Group III metabotropic glutamate receptors as potential targets in the treatment of Parkinson's disease

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Group III metabotropic glutamate receptors as potential targets in the treatment of Parkinson’s disease.

Thesis submitted by
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For the degree of
DOCTOR OF PHILOSOPHY

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Abstract

Parkinson's disease (PD) involves the progressive loss of striatal dopaminergic innervation, resulting in changes in downstream basal ganglia structures. Changes in glutamatergic pathway activity are believed to underlie the cardinal symptoms of PD such as bradykinesia, tremor and rigidity. This thesis has investigated the role of group III metabotropic glutamate (mGlu) receptor agonists as therapeutic agents for PD.

Initial experiments examined whether activation of group III mGlu receptors could reduce glutamatergic transmission within the SNpr. Both broad spectrum agonists (L-SOP and L-AP4) and PHCCC were able to significantly inhibit glutamate release in slices of SNpr. These data suggest group III mGlu receptor agonists can inhibit glutamate release from terminals within the SNpr, by acting at autoreceptors. On the basis of the reversal seen with PHCCC, it is proposed that mGlu4 can mediate this effect.

Secondly, the anti-akinetic potential of group III mGlu receptor agonists were examined in behavioural studies in rodent models of PD. Intranigral injections of L-SOP, L-AP4, PHCCC and (S)-3,4-DCPG were shown to reverse reserpine-induced akinesia, suggesting activation of mGlu4 and mGlu8 may underlie this effect. In a second model of PD, the 6-OHDA lesioned model, antiparkinsonian activity was also observed following intranigral injection of L-SOP. These data support the antiparkinsonian potential of group III mGlu receptor agonists.

For all agents tested the reversal of akinesia was significantly reduced upon second application given 4 hours after the initial efficacious injection. Furthermore, in vitro release studies supported this decrease in efficacy of inhibition of glutamate release with repeated drug exposure. Regardless of mechanism, this phenomenon potentially casts doubt on the long-term usefulness of current group III mGlu agonists for long-term relief of PD symptoms.
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Abstract Publications

2003

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2004

Broadstock M., Francis P. & Duty S. Activation of group III metabotropic glutamate receptors (mGluRs) in the rat substantia nigra reverses reserpine-induced akinesia and leads to behavioral desensitization. Society for Neuroscience 2004 Meeting. Poster 677.9.


Broadstock M. & Duty S. Behavioural desensitization following repeated intranigral injections of mGlu and GABA$_B$ agonists in the reserpinised rat. pA2 online Vol2Issue4abst079P
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Introduction
1.1 The basal ganglia

1.1.1 Introduction

The basal ganglia are a series of interconnected midbrain structures, involved in the processing of motor, cognitive and limbic information. The components of the basal ganglia include the striatum (caudate-putamen), the globus pallidus (GP, external section of the globus pallidus in primates), the internal segment of the globus pallidus (GPi), or in rodents the entopeduncular nucleus (EP), the subthalamic nucleus (STN), and the substantia nigra (SN), which is further subdivided into the pars reticulata (SNpr) and the pars compacta (SNpc). The pivotal role of the basal ganglia nuclei in movement are exemplified by their involvement in the debilitating motor disorders such as Parkinson’s disease, Huntington’s Chorea and Hemiballism (Albin et al., 1989).

1.1.2 Overview of the basal ganglia circuitry

The striatum is considered to be the main input region of the basal ganglia, receiving excitatory glutamatergic inputs from the cortex. In contrast, the output nuclei of the basal ganglia comprise the SNpr and the GPi (EP), which send inhibitory GABAergic projections to the thalamic relay nuclei. The output of the striatum has classically been divided into direct and indirect pathways, and whilst both pathways use GABA as a neurotransmitter, the direct pathway also contains substance P (and / or dynorphin) whilst the indirect pathway also contains enkephalin. In the direct pathway, striatal outputs project monosynaptically to the SNpr / GPi. In contrast, the indirect pathway projects trisynaptically to the SNpr / GPi via the GPe and STN. Initial striatal neurons project to the GPe, which in turn sends inhibitory GABAergic efferents to the STN. The STN then projects via excitatory glutamatergic neurons to the SNpr / GPi, completing the circuit. Figure 1 shows a simplified diagram of the basal ganglia circuitry.
Figure 1: Simplified schematic of the normal basal ganglia circuitry.

Motor Cortex → Striatum (Caudate + Putamen) → SNpc

MRF → SI → PRF → PPN → STN → GPe → GPI (EP) / SNpr → VMT

Abbreviations:
- Enk: Enkephalin
- Dyn: Dynorphin
- RN: Reticular Thalamic Nuclei
- SNpc: Substantia Nigra Pars Compacta
- SNpr: Substantia Nigra Pars Reticulata
- Sub P: Substance P
- GPe: External Globus Pallidus
- GPi: Internal Globus Pallidus
- MRF: Mesencephalic Reticular Formation
- SI: Substantia Innominata
- PRF: Pontine Reticular Formation
- PPN: Pedunculopontine Nucleus
- STN: Subthalamic Nucleus
- VMT: Ventral Motor Thalamus

Neurotransmitter code:
- Glutamate
- GABA
- Dopamine

Figure 1: The relationship between the basal ganglia and other related regions with major connectivity to the basal ganglia.
1.2 Functional anatomy of the basal ganglia

1.2.1 Corticostriatal projections

Cortical input to the striatum occurs from all areas of the cortex, although with differing degrees of intensity; for example, the visual cortex has much fewer projections than the sensorimotor cortex, which makes extensive striatal connections. In most cases, the cruciform axodendritic cortical inputs follow a somatotopic representation, with a high degree of convergence (Parent and Hazrati, 1995f). However, individual striatal neurons do not share more than approximately 1% of cortical afferents, implying each striatal neuron receives a unique cortical input (Kincaid et al., 1998). The striatum itself is a highly homogenous structure, although it may be divided into associative (most of the putamen), sensorimotor (dorsolateral putamen) and limbic (nucleus accumbens and ventral caudate) territories. These different areas receive input from associative (frontal, temporal and parietal lobes), sensorimotor (primary motor cortices and sensorimotor cortex) and limbic (limbic cortices, amygdala and hippocampus) areas respectively (Parent and Hazrati, 1995e). This organisation into functional domains, with overlap is believed to allow integration of cortical information passing through the striatum. The targets of the corticostriatal projections are both major striatal compartments, the striosomes (or patches) and the extrastriosomal matrix, where these projections form asymmetric (excitatory glutamatergic) synapses on striatal medium spiny neurons (Smith and Bolam, 1990).
1.2.2 Nigrostriatal projections

In addition to receiving GABAergic afferents from the striatum, the substantia nigra pars compacta (SNpc) reciprocally projects dopaminergic neurons to the striatum. Similar to that which occurs within the corticostriatal projection, there is a loose topographical organisation within these projections, with medial SNpc neurons associated with limbic system, and lateral and ventral SNpc neurons related to both associative and motor striatal regions (Joel and Weiner, 2000; Haber, 2003). Resembling the corticostriatal pathway, nigrostriatal projections also make connections with striatal medium spiny neurons, although these synapses are symmetrical (generally inhibitory) in nature. In addition, approximately 60% of the dopaminergic nigrostriatal neurons synapse onto the necks of the same striatal neuron spines which are innervated at the heads by the corticostriatal pathway (Parent and Hazrati, 1995d). This arrangement therefore allows modulation of the glutamatergic corticostriatal pathway through both excitatory (via $G_\text{a}$) $D_1$ and inhibitory (via $G_\text{i}$) $D_2$ dopamine receptors. Indeed, recent electrophysiological studies have shown dopamine to exert a powerful influence on corticostriatal activities, through its action on pre-synaptic $D_2$ receptors present on some corticostriatal neurons (Bamford et al., 2004). Activation of these $D_2$ receptors decreases glutamate release from the corticostriatal pathway, and is also selective for terminals with a low probability of dopamine release. Therefore, the less a terminal releases glutamate in response to cortical stimulation, the more dopamine inhibits this release. Post-synaptic $D_1$ receptors are also present at these corticostriatal / nigrostriatal junctions, with $D_1$ receptor activation increasing NMDA responses at glutamatergic synapses (Flores-Hernandez et al., 2002). These studies suggest dopamine may act as a filter, enhancing strong corticostriatal burst activity, whilst reducing those synapses which may have been activated by burst firing erroneously (Horvitz, 2002).

Recent electrophysiological and morphological studies further suggest the presence of a GABAergic nigrostriatal projection, accounting for between 5-8%
of normal nigrostriatal neurons. The activity of this GABAergic nigrostriatal pathway (from the SNpr) was also found to be mediated by the complex actions of dopamine, although the exact significance of this pathway remains to be established (Rodriguez and Gonzalez-Hernandez, 1999).

1.2.3 Striatal cell types

Similar to most other brain nuclei, striatal cell types can be divided into two main types, projection neurons and interneurons, although the structure of the striatum is uncommon within the CNS, due to its larger proportion of projection neurons compared to interneurons. The principal projection neuron has a medium sized cell body (12-20 µm in diameter) from which arise 4-5 dendrites which are heavily laden with spines. These are the aforementioned medium spiny neurons, and account for between 77-98% of all striatal neurons (Tepper et al., 2004a). The axon of these neurons gives rise to several collaterals which generally arborise in two distinct patterns. The most common pattern is the local axonal arborisations, where collaterals contact local dendrites, or even the dendrites of the initial neuron. This allows projection neurons to engage in local circuit interactions within the striatum. The other pattern is that of extensive, non-local arborisation whose axonal collaterals extend well beyond the neuron’s dendritic area. These collaterals make up the mono or tri synaptic direct and indirect projections already discussed. The remaining neurons within the striatum are the interneurons.

1.2.3.1 Intrinsic afferents (interneurons)

Striatal interneurons are morphologically characterised by the absence of spines on their dendrites. These interneurons can be further subdivided into two main categories: (1) giant aspiny interneurons and (2) medium-sized interneurons. The giant aspiny interneurons account for between 1 and 2% of the striatal neuronal population, and have a large (20-50µm diameter) cell body, with extensive axonal collaterals. Being cholinergic in nature, these giant aspiny
neurons represent the major source of acetylcholine within the striatum. They are predominantly innervated by glutamatergic neurons from the corticostriatal pathway, and from the thalamocortical pathway. They also receive a minor dopaminergic input from the nigrostriatal pathway, with dopamine being able to modulate acetylcholine release by actions at the D2-type dopamine receptor (Chang, 1988). Their main connections are made with the projecting medium spiny neurons (Izzo and Bolam, 1988). Similar to that recently found with the nigrostriatal pathway, cholinergic synapses have also been shown to synapse symmetrically onto medium spiny neurons' dendrites and spines, suggesting a role for ACh in the modulation of projection neuron activity. Indeed electrophysiological studies have shown activation of cholinergic interneurons may modulate long-term corticostriatal transmission through a number of mechanisms. One mechanism is 

Medium-sized GABAergic interneurons can again be subdivided into three main categories, based on their neurochemistry and physiology. These are GABA, co-expressed with either parvalbumin, calretinin, or neuropeptide Y, somatostatin and NADPH diaphorase (Kawaguchi et al., 1995). Important species differences exist in interneuron subtype expression with parvalbumin-positive striatal interneurons being the most common subtype in rats, accounting for around 1% of the total; whilst in primates calretinin expressing interneurons are the most plentiful (Levesque et al., 2003a). Parvalbumin-containing interneurons have been the most widely characterised. These interneurons make multiple synaptic connections with medium spiny neurons. These multiple connections permit potent feed-forward inhibition of spiny
neurons, with single interneurons being able to inhibit spike occurrences (Tepper et al., 2004b).

