reduction of ALO AIMs for 1 h post L-DOPA treatment in unilateral 6-OHDA-lesioned rats (Dupre et al., 2008b). These effects are attributed to the anti-glutamatergic effects induced following 5-HT$_{1A}$ receptor activation (Antonelli et al., 2005; Mignon and Wolf, 2005; Dupre et al., 2011). Thus, it is conceivable that combined treatment with subthreshold doses of RGS4 protein inhibitor and amantadine, elicits anti-glutamatergic responses that reach threshold levels to reduce AIMs. Our behavioural data alludes to a signalling mechanism that has been previous identified in an electrophysiological study of rat prefrontal cortical neurons (Gu et al., 2007). Authors of the study showed that blockade of RGS4 proteins potentiated 5-HT$_{1A}$ receptor-mediated inhibition of NMDA-induced excitatory post-synaptic currents (Gu et al., 2007). This signalling mechanism could exist in other cortical areas such as the motor cortex, where direct infusion of 8-OH-DPAT dose-dependently reduces AIMs in unilateral 6-OHDA-lesioned rats (Ostock et al., 2011). Thus,
RGS4 protein inhibition could potentiate the anti-glutamatergic effects of 5-HT$_{1A}$ receptor activation on pyramidal neurons in the cerebral cortex (DeFelipe et al., 2001; Cruz et al., 2004). This could dampen excessive glutamatergic transmission of these neurons in LID, which give rise to corticostriatal afferents (Kunzle, 1975; Kunzle, 1977; McGeorge and Faull, 1989).

### 5.4.3 The effects of RGS4 protein inhibitor treatment on neurotransmitter release in the basal ganglia

The effects of acute RGS4 protein inhibitor treatment, in combination with L-DOPA, on neurotransmitter release in the striatum, GPl and SN of the unilateral 6-OHDA-lesioned rat model of LID are summarised in Table 10.

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<tr>
<th>Neurotransmitters</th>
<th>CCG-2046</th>
<th>CCG-63802</th>
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<tr>
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<td>STR</td>
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<td>DA</td>
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<tr>
<td>DOPAC</td>
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<td>HVA</td>
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<tr>
<td>GABA</td>
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<td>N/A</td>
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<td>Glutamine</td>
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**Table 10.** The effects of acute RGS4 protein inhibitor treatment, in combination with L-DOPA/benserazide (6/15 mg/kg; i.p.), on neurotransmitter release in the striatum (STR), lateral segment of the globus pallidus (GPl), and substantia nigra (SN) of the unilateral 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. Effects of treatment are compared to vehicle treatment in combination with L-DOPA/benserazide. Arrows represent increased or decreased magnitude of change; ↔, no change cf. vehicle treatment; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HT, serotonin; NA, noradrenaline; GABA, γ-aminobutyric acid; N/A, not available.
RGS4 protein inhibitors reduce the dopamine efflux in basal ganglia sub-regions in LID

Acute treatment with RGS4 protein inhibitors, in combination with L-DOPA, reduced the mean DA efflux in the striatum, GPI and SN in L-DOPA-primed unilateral 6-OHDA-lesioned rats.

Consistent with previous in vivo microdialysis studies in L-DOPA-primed unilateral 6-OHDA-lesioned rats, we found a large transient efflux of dopamine in the striatum over 0-3 h post L-DOPA administration (Meissner et al., 2006; Lindgren et al., 2010). Acute treatment with an RGS4 protein inhibitor attenuated DA efflux in basal ganglia sub-regions, which could mediate anti-dyskinetic effects. Indeed, unregulated DA efflux is a characterised pathophysiological feature of LID (Cenci and Lundblad, 2006; Carta et al., 2008; Carta and Bezard, 2011). For example, PD patients with peak-dose dyskinesia have shown a two-fold increase in synaptic dopamine levels in the striatum following L-DOPA treatment, compared to non-dyskinetic PD patients (de la Fuente-Fernandez et al., 2004).

Transient fluctuations of striatal dopamine in LID is caused by raphe-striatal serotonergic afferents, which can catabolise and release dopamine into the striatum (Tison et al., 1991; Arai et al., 1994; Arai et al., 1995; Arai et al., 1996). In support of this, lesion of the serotonergic system in unilateral 6-OHDA-lesioned rats reduced striatal dopamine release by 80% following a single, high dose L-DOPA (50 mg/kg; i.p.) injection (Tanaka et al., 1999). Thus, the serotonergic system plays a fundamental role in the unregulated release of dopamine in LID (Carta and Bezard, 2011). Moreover, it has been shown that the striatum is hyper-innervated by 5-HT neurons, with increased axonal varicosities, following induction of LID in MPTP-lesioned NHPs (Zeng et al., 2010) and PD patients (Rylander et al., 2010b).

Previous behavioural studies showed that directly targeting 5-HT₁A autoreceptors in the dorsal raphe nuclei, dose-dependently reduced AIMs, without compromising the anti-parkinsonian effects of L-DOPA, in unilateral 6-OHDA-lesioned rats (Eskow et al., 2009). Activation of these 5-HT₁A receptors, which are located on the cell bodies of 5-HT neurons (Chalmers and Watson, 1991; Riad et al., 2000), inhibits neuronal firing (Sprouse and Aghajanian, 1987). In turn, this decreases extracellular dopamine release and expression of LID (Lindgren et al., 2010). As mentioned, RGS4 proteins negatively modulate 5-HT₁A receptor signalling (Ghavami et al., 2004) and their regulation of 5-HT₁A receptors on DRN.
neurons controls neurotransmitter efflux into the striatum (Beyer et al., 2004). Thus, our results show that by blocking RGS4 proteins in LID, extracellular dopamine release is attenuated in sub-regions of the basal ganglia. This further supports the role of RGS4 proteins in LID.

**Implications of RGS4 protein inhibitor treatment on basal ganglia circuitry in LID**

Acute treatment with an RGS4 inhibitor, CCG-2046 or CCG-63802, in combination with L-DOPA, in the unilateral 6-OHDA-lesioned rat model of LID, caused significant effects on the release of numerous monoamines and amino acid neurotransmitters in the basal ganglia. Many of these changes are likely to be a secondary effect, following primary ant-dyskinetic effects. Moreover, the different effects induced between the RGS4 protein inhibitors used, CCG-2046 and CCG-63802, are likely to be due to the difference in mechanism of action on, and selectivity for, RGS4 proteins, as discussed in Chapter 3. Here we discuss the effects of RGS4 protein inhibitors on the major neurotransmitters and implications on the basal ganglia circuitry in relation to the pathophysiology of LID.

Previous studies have identified that corticostriatal glutamatergic transmission is enhanced in LID (Robelet et al., 2004; Dupre et al., 2011), exciting the two main striatofugal (‘direct’ and ‘indirect’) pathways of the basal ganglia. In support of this, molecular studies have shown that NMDA (Oh et al., 1998; Hallet et al., 2005; Hurley et al., 2005; Gardoni et al., 2006) and AMPA (Silverdale et al., 2010) receptor subunits are redistributed in the striatum following induction of LID. We found no effect of RGS4 protein inhibitor treatment on striatal glutamine, a by-product of glutamate in the glutamine-glutamate cycle (Behar and Rothman, 2001; Newsholme et al., 2003). Since our behavioural results supported the role of RGS4 protein inhibitors potentiating 5-HT<sub>1A</sub> receptors, a change in glutamine levels was anticipated given that administration of 5-HT<sub>1A</sub> agonist reduces striatal glutamate in LID (Dupre et al., 2011). However, our result is limited since glutamate levels were not directly measured. Moreover, it is also possible that the expression of AIMs is the cause of increased striatal glutamate (Huot and Brotchie, 2010). Previous in vivo microdialysis studies that showed increased striatal glutamate in L-DOPA-treated unilateral 6-OHDA-lesioned rats were conducted in freely moving animals (Robelet et al., 2004; Dupre et al., 2011), while our experiment was conducted in animals under anaesthesia. Taken together, these factors may have limited our findings on the effects of RGS4 protein inhibitor treatment on corticostriatal glutamatergic transmission.
In LID, hyper-inhibition of the basal ganglia output structures (SNr/ GPm) by the striatum, from an overactive ‘direct’ pathway and underactive ‘indirect’ pathway, leads to AIMs (Bezard et al., 2001a). In support of this, dyskinetic MPTP-lesioned NHPs (Papa et al., 1999; Boraud et al., 2001) and PD patients (Merello et al., 1999; Lozano et al., 2000) have reduced GPm neuronal discharge, while dyskinetic unilateral 6-OHDA-lesioned rats show increased levels of GABA in the SN reflecting an over-inhibited state (Mela et al., 2007). In our study, RGS4 protein inhibitor treatment in the dyskinetic state increased extracellular GABA in the striatum. The functional implications would be reduced activity of the overactive GABAergic striatonigral pathway, which is supported by our findings of reduced GABA levels in the SN. This would allow increased activity of basal ganglia output neurons, increasing inhibition of thalamocortical fibres, which would decrease cortical activation, and in turn reduce dyskinesia (Figure 141).