1.2.3.2 Extrinsic afferents

In addition to the corticostriatal and the nigrostriatal pathways described previously, there exist two more extrinsic afferents to the striatum, the glutamatergic thalamostriatal pathway and the serotonergic pathway from the raphe nucleus to the striatum. The main sources of thalamostriatral projections are the intralaminar thalamic nuclei, together with midline and specific relay nuclei. The caudal intralaminar group of thalamic nuclei include the centromedian-parafascicular complex in primates, which projects widely to the striatum. The rostral parafascicular nucleus mainly projects to the limbic striatum, whilst the medial centromedian nucleus is the main source of striatal sensorimotor input (Parent and Hazrati, 1995c). Another important source of thalamostriatal fibres are the ventral motor thalamic nuclei. Ventral motor thalamic nuclei and motor cortices send convergent inputs to the sensorimotor striatum, suggesting interactions between corticostriatal and thalamostriatal inputs in motor behaviour (McFarland and Haber, 2000). A substantial proportion of parafascicular nucleus neurons projecting to the striatum also send sparse collaterals to the cerebral cortex, indicating thalamic pathways may be able to modulate activity of the corticostriatal pathway. Whilst the principal targets of both corticostriatal and thalamostriatal pathways are the medium spiny neurons, these pathways differ in terminal sites, with the corticostriatal pathway terminating on the heads of distal dendritic spines, and the thalamostriatal pathway forming synapses on proximal dendritic shafts. Since ultrastructural studies have suggested neither pathway converges on the same medium spiny neuron (Parent and Hazrati, 1995b), this probably does not represent any hierarchical organisation of control of medium spiny neuron activity. Moreover, approximately 75% of thalamostriatal projections form synapses more frequently with those striatofugal fibres forming the indirect,
rather than the direct pathway (Smith et al., 2004b). Interestingly, thalamostriatal fibres are not closely associated with post-synaptic nigrostriatal targets, supporting the view that the nigrostriatal pathway primarily modulates the corticostriatal input. The other targets of the thalamostriatal pathways are the interneurons, with cholinergic interneurons receiving substantial input from the centromedian-parafasicular complex. GABAergic interneurons also containing parvalbumin are similarly significantly innervated by thalamostriatal neurons in primates, although this thalamic innervation is relatively scarce in rodents (Smith et al., 2004a).

The other major inputs to the striatum are the serotonergic pathways from the raphé nuclei, which form asymmetrical synapses on the dendritic spines of the medium spiny neurons. Due to the presence of multiple 5-HT receptor subtypes on medium spiny neurons and interneurons, activation of these afferents from the raphe nuclei have been shown to profoundly influence both cholinergic and dopaminergic systems within the striatum (Parent and Hazrati, 1995g).

1.2.4 The striatofugal system

The striatum predominantly projects to three other anatomically distinct basal ganglia structures, the internal and external segments of the globus pallidus (GPI and GPe) and the substantia nigra pars reticulata (SNpr). The functional segregation of inputs to the striatum (associative, sensorimotor and limbic) is maintained, leading to topographical maps within striatal output structures too. The SNpr receives greater input from the associative striatal territories versus sensorimotor, whereas the inverse is true for the GPI. For the sensorimotor modality, striato-pallidal projections form rostrocaudal bands within the ventrolateral GPe whereas, in the SNpr, the striatonigral projections formplexuses, some of which make contact with the descending dopaminergic columns from the SNpc. Whilst these pathways have traditionally been divided into those projecting to the GPI, GPe or the SNpr, recent anatomical studies
suggest this is an oversimplification, at least in the primate. Up to 90% of striatofugal fibres in monkeys arborise extensively in all three target structures (Levesque et al., 2003b), although each axon appears to have a preferential target structure, where upon it arborises extensively (Parent et al., 2001a)

1.2.4.1 The direct pathway: striatonigral and striatopallidal (striato-entopeduncular nucleus) projections

The striatonigral projections form “onion-like” lamellae within the SNpr, each associated with a separate striatal modality. Indeed, each part of the striatum has been mapped to well-defined positions within these layers (Deniau et al., 1996). This topographical organisation of the striatonigral projection supports the view that cortical input to the striatum is then functionally subdivided into differing modalities, which when processed return to the cortical area from which they arose – the parallel processing view. Indeed, recent 3D mapping of the dendritic fields of projection neurons in the SNpr show remarkably narrow fields, merely slightly exceeding the striatonigral lamellae, and in some cases overlapping them (Mailly et al., 2001). In addition, due to the ever decreasing numbers of neurons from the striatum to the SNpr (2.79 x 10^6 decreasing to 26,300 in the SNpr (Oorschot, 1996a)) together with the observation that only 3 nigral neurons can occupy the projection field of any striatal district, convergence of striatal inputs within the SNpr is believed to occur. This represents a mechanism for integration of different striatal modalities (Smith et al., 1998).

Similarly, the striatopallidal (striato-entopeduncular nucleus) projections are also topographically organised. Retrograde transport studies using wheatgerm-agglutinin-horseradish peroxidase injected into the EP of rats showed the dorsal striatum projects to the dorsal EP whilst the ventral striatum projects to the ventral EP (Fink-Jensen and Mikkelsen, 1989). In the case of the EP, the reduction in numbers of neurons in the striatum (2.70 x 10^6) to those within the EP (3,200) is striking (Oorschot, 1996b). Once again, this reduction in neuronal
numbers most probably similarly represents convergence of striatal inputs within the GPi (EP).

Activation of the direct pathway results in inhibition of output nuclei of the basal ganglia, due to GABAergic neurotransmission. This, in turn will result in disinhibition of target nuclei in the thalamus / motor cortex and according to the basal ganglia model outlined above (and represented in Figure 1), will result in a net increase in movement.

1.2.4.2 The indirect pathway

In contrast with the direct circuit, the indirect pathway consists of an additional two brain nuclei, through which striatal information flows before passing to the output nuclei. Thus, striatal GABAergic projection neurones connect firstly to the external segment of the globus pallidus (GPe) before passing either monosynaptically to the SNpr / GPi, or to the subthalamic nucleus (STN). The glutamatergic subthalamic neurons then project to the SNpr / GPi, completing the circuit. Activation of the direct pathway results in inhibition of GPe neurones, which in turn alleviates the tonic inhibition of STN neurones, resulting in an increased excitatory drive to the SNpr / GPi. This enhanced activation of the inhibitory output nuclei further inhibits thalamic relay nuclei feedback to the motor cortex, and results in decreased movement. Thus activation of both direct and indirect pathways can modulate movement, though with opposing influences.

1. External segment of the globus pallidus (GPe)

The GPe receives two main inputs: a GABAergic pathway from the striatum, and a glutamatergic pathway from the STN. In addition, less prominent inputs have been observed from the SNpc, the dorsal raphé nucleus and the pedunculopontine nucleus. As with the nigrostriatal pathway, the topographical
organisation of the striatum is maintained within the GPe, with sensorimotor striatal areas projecting predominantly to the ventrolateral GPe (Parent, 1990). The main pallidofugal targets are the STN, the SNpr / G Pi and to a lesser extent, the striatum. On the basis of their axonal targets, GPe neurons can be divided into four main groups: (1) neurons projecting to the G Pi, STN and SNpr (2) neurons projecting to the G Pi and STN (3) neurons targeting the STN and SNpr and (4) neurons projecting to the striatum (Parent et al., 2001b). Phenotypically all these projections are GABAergic in nature.

Classically, the GPe was viewed as being little more than a relay to the STN, permitting the excitatory glutamatergic inputs from the STN to counter-balance the inhibitory GABAergic striatal inputs within the SNpr / G Pi. However, this view has been challenged with the discovery of the pallidostratial and pallidonigral pathways. The pallidostratial pathway projects to the parvalbumin-positive GABAergic interneurons, making contact on their proximal regions. Thus, the pallidostratial pathway can reciprocally control striatofugal pathway activity, via selective inhibition of these interneurons (Bolam et al., 2000). The GABAergic pallidonigral pathway projects to the SNpr / G Pi, and makes symmetrical contact with the proximal dendrites of these neurons. This arrangement may allow pallidal modulation of either striatal or subthalamic afferents within the output nuclei, permitting a further level of modulation of basal ganglia output.

The pallidosubthalamic projection comprises the majority of the remaining GPe efferent neurons. This pallidosubthalamic pathway is again somatotopically and topographically organised, and whilst in rodents virtually the entire STN receives afferents from the GPe this distribution is more complex in primates (Parent and Hazrati, 1995). Of the primate pallidal GABAergic projections, two-thirds contact the cell bodies and proximal dendrites of STN neurons in a symmetrical fashion, with the remainder connecting with the distal dendrites. This pallidosubthalamic pathway is the principal inhibitory projection to the STN (Fonnum et al., 1978). Through these inhibitory connections, the GPe is able to exert powerful
modulatory actions upon the STN, being able to suppress spontaneous firing of
STN neurons and inhibit the excitation of STN neurons through other afferents,
such as corticosubthalamic afferents detailed below (Parent and Hazrati, 1995k)
As the GPe also receives a powerful reciprocal excitatory input from the STN,
this GPe-STN loop is believed to form a shared control mechanism, whereby
activation of one nucleus is able to control the activity of the other. The
importance of this GPe-STN loop can be seen from changes in the activity of
this pathway in disease states such as Parkinson’s disease, discussed in more
detail in section 1.3.2.2.

II. The Subthalamic nucleus

The efferent projections from the subthalamic nucleus (STN) form the basis for
the studies reported in chapters 3-5 of this thesis. Therefore the anatomy of the
STN has been discussed in some detail below.

Similar to other nuclei within the basal ganglia, the STN is topographically
organised, with the dorso-caudal third of the nucleus being related to motor
circuits (Joel and Weiner, 1997). The STN receives two main inputs, the
inhibitory pallidosubthalamic pathway and the excitatory corticosubthalamic
pathway. In addition to these inputs, other pathways have been described
including the glutamatergic thalamosubthalamic, the dopaminergic
nigrosubthalamic, the cholinergic and glutamatergic pedunculopontine-
subthalamic and the serotonergic raphé-subthalamic projections. The main
efferent projections include the subthalamopallidal pathway and the
subthalamonigral projection, although lesser projections to the striatum and
PPN have also been described (Parent and Hazrati, 1995j). The STN is
principally composed of projection neurons whose dendrites are sparsely
covered with spines (Sato et al., 2000).
The direct corticosubthalamic pathway arises predominantly from the primary and supplementary motor areas in the cortex, and is mainly composed of axonal collaterals from the pyramidal tract or the corticostriatal pathway (Hamani et al., 2004b). These axons make asymmetric glutamatergic synapses with the small dendrites of subthalamic neurons which predominantly project to the GPe. Electrical activation of the primary motor cortex thereby results in two excitatory peaks separated by a brief period of inhibition within the STN. The origin of the initial excitatory response is the direct mono-synaptic corticosubthalamic pathway. After the initial excitatory response from the STN, the inhibitory response then arises through reciprocal interactions between the STN and GABAergic efferents of the GPe. The second excitatory response is complex, involving the cortex and nucleus accumbens (Maurice et al., 1998). Since the cortico-subthalamic neurons synapse with the subthalamopallidal projections, there exists no pathway in which cortical inputs may affect the output nuclei of the basal ganglia directly.