We also found increased levels of NA in the SN following acute RGS4 protein inhibitor treatment. The outcome of this on the basal ganglia circuitry is unknown. Speculatively, increased NA release could change neuronal firing of basal ganglia output nuclei, with \( \alpha_1 \) adrenoreceptor activity increasing the firing rate of SNr neurons (Berretta et al., 2000), while activation of \( \alpha_2 \) adrenoreceptors would modulate GABA release in this region (Alachkar et al., 2006). Both of these effects would directly impact the pathophysiology of LID.

Underactivity of the indirect pathway is a major pathophysiological feature of LID (Bezard et al., 2001a). Along this route, hyper-inhibition of the STN is suggested to occur because of increased activity of GABAergic GPi neurons (Mitchell et al., 1992). We found extracellular 5-HT levels are reduced in the GPi following RGS4 protein inhibitor treatment, in combination with L-DOPA, in the unilateral 6-OHDA-lesioned rat model of LID. This effect may have changed the activity of the basal ganglia circuitry to lessen expression of dyskinesia. The globus pallidus receives abundant 5-HT innervations from the raphe nuclei (Pasik et al., 1984; Vertes, 1991; Charara and Parent, 1994) and recent behavioural data showed that microinjection of 5-HT into the GPi potentiated L-DOPA-induced contraversive rotations in unilateral 6-OHDA-lesioned rats (Zhang et al., 2010). This suggests 5-HT release in the GPi can augment L-DOPA-induced behavioural responses. Given that 5-HT depolarises pallidal neurons and increases their firing rate
(Chen et al., 2008), reduced 5-HT in the GPI may help alleviate inhibition of the STN in LID. Thus, increased excitation of GABAergic GPm/ SNr neurons, as a result of increased activity of the STN, would cause increased inhibition of the hyperactive motor thalamic nuclei and a subsequent reduction of dyskinesia (Figure 141).

Taken together, our results suggest that RGS4 protein inhibitor treatment in LID, changes neurotransmitter release in the striatum, GPI and SN, which modulates the basal ganglia circuitry and reduces the expression of dyskinesia.
Mechanisms of action of RGS4 protein inhibitors in LID

Figure 141. A schematic diagram displaying proposed changes to the pathophysiology of L-DOPA-induced dyskinesia (LID) following inhibition of regulator of G-protein signalling protein (RGS) subtype 4. (A) The loss of dopamine input and repeated non-physiological stimulation of dopamine receptors leads to an overactive ‘direct’ pathway and underactive ‘indirect’ pathway. Subsequent disinhibition of motor nuclei leads to increased excitatory input to motor cortical regions producing abnormal involuntary movements. (B) Inhibition of RGS4 proteins mediates increased 5-HT_{1A} receptor signalling, reducing unregulated dopamine efflux and modulation of other neurotransmitters. The resultant effect is reduced overactivity of the ‘direct’ pathway and increased activity of the ‘indirect’ pathway, leading to greater inhibition of motor thalamic nuclei, attenuating the excitatory input to motor cortical regions, thus reducing dyskinesia. Arrow size indicates neuronal activity. Dopamine has opposite effects on the dopamine receptors. D_1R, dopamine D_1 receptor; D_2R, dopamine D_2 receptor; STR, striatum; GPI, lateral globus pallidus; GPm, medial globus pallidus; STN, subthalamic nucleus; SNr, substantia nigra pars reticulata; VL, thalamus, ventrolateral thalamic motor nucleus. Arrows represent (↑ or ↓) increase or decrease of neurotransmitters; DA, dopamine; 5-HT, serotonin; NA, noradrenaline; GABA, γ-aminobutyric acid.
Conclusion
Acute RGS4 protein inhibitor treatment, in combination with L-DOPA, potentiated 5-HT\textsubscript{1A} receptor signalling mechanisms to reduce the behavioural correlates of LID in the unilateral 6-OHDA-lesioned rat model of PD. These effects have a direct impact on the neurotransmitter release in the basal ganglia, which in turn lessens the expression of LID. Thus, RGS4 proteins may be a therapeutic target for future treatments of LID.
Chapter 6
Summary
6.1 Main findings
The main findings of this thesis are summarised below with reference to their involvement in the pathophysiology of LID and possible therapeutic implications.