The subthalamopallidal pathway innervates the dendritic shafts of almost all GPe neurons, forming asymmetric glutamatergic synapses which lie in dense bands. The organisation of these bands is similar to that observed with the striatopallidal pathway, and ultrastructural studies have shown convergence of inputs from both striatopallidal and subthalamopallidal pathways onto the same pallidal output neurons. This projection forms the other half of the reciprocal loop between the GPe and the STN. The other subthalamopallidal (subthalamo-entopeduncular nucleus) pathway is formed from collaterals of STN efferents innervating the GPe. In the rat these single axon collaterals commonly innervate GPe, the EP and the SNpr, whilst in the primate the axonal collateral innervation field is relatively small (Parent and Hazrati, 1995i).

The subthalamonigral projection innervates both segments of the substantia nigra, although the majority of these fibres selectively innervate the SNpr. The remainder innervate the SNpc, and may modulate the subsequent activity of the
nigrostriatal pathway. Upon entry to the SN, subthalamo-nigral neurons arborise extensively, forming local collaterals which predominantly contact dendritic shafts of nigral neurons (Hamani et al., 2004a). Whilst this pathway accounts for the majority of glutamatergic neurotransmission within the SN, overall glutamatergic synapses only account for a minority of the total synapses present, with those immuno-positive for GABA accounting for the majority (Rinvik and Ottersen, 1993). Similar to that observed with other basal ganglia pathways, the subthalamonigral projection neurons have also been shown to converge with striatonigral and pallidonigral inputs, upon single nigral target neurons (Kolomiets et al., 2003). This convergence of inputs could allow interactions between parallel pathways within the basal ganglia.

1.2.5 The thalamocortical loop

Thalamocortical loops from the ventral anterior and ventral lateral thalamic nuclei innervate distinct cortical areas. The ventral anterior pathways predominantly innervate caudal cortical regions and are primarily involved in movement, whilst those projections from the ventral lateral nuclei innervate the rostral motor areas and are primarily involved in cognitive aspects of motor function, including motor learning (Haber and McFarland, 2001b). Whilst the exact mechanisms by which the thalamocortical loop serves to modify motor behaviour are not yet known, this final link within the basal ganglia is well placed to integrate limbic, associative and sensorimotor inputs to produce the desired outcome.

1.2.6 Net result of basal ganglia function

The initiation of movement is brought about through activation of the 'direct' cortico-striato-pallidal circuit. Activation of these projections results in inhibition of the output nuclei, and resultant disinhibition of thalamic nuclei, which in turn
leads to activation of the thalamo-cortical loop, returning to the motor cortex. Conversely, cessation of movement results from activation of the 'indirect' cortico-striatal-pallidal-subthalamic circuit. This leads to stimulation of the output nuclei, resulting in inhibition of thalamo-cortical afferents. The temporal balance between these two pathways is such that following direct pathway activation, and resulting initiation of movement, indirect pathway activation serves to either focus the resultant movement, or inhibit further unwanted movements (Haber and McFarland, 2001a). This complex interplay of pathway activation is dependent on integration of the actions of many neurotransmitters, loss of which results in basal ganglia dysfunction and resultant aberrations in motor control, as exemplified by Parkinson's disease.

1.3 Basal ganglia dysfunction: Parkinson's disease.

Parkinson's disease (PD) is a common neurodegenerative disorder, second only in prevalence to Alzheimer's disease. It was first described by James Parkinson in 1817 in his seminal 'Essay on the Shaking Palsy'. PD is a progressive disorder, with incidences increasing in the U.K. from 4-12.5 per 100,000 at age 49 to 664-708 per 100,000 at age 70 (von Campenhausen et al., 2005). Pathologically, the disease results from loss of nigrostriatal dopaminergic terminals and extensive degeneration of this pathway is required for clinical signs to be apparent. Even relatively mild forms of the disease are only present once 70-80% of dopaminergic terminals are lost within the striatum, and approximately 50% of dopaminergic cells lost within the SNpr (Zigmond et al., 2002). Whilst any disease that affects striatal dopaminergic transmission may lead to parkinsonism, PD is the most common, accounting for 80% of cases. PD is characterised by tremor at rest, rigidity, bradykinesia, hypokinesia or akinesia, postural instability and freezing. In addition to these cardinal symptoms, paucity of normal facial expression (hypophonia), decreased size of writing (micrographia) and decreased stride length occur. Abnormalities of affect and
cognition are also present, with many patients suffering depressive symptoms and slowness of cognition (bradyphrenia). Dementia, especially in the elderly may also be present (Dauer and Przedborski, 2003a). Histologically, neurons from Parkinson's disease patients have intracellular inclusions called Lewy bodies, which are characteristic of the disease. 95% of PD cases are believed to be unaffected by genetics, with the remainder having some form of genetic inheritance.

1.3.1 Pathogenesis of Parkinson's Disease

Whilst the degeneration of dopaminergic neurons has been well characterised in PD, the underlying causes of the cell death only now being disclosed. At least four different mechanisms have been proposed to be involved in nigral cell death. These include: mitochondrial dysfunction; oxidative stress; excitotoxicity and proteasome dysfunction. More recently these differing modalities have been interlinked and it would now appear that all have some role to play in the initiation of PD.

Mitochondrial dysfunction and oxidative stress are both hallmarks of PD. The environmental toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes PD symptoms in both human illegal drug abusers and animal models of PD. Astrocytes metabolise MPTP to 1-methyl-4-phenyl pyridium (MPP+) via monoamine oxidase B. MPP+ is then selectively taken into neurons by the dopamine transporter where it inhibits mitochondrial respiration at complex I (Bossy-Wetzel et al., 2004). This inhibition of complex I interrupts the flow of electrons along the electron transport system, and leads to an increased production of reactive oxygen species. Mitochondrial complex I inhibition also results in a loss of cellular ATP. Since the packaging of dopamine into cellular vesicles is an ATP-dependent process, depletion of ATP inhibits the vesicular monoamine transporter VMAT2. This reduction in VMAT2 activity results in increased intracellular dopamine which may in turn result in increased oxidative
stress through the generation of dopamine oxidation by-products such as dopamine quinones. These quinones can bind to the antioxidant glutathione, reducing the unbound reduced glutathione \(N\)-(N-L-gamma-glutamyl-L-cysteinyl-glyceine, or GSH) pool and hence reducing the ability of neurons to protect against oxidative damage. Indeed, deficits in complex I activity have been found in the brains and platelets of PD patients (Dawson and Dawson, 2003). Impaired electron transport systems may also lead to indirect excitotoxicity, since maintenance of membrane polarity is an energy dependent process. If this process is impaired, for example through a fall in cellular ATP levels as a result of mitochondrial dysfunction, normally non-toxic levels of glutamate may activate NMDA receptors (in the absence of voltage-gated Mg\(^{2+}\)-block) and lead to Ca\(^{2+}\) influx with subsequent excitotoxicity (Greenamyre, 2001c). When compared against the rest of the brain, neurons of the SNpc appear to have higher levels of oxidative stress (Blandini et al., 2000b) and are preferentially damaged by complex I inhibitors such as rotenone (Bywood and Johnson, 2003). Thus, in response to increased oxidative stress or mitochondrial deficits, the SNpc may degenerate, initiating the pathophysiology of Parkinson’s disease within the basal ganglia.

More recently, failure of the ubiquitin-proteasome system (UPS) has been implicated in the pathogenesis of PD. The UPS is a system involved in the clearance and degradation of damaged proteins within neurons, whereby damaged proteins are tagged with ubiquitin molecules and transported to a 26/20S proteasome, where degradation occurs (Ciechanover, 2005). Failure of this system results in protein aggregation, including the formation of Lewy bodies, although it is not yet clear whether this process is toxic per se, or a cytoprotective response to other toxic events (McNaught et al., 2006a). However, treatment of dopaminergic neurons with proteasome inhibitors leads to protein accumulation and the appearance of Lewy body inclusions which contain \(\alpha\)-synuclein together with ubiquitin and damaged proteins (McNaught et al., 2006b). Since oxidative stress and excitotoxicity may increase the number of
damaged proteins and induces failure of the ATP-dependent UPS, these three potential mechanisms may converge creating a condition of proteolytic stress, which may lead to death of the neuron or cellular dysfunction (McNaught et al., 2006c).

1.3.2 Aetiology of Parkinson’s Disease: genes and environmental factors

Whilst the relative contributions of genetic and environmental agents are still hotly debated, there appears to be a consensus amongst authors that each component may have a role to play in the disorder. Supporting the view that genetic factors may be involved include research showing the occurrence of rare clusters of families with early onset (<40 years of age) PD and familial clusters of PD (Jenner, 1999). Indeed four genes have now been identified which occur in multiple families with PD, and many others in single families.

Of the four multiple-family genes identified, mutations in α-synuclein (PARK1) have received much attention recently, following the discovery of an Italian-Greek family carrying mutations in this gene who then developed early-onset parkinsonism. This autosomal dominant form of PD was associated with a single point mutation, substituting threonine for alanine at position 53. This initial discovery was then followed by the identification of α-synuclein as the major component of Lewy bodies in brains of both familial and sporadic PD patients (Bennett, 2005). In addition to the Italian family mutation, other point mutations have been discovered in unrelated German and Spanish families, and duplication or triplication copies of the gene have also been found in unrelated families typically exhibiting early-onset PD (Healy et al., 2004a). In addition, the existence of a micro-satellite repeat within the gene for α-synuclein has recently been suggested to lead to an increased incidence of PD (Mellick et al., 2005). Taken together, these data suggest either mutations or over-expression of α-synuclein to be involved in the pathogenesis of PD, and therefore reducing the expression of α-synuclein may be of therapeutic benefit in PD (Singleton, 2005).
α-synuclein is a soluble 140 amino acid protein abundant in pre-synaptic nerve terminals. Multiple proteins have been described as associating with α-synuclein, including TH, the cytoplasmic chaperones 14-3-3, tubulin and tau. It appears the toxic accumulation of α-synuclein within nerve terminals can also be linked with another autosomal dominant mutation in PD, that of Parkin (PARK2).

Mutations in the gene encoding the protein parkin were discovered to be present in approximately 50% of autosomal recessive young-onset PD (patient age <40 years). To date, there are some 1000 single nucleotide variants in the parkin gene, which are found in approximately 5% of young adults with PD. Parkin is a member of the ubiquitin ligase family, serving to attach short ubiquitin chains to damaged proteins. Thus a loss of function of parkin may result in excessive levels of α-synuclein within neurons, which may aggregate to form Lewy bodies.

Other genes in which mutations may lead to PD include DJ-1 (PARK7). DJ-1 is a ubiquitous brain protein, whose main effect appears to be protection of neurons from oxidative stress. This view is supported by experimental evidence showing down-regulation of DJ-1 lead to increased cell death from oxidative stressors, such as hydrogen peroxide, which would be rescued by overexpression of wild-type DJ-1 but not of the mutant L166P form of DJ-1 (Yokota et al., 2003).

The most recent gene to be identified as a risk factor for PD is PINK1 (PARK6). Currently, the protein product of this gene is unknown, but it is believed to encode a protein kinase. Wild-type PINK1 is believed to protect cells from mitochondrial dysfunction, this effect was reduced when cells were transfected with mutated PINK1, as found in human PD patients (Valente et al., 2004). Interestingly, as PINK1 is normally found within the mitochondria, this may form a link between mitochondrial dysfunction and PD.
Other genes which may be involved with familial PD include mutations in UCHL1 (PARK5), neurofilament medium and Synphilin-1. Autosomal dominant UCHL1 mutations have been reported for a single family in Germany, although a common variant in UCHL1 is associated with decreased PD susceptibility in the general population. UCHL1 again belongs to the ubiquitin-proteasome pathway, being an enzyme involved in de-ubiquitination, and is found in Lewy bodies. Whether mutations in either neurofilament medium or Synphilin-1 are involved in PD is currently unclear, with mutations found in only 3 people so far (Healy et al., 2004b).

Whilst these genetic mutations may account for a small minority of PD cases, the remaining causes of PD have not yet been found. With the discovery that contaminants in a synthetic meperidine analogue (MPTP) can lead to juvenile onset PD, the role of environmental agents has also been suggested. The environmental agents currently believed to increase the likelihood of developing PD include: pesticide exposure, rural living, well-water consumption and heavy metal exposure. On the other hand, both smoking and caffeine intake have been negatively correlated with PD.

The potential for pesticide exposure to lead to PD was made more intriguing by the finding that the structure of MPP⁺ (the ultimate ion responsible for nigral damage induced following MPTP exposure) closely resembles that of the commonly used pesticide paraquat. Some studies have suggested a role for paraquat in nigral cell loss following chronic exposure (Di Monte et al., 2002). Furthermore, recent studies have shown up-regulation of α-synuclein following chronic paraquat exposure in the substantia nigra of mice, suggesting an a commonality between paraquat-induced nigral damage and genetic processes (Manning-Bog et al., 2002). Additionally a naturally occurring pesticide, rotenone has been used to develop a rodent model of PD, showing behavioural deficits, nigro-striatal pathway damage, and cytoplasmic inclusions of α-
synuclein (Betarbet et al., 2000). In common with paraquat, rotenone can also induce α-synuclein fibrillation; both agents appear to exert their effects through increasing the concentration of a partially-folded intermediate of α-synuclein, which subsequently leads to aggregation (Uversky et al., 2002). Since both MPP⁺ and rotenone are inhibitors of mitochondrial complex I, this raises the possibility that oxidative stress plays an important role in the pathogenesis of PD, as previously mentioned.

Both rural living and well-water consumption have been examined as risk factors for developing PD, although the epidemiological studies are so far unequivocal (Lai et al., 2002). This spread of epidemiological data makes interpreting the effects of rural living and well-water consumption difficult.

Contrastingly, the suggestion that chronic exposure to metals increases the risk of PD has been supported by most epidemiological data. Studies have shown increased association of PD with iron, copper, aluminium, manganese, zinc and lead in metal workers exposed to these metals for more than 20 years (Gorell et al., 1997). It is thought that some of these, e.g. zinc, iron and copper may exert their effects through production of free radicals, which in turn enhance oxidative damage to neurons (Veldman et al., 1998b).

Numerous epidemiological studies have suggested a decreased risk of PD amongst smokers. The combined risk of developing PD is half in smokers, when compared against non-smokers. A variety of hypotheses have been suggested to explain the protective action of smoking including: decreased MAO-B, stimulation of dopamine release and up-regulation of nicotine receptors. Decreased MAO-B, as found in the brains of smokers may inhibit some, as yet unidentified, toxic environmental component (Veldman et al., 1998a); since MAO-B is involved in the conversion of MPTP into the toxic metabolite MPP⁺. Nicotine is also capable of stimulating dopamine release within the striatum (Visanji et al., 2005) and may itself be neuroprotective (O'Neill et al., 2002). The
other environmental agent which may reduce the risk of PD is caffeine. The risk of PD in non-coffee drinkers was more than double that compared to coffee-drinkers (Siderowf and Stern, 2003). The actions of caffeine may be due to its pharmacological antagonism of adenosine $A_{2A}$ receptors.

1.3.3 The Parkinsonian basal ganglia

Following degeneration of the nigrostriatal pathway, a number of alterations are initiated within the basal ganglia circuitry (summarised in figure 2). Loss of striatal dopamine results in changes in activity within both the direct and the indirect pathway. The direct striato-nigral pathway loses the $D_1$-like mediated excitation, and thus becomes under-active. By comparison the striatopallidal pathway loses the $D_2$-like mediated inhibition, thus this portion of the indirect pathway becomes overactive. This over-activity results in inhibition of the GPe, which subsequently results in disinhibition of the STN, hence the STN becomes overactive. The over-activity of the excitatory subthalamonigral pathway, coupled with the loss of the inhibitory striatonigral pathway results in a net increase in activity of the GPi / SNpr. The over-activity of the output nuclei subsequently inhibits thalamic relay nuclei and thus inhibits thalamocortical feedback. It is this inhibition of thalamocortical feedback that is believed to underlie the bradykinetic / akinetic symptoms of PD.
Figure 2: Simplified diagram showing changes in the basal ganglia following nigrostriatal degeneration.

Figure 2: Changes in basal ganglia neurotransmission following dopaminergic denervation of the striatum.
1.3.3.1 Consequences of dopamine denervation on the striatum

Loss of dopamine has several actions within the striatum including: alteration of the corticostriatal pathway and modulation of both the direct and indirect pathways, as outlined in section 1.3.2. Loss of endogenous dopamine results in decreased activation of the inhibitory pre-synaptic corticostriatal D$_{2}$-like receptors. This loss of control of glutamatergic transmission then results in increased excitation of striatal neurons both directly, through increased AMPA and NMDA mediated excitation and indirectly, through loss of dopamine mediated cortico-striatal LTD (Calabresi et al., 1996). Slices obtained from dopamine-denervated animals reinforce this view, since glutamate-mediated depolarisations occur more often than in control animals (Calabresi et al., 1993).

In addition to the influences on the activity of the GABAergic striatofugal pathways described above, dopamine depletion also leads to alterations in the levels of peptides within these pathways. In the direct pathway, the loss of excitatory effects of dopamine mediated by the D$_{1}$-like receptors results in decreased expression of both Substance P and dynorphin. Conversely, the loss of D$_{2}$-like-mediated inhibition in the indirect pathway has been linked with an increase in enkephalin expression. These alterations in peptides can also be reversed by selective dopamine agonist application, indicating the alterations in marker levels within the striatofugal neurons may reflect changes in dopaminergic input to the striatum (Gerfen, 2000).

The alterations in activity within the striatofugal pathways proceed to influence the other basal ganglia structures including the GP and STN. This over-activity of the STN underpins much of the experimental chapters described later in this thesis and therefore this particular aspect of the parkinsonian basal ganglia will be discussed in more detail than other sections.
1.3.3.2. Consequences of dopamine denervation on the globus pallidus and the subthalamic nucleus.

Alterations in STN activity resulting from loss of dopaminergic innervation, underpin much of the experimental data presented in later chapters, for this reason this particular part of the basal ganglia will now be discussed in more detail.

Ever since the model of basal ganglia anatomy and function was proposed by Albin and colleagues in 1989, the fate of the GP and the STN following loss of striatal dopamine has been contentious. According to this model, loss of the dopaminergic nigrostriatal pathway results in reduced activity of the GPe which in turn disinhibits the STN, resulting in STN over-activity. Initial experimental evidence suggested this view was correct, as uptake of 2-deoxyglucose (2-DG), a marker of synaptic afferent activity, is decreased by 17-26% in the STN of MPTP-treated monkeys compared to controls (Mitchell et al., 1989). This suggested a decreased inhibitory input from the GPe to the STN in line with the suspected decrease in activity of the GPe. In addition recordings from the GPe of MPTP-treated monkeys showed reduced average firing rates compared to normal animals (Filion and Tremblay, 1991). Together these results suggest the GPe is under-active in animal models of PD. Since the GPe has close reciprocal anatomical connections with the STN, (outlined in section 1.2.4.2), under-activity of the GPe is predicted to directly underpin the over-activity of the STN.

Over-activity of the STN has been well documented both in animal models of PD and in PD patients. Thus, in monkeys, the spontaneous firing rate of STN neurons increased from 19 to 26Hz following MPTP treatment (Bergman et al., 1994b). In reserpine and α-methyl-para-tyrosine treated rats, where striatal dopamine levels were acutely decreased by 94% concomitant activity of STN neurons was increased by 53% (Robledo and Feger, 1991). Following chronic striatal dopamine depletion by 6-OHDA, STN neurons increased their discharge
rates even further, to 233.3% compared to non-lesioned animals (Magill et al., 2001).

Thus increases in cytochrome oxidase subunit-1, CO-I (a marker of metabolic activity) have been observed in the STN of MPTP-treated monkeys. Interestingly, this increased CO-I expression was reversed by treatment with the antiparkinsonian medication, L-DOPA. (Vila et al., 1997). In the same study, CO-I levels in the STN from PD patients (who were treated with L-DOPA prior to morbidity) showed no changes compared to control, suggesting that normalising striatal dopamine may restore the striatopallidal pathway, and hence restore inhibition of the STN. A direct 10 to 16 fold increase in glutamate levels (measured by microdialysis) in the output nuclei of the STN, (the SNpr and the GP) has also been noted in 6-OHDA lesioned rats, compared to non-lesioned animals (Windels et al., 2005b).

That this over-activity of the STN, and subsequent increase in glutamatergic transmission in the GPi / SNpr contributes to PD symptoms is supported by the anti-parkinsonian efficacy of subthalamotomy, which serves to reduce excessive glutamatergic transmission to the SNpr (Alvarez et al., 2005c). In further support, systemic administration of the N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) antagonists, MK-801 and LY293558 either alone or in combination with L-DOPA in 6-OHDA lesioned rats produced anti-parkinsonian efficacy (Vila et al., 1999). Indeed, targeting of over-active glutamatergic subthalamic terminals is the rationale behind various therapeutic approaches, discussed in sections 1.3.3.3 and 1.4.

Despite this certainty that over-activity of the STN is evident following dopamine denervation, the pivotal role played by under-activity of the GP is now under close scrutiny for a number of reasons. Whilst decreased firing rates were reported for GPe neurons of MPTP-treated monkeys, this was associated more specifically with alterations in firing pattern to a burst-type firing. This switch of
firing is indicative of more excitatory stimulation when compared to normal. Similar alterations in firing patterns have also been observed in 6-OHDA lesioned rats, which only displayed modest (approximately 20%) decreases in pallidal activity three weeks post-lesion (Levy et al., 1997a). Therefore it is possible that the GP is not firing at a decreased rate in PD, rather that the loss of dopamine modifies the pattern of firing in GPe neurons.