In Chapter 2, the spatio-temporal distribution of RGS protein gene expression was investigated in the unilateral 6-OHDA-lesioned rat model of PD. Following 21 days L-DOPA treatment in unilateral 6-OHDA-lesioned rats, RGS4 protein mRNA levels were specifically increased in the lateral regions of the rostral striatum, which are the sensorimotor regions (McGeorge and Faull, 1969). RGS4 mRNA remained up-regulated at 1 h post L-DOPA injection, and for at least 24 h following the final dose of L-DOPA. These elevated levels of striatal RGS4 mRNA positively correlated with both behavioural (AIMs scores) and molecular (PPE-B mRNA) markers of LID.

Given that RGS4 proteins have a general role in the production of motor behaviours (Grillet et al., 2005; Lerner and Kreitzer, 2012), increased RGS4 protein activity may be directly associated with the expression of hyperkinetic movements. Thus, selectively targeting RGS4 proteins may be beneficial in reducing LID, without compromising the anti-parkinsonian effects of L-DOPA. The behavioural effects of novel RGS4 protein inhibitors were investigated in the following Chapter to uncover the roles of RGS4 proteins in the expression of LID.

In Chapter 3, the acute behavioural effects of novel RGS4-Gαo interaction inhibitors (CCG-2046 and CCG-4986) and reversible RGS4 protein inhibitors (CCG-63802 and CCG-63808), in combination with L-DOPA treatment, were investigated in the unilateral 6-OHDA-lesioned rat model of LID. Treatment with RGS4 protein inhibitors, via direct intrastriatal infusion or systemic administration, reduced expression of L-DOPA-induced AIMs and increased overall motor ability.

These behavioural data support the involvement of increased RGS4 protein activity in mediating the expression of LID. However, the roles of RGS4 proteins in L-DOPA priming, i.e. the induction of LID, have not been studied. In the following Chapter, the behavioural and molecular effects of RGS4 protein inhibition during repeated L-DOPA treatment were investigated.
In **Chapter 4**, the effects of 28 days and 7 days RGS4 protein inhibition, in combination with L-DOPA treatment, were investigated in the unilateral 6-OHDA-lesioned rat model of PD. *De novo* treatment with RGS4 protein inhibitors (CCG-2046, CCG-63802, or RGS4 antisense oligonucleotides) in combination with L-DOPA, reduced L-DOPA priming and subsequent induction of AIMS in unilateral 6-OHDA-lesioned rats. Moreover, 28 days RGS4 protein inhibition and RGS4 mRNA knockdown dampened the expression of molecular markers of LID, reducing the up-regulation of striatal PPE-B mRNA (Cenci et al., 1998; Henry et al., 2003), and decreasing the supersensitivity of striatal dopamine receptors (Aubert et al., 2005). 7 days RGS4 protein inhibition in L-DOPA-primed unilateral 6-OHDA-lesioned rats attenuated the expression of AIMS, and reduced the supersensitivity of striatal dopamine receptors, but did not reverse the increased expression of striatal PPE-B mRNA.

These behavioural and molecular data suggest that repeated *de novo* treatment with RGS4 protein inhibitors, in combination with L-DOPA treatment, in PD can reduce L-DOPA priming and induction of LID. However, 7 days treatment with RGS4 protein inhibitors can only reduce the expression, but cannot reverse L-DOPA priming and established LID. To further understand the roles of RGS4 proteins in the pathophysiology of LID, the mechanisms of RGS4 protein inhibitors were investigated in the following Chapter.