Moreover, the reduction in 2-DG uptake in the STN previously described may reflect changes in intrinsic neuron activity, rather than changes in GABAergic inputs from the GPe. It has been suggested that 2-DG uptake cannot differentiate between excitatory or inhibitory inputs to a nucleus, nor distinguish between pre-synaptic or post-synaptic transmission (Parent and Cicchetti, 1998). Furthermore, no decrease in GPe GAD₆₇ mRNA (a marker of GABAergic transmission) was observed in either PD patients, or MPTP-treated monkeys, although this was the case for the SNpr and GPi, in support of GAD₆₇ as a marker of GABAergic neurotransmission (Levy et al., 1997b).

Additionally, 3 weeks after intranigral 6-OHDA administration, activity levels of the STN neurons were increased by 105.7% and were similarly associated with increased burst-type firing. In contrast, lesion of the pallidosubthalamic pathway alone only produced modest increases in STN firing rates (19.5%), without associated switching to burst firing (Hassani et al., 1996). Therefore it appears that the activity levels of the STN are not solely controlled by the under-activity of pallidal neurons, and therefore suggests the involvement of other brain nuclei.

Whilst pallidal activity alone is no longer believed to underpin the changes in overall activity of the STN, recent reports have suggested an involvement of the GPe in the generation of subthalamic burst firing. Under normal resting conditions, STN neurons are spontaneously active, with an irregular firing pattern and low-frequency oscillations between 10 and 30 Hz (Wichmann et al., 1994). By contrast, both in animal models of PD and PD patients there is a
switch to rhythmic burst firing which is apparently related to tremor (Bergman et al., 1994a; Levy et al., 2000). Studies performed in STN-GPe cultures in the absence of dopamine suggest the GPe does indeed have a crucial role in the generation of synchronous bursting of STN neurons (Plenz and Kital, 1999). In unlesioned mouse parasagittal brain slices, elevated pallidosubthalamic transmission brought about by high-frequency stimulation of pallidal fibres, resulted in post-synaptic GABA\textsubscript{A} and GABA\textsubscript{B}-receptor mediated hyperpolarisation of STN neurons followed by a GABA\textsubscript{B}-receptor mediated subsequent rebound STN depolarisations, possibly accounting for the generation of burst firing within the STN (Hallworth and Bevan, 2005).

If a decrease in inhibitory transmission from the GPe does not underlie the increase in STN activity, is it possible that this is brought about by increases in excitatory inputs? Excitatory glutamatergic inputs to the STN arise from the PPN, the PF complex within the thalamus and the cerebral cortex (section 1.2.4.2). In support of an increase in glutamatergic transmission being a serious contender, the level of CO-I in PPN and PF neurones projecting to the STN in 6-OHDA lesioned animals was significantly increased (123 and 62% respectively) compared to controls (Orieux et al., 2000). These data suggest increased glutamatergic transmission from thalamic / pedunculopontine nuclei may indeed underlie some of the increased STN activity. Additional support for an overactive PPN in PD comes from electrophysiological studies showing increased PPN firing rates following a 6-OHDA lesion of the SNpc. Furthermore following STN ablation, these observed increases were reduced to baseline levels (Breit et al., 2001). This increased activity of the PPN can be explained through the reciprocal connections from the STN, itself overactive following dopamine depletion. This explanation would also account for the increased 2-DG uptake seen in the PPN following 6-OHDA lesion (Hirsch et al., 2000). However, since the PPN appears to be under the influence of the STN, it is difficult to reconcile how STN over-activity initially arises. One possibility is that following IPSP generation from SNpr terminals in the PPN, the PPN generates strong rebound
spike generation (Kang and Kitai, 1990), which initiates STN over-activity. Support for this hypothesis is the finding that the GABAA-receptor antagonist, bicuculline, when directly injected into the PPN is able to alleviate akinesia in the MPTP-treated monkey, with the magnitude of this effect being comparable to the effects of oral L-DOPA (Nandi et al., 2002). Nevertheless, the PPN itself degenerates in PD, with post-mortem PD tissue showing only 43% PPN neurons compared to control. Furthermore, thalamic nuclei also degenerate in PD, with significant reductions in the CM-PF complex in post-mortem human PD brains compared to age-matched controls (Henderson et al., 2000). One possible explanation for these striking decreases in neurons is the over-activity of the STN, once initiated, leads to excitotoxicity of the remaining PPN and thalamic neurons, in a similar fashion to the excitotoxic hypothesis of degeneration of the SNpc. It is therefore difficult to reconcile the involvement of the PPN in the generation of STN over-activity, suggesting alterations in transmission of a number of other structures may also be involved.

Whilst the PPN-STN pathway appears to be over-active following loss of dopamine, the reverse appears to be true for the corticosubthalamic pathway. Following 6-OHDA lesion of the SNpc, CO-I expression in the motor and dorsal insular cortex neurons projecting to the STN was significantly decreased compared to sham-lesioned animals (Orieux et al., 2002). It is therefore likely that the corticosubthalamic pathway is under-active (if changed at all) following dopamine depletion. This decreased activity may be due to decreased thalamocortical feedback or degeneration of this pathway, as has recently been demonstrated to occur in the corticostriatal pathway of PD patients (Stephens et al., 2005).

One further possibility for the over-activity of the STN is that the degeneration of the SNpc initiates changes in dopaminergic transmission in extra-striatal regions. The STN receives a dopaminergic input from the SNpc and degeneration of this pathway may result in similar changes to those observed in
the striatum. Both D₁ and D₂ receptor types have been shown to be present within the STN (Hassani and Feger, 1999). In rats with a 6-OHDA lesion intrasubthalamic injections of apomorphine (a mixed D₁ and D₂ agonist) and quinpirole (a selective D₂ agonist) increased STN neuron discharge rates together with induction of c-Fos (an immediate early gene, taken here as a marker of neuronal activity to dopamine agonists). In contrast, the D₁ selective agonist, SKF 82958 decreased the discharge rate and only induced a few c-Fos-positive nuclei (Hassani and Feger, 1999). These authors suggest that this rather paradoxical response to dopamine receptor agonists is brought about by D₁-mediated pre-synaptic inhibition of glutamate release from the cortico-subthalamic neurons, coupled with a D₂-mediated inhibition of pre-synaptic GABA release from GPe neurons. The predominant role of D₂ receptors has also been demonstrated in binding studies, with 6-OHDA lesioned rats having increased [¹²⁵I]sulpiride binding to D₂ receptors in the STN, with a reversal of this lesion-induced increase by oral L-DOPA (Murer et al., 1999). Taken together these data suggests a complex co-ordination of basal ganglia function through the actions of dopamine within the STN, in conjunction with alterations in both the type and rate of transmission in pallidosubthalamic and pedunculopontinesubthalamic pathways following degeneration of the SNpc. Clearly however, much remains to be resolved regarding STN overactivity in PD.

Regardless of the mechanism underlying the over-activity of the STN, increases in activity of both the STN and PPN neurons projecting to the SNpc have been shown in the pre-symptomatic phase of PD (Bezard et al., 2003).

1.3.3.2 Effects of dopamine denervation on the output nuclei of the basal ganglia (SNpr and GPi) and thalamocortical feedback.

The changes discussed above bring about profound alterations in the output nuclei of the basal ganglia. Since the SNpr and the GPi both receive increased
glutamatergic transmission from the over-active STN, coupled with decreased GABAergic transmission from the striatum, the net result proposed by the model is an increased GABAergic output from the SNpr and the GPi. Support for this view comes from electrophysiological recordings from the GPi of MPTP-treated monkeys, where one-third of GPi neurons developed burst firing and increased frequency of firing, when compared to control monkeys that never developed burst firing (Bergman et al., 1998). Furthermore, recordings were taken from the GPi of patients undergoing pallidotomy for advanced PD; following doses of apomorphine which reversed akinesia and tremor, the firing rates in GPi neurons were reduced by approximately one half (Lozano et al., 1998b). These results suggest reversal of PD symptoms is associated with a decrease in firing rates of GPi neurons following dopamine agonist injection, and therefore support an increase in firing underlies the symptoms per se. In rats lesioned with 6-OHDA, neurons of the SNpr have indeed been shown to increase the proportion of burst firing, an effect which can be reversed by kainic acid lesions of the STN (Tseng et al., 2000). These results support the view that STN neurones have an important influence on SNpr neurons.

Ultimately, increased activity in the SNpr and the GPi will result in increased inhibition of thalamic relay nuclei and a consequent loss of thalamocortical feedback to the motor cortex. It is this reduction in thalamocortical transmission that is believed to underpin the akinetic aspects of PD (Lang and Lozano, 1998).

1.3.3.3 Current therapies in use for Parkinson’s Disease

1.3.3.3.1 Symptomatic approaches

Since the underlying pathophysiology of PD involves the loss of dopaminergic innervation within the striatum, current therapies have focussed on dopamine replacement or replicating the actions of dopamine at dopamine receptors. The
aims are to closely mimic the endogenous transmission that degenerates in PD. The current gold-standard for PD therapy is treatment with the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA). Unlike dopamine itself, L-DOPA can effectively cross the blood-brain barrier where it is converted by DOPA decarboxylase into dopamine by the remaining dopaminergic neurons. Concomitant treatment with the peripheral DOPA decarboxylase inhibitor, carbidopa can increase the amount of levodopa entering the brain from 1 to 10% of the administered dose. As the medullary vomiting centre is not enclosed by the blood-brain barrier this has the additional benefit of preventing levodopa conversion in the medulla, thus attenuating the side-effects associated with elevated levels of dopamine in this region, such as vomiting and anorexia (Jankovic, 2002). Other advantages of levodopa include a relatively rapid onset of action and when introduced slowly and with increasing dosages, it is relatively well tolerated in patients. Despite these advantages however, levodopa does have a number of side-effects such as dietary restraints (other dietary proteins can compete with the carrier system for levodopa entry into the brain, necessitating low-protein meals when taking levodopa), hallucinations, sedation, hypersexuality, a gradual wearing off of the efficacy of L-DOPA (with patients experiencing “on” and “off” periods) and the gradual emergence of debilitating motor movements known as dyskinesia (Obeso et al., 2000a). Both motor fluctuations and dyskinesia are viewed as almost inevitable consequences of long-term levodopa therapy with around half of patients taking levodopa for more than 5 years developing motor fluctuations, of whom half again developed dyskinesia (Nutt, 2001a). The emergence of dyskinesia is believed to reflect the relentless loss of dopaminergic terminals as a consequence of disease progression. Alongside this, the sites of L-DOPA decarboxylation may change from striatal terminals to non-dopaminergic sites, such as interneurons and glia. These shifts in decarboxylation sites may contribute towards the development of dyskinesias (Guigoni et al., 2005). In addition to this putative shifting of L-DOPA decarboxylation, the unrelenting neurodegeneration results in the loss of constant dopamine stimulation (as found in the normally innervated striatum)
and shifts towards a more pulsatile stimulation of receptors. This shift towards a more pulsatile stimulation alters the electrophysiological firing patterns of basal ganglia nuclei and may also underlie the mechanism of action of the on-off effect and dyskinesias (Olanow et al., 2004b). Indeed, both short acting D₁ agonists such as SKF 82958 and D₂ agonists given to MPTP-treated monkeys by repeated pulsatile administration gives rise to increased levels of dyskinesia, when compared to continuous receptor stimulation brought about by drugs with a longer half-life (Calon et al., 2000; Jenner, 2000).

Whilst dopamine replacement therapy with L-DOPA has been shown to be highly efficacious in PD patients, some controversy remains about its possible toxicity. Both L-DOPA and dopamine are known to undergo auto-oxidation which can lead to increased levels of reactive oxygen species and free radicals (as described earlier in section 1.3.1.). These agents can then lead to lipid peroxidation and damage to DNA, possibly resulting in the apoptotic death of the neuron. A number of in vitro studies have suggested high levels of levodopa to be toxic (Fahn, 1996). However, there are many criticisms of these studies such as the lack of supporting glial components and the absence of the antioxidant, ascorbate, normally found within the CNS. Once these factors are present, it appears levodopa is no longer toxic to cultured dopaminergic neurons (Mena et al., 1997). Indeed, almost no in vivo data support the hypothesis that levodopa is toxic, as no reductions in dopaminergic SNpc neurons were found in normal rats or mice treated with L-DOPA for up to 18 months (Olanow et al., 2004a), nor in patients given L-DOPA for up to 26 years (Rajput et al., 1997).

An alternative to dopamine replacement with L-DOPA is the use of dopamine receptor agonists. Current dopamine agonists such as pramipexole, ropinirole and cabergoline have been designed with longer half lives when compared to L-DOPA, in an attempt to avoid the generation of dyskinesias whilst maintaining antiparkinsonian efficacy. Indeed, such compounds have been demonstrated to control the motor symptoms of PD for up to 5 years, and with a lower incidence.
of dyskinesias when compared to levodopa therapy (Rascol et al., 2000). Whilst controversial, as levodopa therapy is most effective in early disease states, many neurologists recommend initial therapy with dopamine agonists, followed by supplementation with levodopa as necessary in an effort to reduce dyskinesias associated with the priming effect (Kondo, 2002). All current dopamine receptor agonists exert their therapeutic effects through their actions at the D$_2$ receptor, although compounds showing affinity for the D$_3$ receptor are also in use. Indeed, pramipexole has no affinity for the D$_1$ receptor, high for D$_2$ and highest for D$_3$ receptor (Jenner, 2002). Furthermore, partial agonists for D$_1$ and D$_2$ receptor classes are in development in an effort to reduce dopamine-mediated side effects such as hallucinations and psychosis. These agents are predicted to exert maximal effect in the denervated striatum, but compete with endogenous dopamine from the intact mesolimbic and mesocortical pathways, thus exerting a more modulatory action than full agonists (Albanese and Colosimo, 2003). Injection of a dopamine D$_3$ partial agonist has been shown to significantly reduce levodopa-induced dyskinesias by 66% in MPTP-treated dyskinetic monkeys, whilst having no adverse effect on the symptomatic improvement brought about by levodopa (Guillin et al., 2003).

An alternative approach to directly acting dopamine agonists, is to indirectly increase the amount of dopamine available, either through inhibition of L-DOPA degradation, prevention of dopamine re-uptake, or inhibition of dopamine metabolism. The inhibitors of L-DOPA metabolism include the catechol-O-methyltransferase inhibitors tolcapone and entacapone. Both of these agents increase the area under the levodopa plasma curve and thus prolong the drug action without increasing plasma levels, resulting in decreased “off” time. However, there are concerns with tolcapone and fatal hepatotoxicity (Smith et al., 2003). The MAO-B inhibitors, selegiline (l-deprenyl) and rasagiline prevent the degradation of dopamine and therefore increase its concentration, resulting in decreased doses of L-DOPA required. In addition to this mechanism of action, rasagiline has been reported to have a multitude of putative
neuroprotective actions, including down-regulation of pro-apoptotic Bax, whilst up-regulating anti-apoptotic Bcl-2 and PKC (Mandel et al., 2005). Indeed, rasagiline has recently been licensed as a monotherapy in the treatment of PD.

1.3.3.3.2. Non-dopaminergic pharmacological approaches.

The wide variety of different neurotransmitters and receptor types present in the basal ganglia present a number of possible targets for therapeutic intervention following nigrostriatal degeneration.

Intervening with the proposed pathological mechanisms e.g. oxidative stress and mitochondrial dysfunction in PD has also recently been investigated. A phase II clinical trial of the antioxidant and key component of the mitochondrial electron transport system, Co-enzyme Q10 with early untreated PD patients demonstrated a dose-dependent trend towards a reduction in PD progression. Based on these data, a further phase III study of coenzyme Q10 was planned for starting late 2005 (Shults, 2005).

1.3.4.3 Surgical approaches in clinical use.

1.3.4.3.1 Subthalamotomy and DBS.

Surgical therapies for Parkinson's disease commenced in the 1930s with such procedures as resection of the primary motor cortex, although these methods had unacceptably large morbidity occurrences and were subsequently abandoned. With the advent of levodopa therapy in the 1960s and 70s, interest in surgical approaches for PD therapy waned, but has recently gained enthusiasm, as a consequence of increased understanding of the pathophysiology of PD, coupled with advances in medical imaging and surgical techniques. Given the relative success of levodopa therapy, surgical
approaches are generally reserved for those patients for whom the side-effects of levodopa therapy become untenable. Three main surgical targets have been evaluated, the thalamic nuclei, the GPi and the STN, with irreversible ablative procedures now being superseded by reversible deep brain stimulation (DBS). Thalamic stimulation has been shown to be highly effective in controlling tremor, although most studies have found it to be ineffective against other motor symptoms of PD (Wafter and Vitek, 2004a). In contrast, DBS of the STN or GPi has been shown to be highly effective in treating most motor symptoms of PD, allowing reductions in levodopa dosage (and thus reducing incidences of dyskinesias), improving tremor, bradykinesia and gait disturbances (Krack et al., 2000). Of these two target areas, bilateral DBS of the STN is now more commonly performed, due to increased attenuation of symptoms (Peppe et al., 2004), being technically simpler and the smaller area stimulated means a smaller voltage can be used, with associated increased battery life of the implanted stimulator compared with DBS of the GPi.

As mentioned earlier, the STN forms the major focus of this thesis and hence it is pertinent here to expand a little on DBS. At 12 months of DBS of the STN, typical clinical findings show 50% reductions of L-DOPA dose intake, together with improvements in tremor (80%), gait (64%) and off-period dyskinesias reduced by 94% (reviewed in (Hamani et al., 2005a). Whilst undoubtedly effective in reducing severity of PD symptoms, the mechanism of action of DBS remains uncertain. Since the overall effects of DBS are similar to those achieved with subthalamotomy (discussed later), there appears to be a paradox; with high frequency (>100Hz) stimulation resulting in effects indicative of ablation of the nucleus. The main hypotheses for the mechanism of action of STN DBS include: (1) depolarization blocking of neuronal transmission, through inactivation of voltage-dependent ion channels, (2) prevention of altered firing patterns through imposing high-frequency derived firing patterns onto STN neurons (Hashimoto et al., 2003) and (3) synaptic inhibition following stimulation of inhibitory STN afferents (Breit et al., 2004).
Recently, Windels and colleagues reported increased levels of GABA in the SNpr following DBS (or High Frequency Stimulation, HFS) in 6-OHDA-treated hemi-parkinsonian rats. The authors suggest STN HFS brings about this increase in GABA via a direct stimulation of pallidonigral neurons rather than indirectly via stimulation of the subthalamopallidal pathway, as no increases in glutamate within the GPe (or the SNpr) were detected. This increased GABA activity to the SNpr, if of sufficient magnitude, may override the effect of the excitatory subthalamonigral pathway and result in net inhibition of the SNpr, thus normalising motor loops (Windels et al., 2005a). This mechanism may also account for the reduction in GAD$_{67}$ mRNA expression seen in the SNpr and the GPi, but not in the GPe following STN HFS in hemi-parkinsonian rats (Salin et al., 2002).

In addition to the hypotheses offered above, the influence of other brain regions on the beneficial effect of STN DBS is unclear. Due to the relative small size of the nuclei during DBS current effects may spread to encompass adjacent structures such as the zona incerta and H1 fields of Forel (although no data suggests these effects may spread to the GPe). HFS of the zona incerta has recently been suggested to underlie at least some of the effects of STN DBS, since HFS of this structure reversed the metabolic changes in the SNpr and GP due to dopamine depletion in a 6-OHDA model of PD (Benazzouz et al., 2004).

Whilst highly effective, DBS is expensive in terms of both labour and equipment needed, therefore an alternative is bilateral subthalamotomy. Subthalamotomy may therefore be advantageous in a clinical subset of patients such as those living in remote areas and in countries unable to afford DBS equipment. However, this procedure has the disadvantage of being irreversible and may also result in hemiballism. In 2005, Albarez and colleagues reported the long term effects of bilateral subthalamotomy on 18 patients with advanced PD. Significant reductions in levodopa dosage (47%), levodopa-induced dyskinesias
(50%), and "off" time (49.5%) were reported compared to baseline levels after 2 years, some lasting for 3-6 years. Side-effects from the surgery were generally minimal and transitory in nature, e.g. dyskinesia in 3 patients, which resolved within 3 months whilst motor improvements were present for the following 4 years (Alvarez et al., 2005b).

Lesioning of the STN has also been suggested to reduce the over-activity of the glutamatergic corticostriatal pathway brought about by dopamine depletion in 6-OHDA treated rats, which is similar to that brought about by L-DOPA treatment (Centonze et al., 2005). Like DBS, lesioning of the STN has also been investigated from a neuroprotection viewpoint. Since over-activity of this glutamatergic pathway may result in excitotoxicity, subthalamotomy may offer a potential symptomatic and neuroprotective therapy. Unfortunately, this does not appear to be the case, as a lesion of the STN did not affect the number of dopaminergic neurons remaining after 6-OHDA lesion. However, STN lesion did significantly increase the number of tyrosine hydroxylase (TH, a marker of dopaminergic phenotype) positive neurons, which lead to a partial behavioural recovery (Paul et al., 2004). Similarly, a recent study has described the lack of neuroprotection following 16 months of STN DBS in PD patients, quantified by $^{18}$F-fluordopa PET (Hilker et al., 2005).

1.3.4.3.2 Stem cells and tissue transplants.

Transplantation of dopaminergic tissues involving a striatal infusion of autologous adrenal medullary cells, and later fetal mesencephalon tissue was pioneered by Swedish surgeons in 1982. Initially, these results were highly promising, although later, double-blinded trials have failed to replicate these initial findings. In one study, forty patients with severe PD were randomly assigned to receive transplants of fetal mesencephalic tissue or sham surgery and followed for a year. This study found no difference between groups, although there was a significant improvement for younger (<60 years old) patients receiving fetal implants. Moreover, fiber outgrowth was present in 17
out of 20 patients, indicated by an increase in $^{18}$F-fluorodopa uptake (Tintner and Jankovic, 2002). In another blinded study, no significant benefit from transplantation was observed. This study also reported significant increases in dyskinesias (occurring in 56% of patients, compared to 15% reported previously) which persisted even when drug therapy was withdrawn (Walter and Vitek, 2004b). Problems associated with the use of fetal transplantation studies include: the poor neuronal survival (typically around 90% loss after one week of transplantation) necessitating the use of tissue from multiple fetuses; a highly expensive and ethically questionable approach, not to mention one which is fraught with problems of tissue supply. The problem of poor neuronal survival may be addressed by providing neurotropic factors together with transplanted tissue. Indeed a recent study in 6-OHDA lesioned rats has shown that human embryonic mesencephalic tissue, when concomitantly implanted with polymer-encapsulated genetically modified cells releasing glial cell line-derived neurotrophic factor (GDNF), produced significant fiber outgrowths compared to control (Ahn et al., 2005).