In **Chapter 5**, the mechanisms of action of RGS4 protein inhibitors were investigated in the unilateral 6-OHDA-lesioned rat model of LID. Given that RGS4 proteins negatively modulate 5-HT$_{1A}$ receptors *in vivo* (Beyer et al., 2004), the acute behavioural effects of combined subthreshold doses of an RGS4 protein inhibitor and a selective 5-HT$_{1A}$ receptor agonist, or non-selective NMDA receptor antagonist, were tested in L-DOPA-treated unilateral 6-OHDA-lesioned rats. Combined treatment regimens caused a synergistic reduction of AIMS and increased overall motor ability, while the anti-dyskinetic effects induced by intrastralatal infusion of an RGS4 protein inhibitor were blocked following antagonism of 5-HT$_{1A}$ receptors. Next, the acute effects of RGS4 protein inhibitor treatment on neurotransmitter release in basal ganglia were investigated using *in vivo* microdialysis in the unilateral 6-OHDA-lesioned rat model of LID. Acute RGS4 protein inhibitor treatment, in combination with L-DOPA, reduced unregulated dopamine efflux in the striatum, GPl and SN of the basal ganglia.
These data suggest that RGS4 protein inhibitors elicit anti-dyskinetic effects via direct modulation on 5-HT$_{1A}$ receptors. Moreover, RGS4 proteins operate as part of a signalling mechanism, with 5-HT$_{1A}$ and NMDA receptors, in the pathophysiology of LID. Acute RGS4 protein inhibitor treatment, like 5-HT$_{1A}$ receptor agonist treatment (Lindgren et al., 2010), reduced dopamine efflux in the basal ganglia, which is likely to be the mechanism of action for mediating anti-dyskinetic effects.

**Final Conclusion**

The data present in this thesis support the roles of RGS4 proteins in the pathophysiology of LID. These proteins become up-regulated following repeated L-DOPA treatment in PD. RGS4 proteins mediate the expression of AIMs through regulation of certain GPCR signalling pathways. The blockade of RGS4 proteins in LID, results in modulated neurotransmitter release in the basal ganglia, which lessens the expression of AIMs. Thus, inhibition of RGS4 proteins and associated signalling mechanisms could be beneficial in the treatment of LID in PD patients.
6.2 Implications of research – ‘The bigger picture’

As summarised above, the work described in this thesis shows that RGS proteins are involved in the pathophysiology of LID. The implications of these findings are that RGS proteins may represent novel therapeutic targets for treatment of neurological disorders. As scientific research starts to uncover G-protein signalling mechanisms, RGS proteins are shown to play a much more valuable role in modulating signal transduction than originally thought (Hepler, 1999; Bansal et al., 2007). Given that GPCRs represent a major portion of therapeutic targets (~60%) in the pharmaceutical industry, modulating RGS proteins may be the next step forward in treatment of disease. Following G-protein activation, RGS proteins modulate a variety of downstream responses and mediate cross-talk with multiple cellular receptors. Thus, RGS proteins are factors whereby signalling pathways converge, offering target specificity that cannot be achieved with traditional GPCR agonists/antagonists. This intracellular convergence may be a key factor for treatment of a disease where there are multiple receptors or signal transduction mechanisms involved. However, this also represents a ‘double-edged sword’, as RGS proteins could induce a number of adverse effects, incorporating non-disease affected signalling pathways. Moreover, ubiquitously expressed RGS proteins may be unsuitable therapeutic targets for this reason.

One way to avoid inducing adverse effects is to target RGS proteins, or their splice variants, which show a selective pattern of cellular distribution. The ideal scenario would be targeting RGS proteins only expressed in ‘disease affected areas’, which could provide therapeutic effects with few associated side-effects. A second approach would be conjunctive treatment regimens with current pharmacological agents. For example, a subthreshold dose of GPCR agonist combined with a subthreshold dose of a selective RGS protein inhibitor. Ideally, in this scenario, the therapeutic response is reached as a result of a synergistic effect from the RGS protein inhibitor potentiating GPCR agonist-mediated signalling in disease affected areas, while side-effects are avoided due to non-overlapping expression of the GPCR and RGS protein in unaffected areas. Thus, research into identifying specific RGS proteins in disease states would be invaluable for developing future treatments. However, care must also be taken when targeting RGS proteins since they have complex mechanisms of action. Indeed, they have been shown to positively modulate signalling in one neuroanatomical region and negatively modulate signalling events in another, depending on the agonist bound at the GPCR (Han et al., 2010).
The work presented in this thesis offers evidence to the potential of targeting RGS proteins in disease and is worthy of further experimental investigation. These RGS proteins may offer a novel approach in modulating signal transduction pathways for future treatments of disease and, importantly, may help us further understand chemical signalling in neuroscience and other fields, adding to our knowledge of ‘how the brain and body works’.
Chapter 7
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