Combining the approach of embryonic stem (ES) cells and gene therapy, genetically modified human ES cells encoding the genes for TH and GTP cyclohydrolase 1 (involved in the synthesis of the cofactor tetrahydrobiopterin, required for TH activity) produced significant amounts of L-DOPA and relieved dopamine-depleted behaviour in 6-OHDA lesioned rats. Post-mortem immunohistochemical stains also indicated these ES cells survived and expressed TH for the duration of the experiment (6 weeks) (Park et al., 2003). If these findings can be safely replicated in other animal models of PD, these techniques could provide a novel way for the treatment of PD. One possible alternative is the use of human embryonic stem cells. This approach is still in relative infancy, however, despite the mass media attention and heavy investment this approach has received...watch this space eagerly.
The use of growth factors and gene therapy in PD has been steadily growing, although hopes for continuous GDNF intraputaminal infusions have been dashed by its withdrawal from the market following safety concerns (Slevin et al., 2006). However in a gene-therapy study using HSV-1 vector, GDNF was shown to be more efficacious than brain-derived neurotrophic factor (BDNF) in both correcting behavioural deficits and protecting dopaminergic neurons in 6-OHDA lesioned rats (Sun et al., 2005).

Given the noted efficacy of both STN DBS and subthalamotomy in the treatment of PD together with the fact that the STN is glutamatergic in nature, attention has recently been focussed on the potential use of glutamatergic agents in the treatment of PD.

1.4 Role of glutamate in PD.

The role of glutamatergic transmission in both the pathogenesis and pathophysiology of PD has been described previously (section 1.3.2.2.) Given the pivotal role of the glutamatergic transmission in PD, one may expect this system to be targeted for rational pharmaco-therapies in PD. However, since glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system, any manipulation of this system is likely to have widespread side-effects, which may limit the usefulness of this approach in the treatment of PD.

1.4.1 Glutamatergic neurotransmission.

Since there does not appear to be any enzyme present extracellularly that can metabolise glutamate to any significant degree, termination of glutamatergic signalling relies on removal of glutamate from the active synapses or
desensitisation of glutamatergic receptors (Sinclair et al., 2003; Schousboe et al., 2004a; Waagepetersen et al., 2005). The extracellular concentration of glutamate must be kept low for a number of reasons including: possible excitotoxicity following exposure to high concentrations of glutamate, a reduction in background transmission permitting high signal to noise ratios required for neurotransmission and conservation of glutamate used. Glutamate re-uptake is mediated by five \( \text{Na}^+ \) and \( \text{K}^+ \)-driven transporters, known as excitatory amino acid transporters (EAATs) 1-5, although EAAT1 was previously known as GLAST (glutamate-aspartate transporter), and EAAT2 as GLT-1 (glutamate transporter). Each transporter shares approximately 50-60% amino acid homology with each other and 30-40% with other amino acid carriers. EAAT2 is the major glutamate transporter within the CNS (apart from the cerebellum, the inner ear, the retina and the circumventricular organs where EAAT1 predominates). Both EAAT1 and EAAT2 are expressed on astrocytes, whilst EAAT3 is expressed on both glutamatergic and non-glutamatergic neurons, together with astrocytes and oligodendrocytes. EAAT4 has a relatively specific (and low-level) distribution on Purkinje cells of the cerebellar molecular layer, whilst the distribution of EAAT5 is restricted to Müller cells in the retina (Schousboe et al., 2004b). Following uptake into astrocytes, glutamate may be metabolised in two different manners. It may be amindated to glutamine by the ATP-dependent, glial specific enzyme glutamine synthetase or converted to \( \alpha \)-ketoglutarate by glutamate dehydrogenase, which can then enter Krebs' cycle and lead to lactate formation. Both glutamine (which is non-toxic) and lactate are then exported from astrocytes and taken up by neurons. Glutamine is then hydrolysed by glutaminase within neurons to form glutamate (Broer and Brookes, 2001). This reformed glutamate is then packaged into vesicles by another transporter system, the vesicular glutamate transporters (VGLUT1-3). These \( \text{Na}^+ / \text{K}^+ \) independent transporters are driven by the internal positive membrane potential generated by vacuolar \( \text{H}^+ \)-ATPase and achieve intraneuronal vesicular concentrations of around 150mM (Danbolt, 2001). This re-packaged glutamate is then able to be released in a calcium-dependent
manner and act on a variety of glutamate receptors at the synapse to bring about physiological responses.

Glutamate receptors can be broadly divided into two main classes, the "ionotropic" and "metabotropic" families. Activation of ionotropic receptors leads to an opening of ion channels, whilst activation of metabotropic receptors produces changes in G-protein linked effects. Whilst the metabotropic receptors form the main focus of this thesis, consideration will first be given to the ionotropic class of receptors.

1.4.1 Ionotropic glutamate receptors, structure and distribution

Ionotropic receptors can be further subdivided into N-methyl-D-aspartate (NDMA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), or Kainate based on their selective affinities for these agonists. Pentameric AMPA receptors respond rapidly to agonist application, and also display rapid (2 – 14 msec) desensitisation to agonist exposure. Homomers or heteromers of subunits GluR1 to GluR4 form the AMPA ion channels, although the key subunit in determination of ion channel permeability is the GluR2 subunit, incorporation of which provides low Ca\(^{2+}\) permeability to the resultant channel, such as seen in the mammalian CNS. Homomers or heteromers of GluR1, GluR3 or GluR4 in the absence of GluR2 form ion channels that are permeable to the divalent cations Ca\(^{2+}\), Mg\(^{2+}\), and Ba\(^{2+}\) (Michaelis, 1998a). The GluR1-4 subunits may also be alternatively spliced near the fourth trans-membrane domain, resulting in "flip" and "flop" forms of GluR1-4, the flip form being present pre-natally, whilst the flop form is present post-natally in the CNS, and is believed to underlie the rapid desensitisation seen following agonist activation of these receptors (Sommer et al., 1990). AMPA receptors are widely distributed throughout the CNS, although those receptors containing the GluR4 subunit have a more restricted expression, predominantly in the cerebellar cortex and the dentate gyrus. Within the basal ganglia there are high levels of AMPA receptors containing GluR1 and GluR2, especially in the striatal medium spiny neurons,
although also in the GPi and SNpc. Those containing the GluR4 subunit are predominantly expressed in the GPi and SNpr along with glial cells (Greenamyre, 2001b).

Within the basal ganglia, the complete distribution of kainate receptors is currently unknown. However, pre-synaptic kainate receptors have recently been shown to be present on striatopallidal terminals. Activation of these receptors may serve to modulate GABA release in an activity-dependent manner (Kane-Jackson and Smith, 2003). Similar to AMPA receptors, Kainate receptors also show fast desensitisation of responses to the agonist, kainate. They are composed of GluR5-7 subunits in combination with either KA-1 or KA-2. Whilst GluR7 and KA-1 or KA-2 cannot form homomers, they are capable of forming heteromeric channels with either GluR5 or GluR6. Kainate receptors are widely distributed throughout the CNS, with subunits containing GluR7 and KA-2 being very highly expressed in the frontal, parietal and entorhinal cortex (Michaelis, 1998b).

By contrast to the AMPA and Kainate receptors, NMDA receptors are not believed to participate in rapid synaptic transmission, since their pore forming units are tonically inhibited by Mg$^{2+}$ present in extracellular CSF. Relief of the Mg$^{2+}$ block is required for NMDA receptor activation to occur. This can be achieved through the rapid activation of AMPA or kainate receptors, leading to Na$^{+}$ influx and EPSP generation, which if sufficient in magnitude can remove the voltage-dependent Mg$^{2+}$ blockade. It is this mechanism which gives NMDA receptors a central role in learning and memory (Hallett and Standaert, 2004d). In addition NMDA receptors desensitise relatively slowly and are permeable to Ca$^{2+}$, a feature which may lead to excitotoxicity and subsequent apoptosis (as described in section 1.3.1.). There are three families of NMDA subunit proteins, NR1, NR2 and NR3 which assemble in heteromeric complexes. Eight forms of the NR1 subunit (a-h) result from alternate splicing of the NR1 mRNA. Four forms (a-d) of the NR2 subunit are currently known, these are encoded on a
separate genes. Furthermore, two forms of the NR3 subunit (a-b) are also known to occur. Functional NMDA receptors are heteromers with at least one NR1 and one NR2 subunit, and whilst the total number of subunits needed to form a receptor are not yet known, current models favour four or five subunits (Hallett and Standaert, 2004c). Within the CNS, NR1 subunits are widely expressed in both neurons and glial cells, whilst NR2 subunits appear to have a distinct pattern of expression. NR2A mRNA is the most widespread, being present in the cerebral and cerebellar cortices, and the hippocampus. The NR2B is predominantly expressed in the telencephalon, NR2C expressed in the granule cells of the cerebellar cortex and the NR2D chiefly expressed in the diencephalon. NR3 subunits are scare in the adult brain, apart from motor neurons of the spinal cord (Nishi et al., 2001). Within the basal ganglia, expression of a wide variety of NMDA subunits can be found. The NR1 containing receptors are present in all structures of the basal ganglia, in keeping with their overall distribution in the CNS, NR2 containing subunits tend to be compartmentalised within the motor circuit. NR2A and NR2B containing receptors are found in the striatum (both projection and interneurons), NR2C are only found in the SNprc, whilst NR2D are found in all structures of the basal ganglia, but predominate in the striatal interneurons (Greenamyre, 2001a; Hallett and Standaert, 2004b).

In addition to the ionotropic glutamate receptors (iGluRs) outlined above, there exists another glutamate receptor, which based on its amino acid similarity to iGluRs, was classified as an ionotropic receptor, but fails to form channels when expressed either alone or in combination with other iGluRs. This is the δ2 glutamate receptor, and is predominantly expressed on Purkinje cells and is involved in cerebellar function, since knock-out mice for the δ2 glutamate receptor develop ataxia and impaired long-term depression (Yuzaki, 2003). However, the mechanisms of δ2 glutamate receptor signalling have yet to be elucidated, as does its presence in the basal ganglia.
1.4.2.1. Ionotropic glutamate antagonists as potential therapeutic approaches for PD.

NMDA antagonists have been investigated as possible therapies for PD, ever since the role of glutamate within the basal ganglia was established. Low oral doses of systemically active NMDA antagonists, such as MK-801, amantadine and remacemide have been shown to improve dopamine-depletion induced akinesia (Greenamyre et al., 1994). The sites of action of these systemically active compounds have not yet been clearly elucidated, although both direct intra-striatal (Nash and Brotchie, 2002b) and intra-subthalamic (Allers et al., 2005) injections of these antagonists have been reported to have anti-parkinsonian effects. These sites of action would fit well with the premise that the drugs bring about their systemic effects through blockade of voltage-dependent NMDA receptors in regions known to have hyperactive glutamatergic transmission in PD. Whilst systemic injections of MK-801 are relatively well tolerated in rodents, in primates they produce sedation of parkinsonian macaques similar to that seen with another NMDA antagonist, ketamine (Hallett and Standaert, 2004a). Indeed, in one early study, intramuscular MK-801 given following L-DOPA treatment in an MPTP-treated monkey antagonised the anti-parkinsonian action of L-DOPA, possibly through these sedative effects (Crossman et al., 1989). The advent of more specific NMDA-subunit antagonists may also counter the unacceptable side-effects that have limited the use of broad-spectrum NMDA receptor antagonists. These side effects encompass conditions such as psychostimulation, impairment of learning and memory and motor disturbances. Thus more selective antagonists have recently been investigated, with the main target being the NR2B subunit, owing to its high levels of expression within the striatum (Marino et al., 2003a). Systemic oral treatment with CI-1041, a selective NR1A / NR2B subunit NMDA receptor antagonist has recently been shown to prevent dyskinesias when co-administered with L-DOPA in a MPTP-lesioned primate model of PD (Hadj Tahar A. et al., 2004). However, another systemically administered NR1 / NR2B-selective NMDA antagonist, CP-101,606 was found to be ineffective as
monotherapy, provided only mild potentiation of levodopa-induced motor movement and, worryingly augmented levodopa-induced dyskinesias in MPTP-treated marmosets (Nash et al., 2004).

Whilst various NMDA antagonists have been shown to alleviate akinetic symptoms of PD in animal models, AMPA antagonists have not had the same effect. Systemic administration of the AMPA antagonist NBQX did not affect either reserpine-induced akinesia, or behavioural responses to 6-OHDA lesion in rodents although it was found to potentiate the effect of levodopa in the latter (Starr, 1995). In a study investigating the role of AMPA receptors in mediating L-DOPA-induced dyskinesias, a selective non-competitive inhibitor of the AMPA allosteric modulatory site, LY300164 did not reduce the severity of parkinsonian signs in primates, but did attenuate levodopa-induced dyskinesias. On the other hand, sub-cutaneous injections of an AMPA selective agonist, CX516 alone similarly possessed no antiparkinsonian efficacy, but potentiated levodopa-induced dyskinesias (Chase et al., 2000). These results indicate the involvement of AMPA receptors in the genesis of L-DOPA-induced dyskinesias. More recently, systemically active and selective AMPA potentiators such as LY404187 have been reported to reduce both MPTP-induced toxicity in mice and lead to functional, histological and neurochemical improvement following 6-OHDA lesion in rats, although these actions are thought to relate to the ability of these drugs to increase BDNF production, rather than their direct action on AMPA receptors (O'Neill et al., 2004).

Another mechanism by which glutamatergic neurotransmission could be modulated is through the actions of another group of glutamatergic receptors. This group, the metabotropic glutamate (mGlu) receptors, are found within the basal ganglia and have recently shown promise in effecting the modulation of glutamatergic transmission. Indeed of these groups, the metabotropic glutamate receptors form the main focus of the studies outlined in this thesis.
1.4.2 Metabotropic glutamate receptors

1.4.2.1 Structural features

In contrast to the ionotropic glutamate receptors, mGlu receptors are not linked to ion channels, rather they are G-protein coupled receptors (GPCRs). On the basis of sequence homology they are classified as belonging to the class 3 family of GPCRs. Other members of this family include the metabotropic GABA (GABA\(_B\)) receptor, Ca\(^{2+}\)-sensing receptors and certain pheromone receptors (Schoepp, 2001). Typically these receptors have a large extracellular ligand binding site together with seven transmembrane spanning regions with G-protein binding occurring in the second intracellular loop. GPCRs activate G-proteins through stimulating the exchange of bound GDP in the \(\alpha\) subunit of the G-protein complex for GTP. Binding of GTP then allows dissociation of the \(\alpha\) subunit from the \(\beta\gamma\) dimer. Depending on the type of GPCR, the dissociated \(\alpha\) and \(\beta\gamma\) subunits are then able to regulate enzymes or ion channels, with resultant cellular effects. The extracellular ligand binding site for mGlu receptors is believed to be hinged, with two lobule-like domains, similar to a Venus fly-trap configuration (Conn and Pin, 1997). mGlu receptors can further be subdivided into groups I, II and III based on their second messenger coupling. The formation of dimers has been established (and is indeed functionally required) for some class III GPCRs, (e.g. the GABA\(_B\) receptors), and is also believed to occur in the mGlu family. The group I mGlu receptor, mGlu1A has also been reported to form a heterodimer with calcium-sensing receptors and adenosine A1 receptors, although such complex dimerisation has not yet been demonstrated for either group II or group III mGlu receptors (Jingami et al., 2003).
1.4.2.2 Group I mGlu receptors: Signal transduction mechanisms, distribution and potential as targets for the treatment of PD.

The predominantly post-synaptically located group I mGlu receptor family includes the mGlu1 (variants a-d) and mGlu5 (variants a & b) receptors. All members of the group I mGlu receptor family couple via Gq to phospholipase C to increase levels of diacylglycerol and inositol trisphosphate. These second messengers activate protein kinase C and lead to a release of Ca$^{2+}$ from internal stores respectively (Cho and Bashir, 2002), thus increasing neuronal excitability. The mGlu1a and mGlu5a & b variants all contain a long intracellular C-terminus end, which is the binding site for their association with Homer proteins. Homer connects these mGlu receptors to endoplasmic reticulum transmembrane proteins such as Ins(1,4,5)P$_3$ and ryanodine receptors, transient receptor potential channels and to small GTP-binding proteins (Guhan and Lu, 2004). This huge protein complex therefore provides an ideal intracellular organisation for the release of Ca$^{2+}$ following group I mGlu receptor activation. In addition, Homer proteins connect to both AMPA and NMDA ionotropic glutamate receptors via interaction with the anchoring protein Shank. This interaction can lead to potentiation of NMDA receptor responses, an effect which is driven by increased intracellular calcium ions (Fagni et al., 2000). This combined activation of NMDA and group I mGlu receptors can lead to increased phosphorylation of transcription factors such as cAMP response element-binding protein (CREB) and Elk-1 (Choe et al., 2002; Choe and Wang, 2002), together with activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) via activation of the mitogen-activated protein kinase (MAPK) pathway (Ferraguti et al., 1999). This activation of MAPK and CREB may underlie the alterations in LTP and depotentiation seen following group I mGlu receptor activation (Delgado and O'dell, 2005).

Within the basal ganglia, Group I mGlu receptors are preferentially expressed within post-synaptic constituents of the cortex, striatum, globus pallidus, subthalamic nucleus and substantia nigra pars reticulata. mGlu$_1$ is abundantly
expressed in the substantia nigra pars compacta, whilst mGlu_{5} has increased mRNA expression compared to other basal ganglia regions in the parafascicular nucleus, the striatum and the subthalamic nucleus (Testa et al., 1994a; Messenger et al., 2002e). mGlu_{1} receptors are expressed by the dopaminergic neurons of the SNpc (Fiorillo and Williams, 1998c). Surprisingly, activation of mGlu_{1} within the SNpc can lead to both depolarisation and hyperpolarisation of these dopaminergic neurons, although hyperpolarisation appears to be less common than depolarisation (Fiorillo and Williams, 1998b). The authors suggest that this hyperpolarisation could be brought about through a rise in intracellular Ca^{2+}, which can then lead to activation of a calcium-sensitive potassium conductance (Fiorillo and Williams, 1998a). Within the striatum, group I receptors are also present pre-synaptically on dopaminergic terminals that converge with glutamatergic synapses on medium spiny neurons (Smith et al., 2000) (see section 1.2.2.). Here, under conditions of elevated glutamate release, activation of group I mGlu receptors has been shown by fast-scan cyclic voltammetry to inhibit dopamine release on medium spiny neurons within the striatum (Zhang and Sulzer, 2003). On medium spiny neurons, activation of mGlu_{5} receptors amplifies NMDA receptor-mediated currents (Pisani et al., 2001). This enhancement of NMDA receptor function may be brought about through the interaction of both receptor types with Homer proteins. Conversely, activation of NMDA receptors can similarly lead to an augmentation of mGlu_{5} receptor signalling, through inhibition of receptor desensitisation following activation of calcineurin (a protein phosphatase) (Alagarsamy et al., 1999a; Alagarsamy et al., 1999c; Alagarsamy et al., 2002).

Group I mGlu receptors are also found on those medium spiny neurons that project to the GPe (Smith et al., 2001). Other receptor types found on these projection neurons include the G_{i}-coupled dopamine D_{2-like} receptors and the G_{s}-coupled, adenosine A_{2A} receptors that respectively inhibit and activate adenylate cyclase. mGlu_{5} receptors are able to physically interact with adenosine A_{2A} receptors and promote a series of downstream events including
MAP kinase activation (Nishi et al., 2003). Group I mGlu receptors have also been demonstrated to interact with adenosine A$_{2A}$ receptor-mediated signalling following intra-striatal and intra-subthalamic injection of the broad-spectrum group I mGlu receptor agonist, 1(S),3R-ACPD (Kearney and Albin, 1995), via increases in intracellular calcium-mediated adenylate cyclase activation. Therefore, at these striatopallidal synapses, group I mGlu receptor activation appears to act in concert with NMDA & A$_{2A}$ receptor activation to antagonise the overall effects of dopamine on the indirect pathway. Furthermore, group I mGlu receptor activation leads to downstream activation of casein kinase 1, which phosphorylates dopamine- and cAMP-regulated phosphoprotein (DARPP-32). Under conditions where group I mGlu receptors are not activated, following dopamine D$_1$ receptor activation (and subsequent PKA activation) DARPP-32 acts as an inhibitor of protein phosphatase 1, augmenting the D$_1$ signal. However, when group I mGlu receptors are activated, casein kinase 1 activation alters the phosphorylation of DARPP-32, and converts its actions to an inhibitor of PKA – thus attenuating the D$_1$ receptor-mediated signal (Conn et al., 2005). Furthermore, behavioural and c-Fos studies show a selective increase in the activity of the indirect striatopallidal pathway following intrastratal injection of group I mGlu receptor agonists (Kearney et al., 1997). Taken together, these observations suggest that activation of group I mGlu receptors serves to antagonise the effect of dopamine in the indirect pathway.

Activation of group I mGlu receptors also increases activity through the indirect circuit in other basal ganglia nuclei, thus counteracting the negative effects of dopamine on this circuit. For example, electrophysiological studies using group I mGlu receptor agonists have demonstrated their ability to depolarise SNpr neurons \textit{in vitro}, and this effect is mediated by the mGlu1 receptor alone in normal rats (Marino et al., 2001). In haloperidol-treated rats however, this depolarisation of the SNpr is mediated by both mGlu1 and mGlu5 receptor types, suggesting a dopamine-mediated regulation of group I mGlu receptor function (Marino et al., 2002b). This alteration in group I function could possibly
be brought about via alterations in receptor coupling to Homer or NMDA-mediated alterations in group I mGlu receptor-mediated responses (Marino et al., 2002a).

On the basis that group I mGlu receptor activation largely opposes the actions of dopamine, it is not surprising that group I receptor antagonists have been investigated for anti-parkinsonian efficacy. The systemically active negative allosteric modulator of the mGlu5 receptor, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) has been shown to have antiparkinsonian efficacy in a number of studies. Chronic MPEP treatment in bilaterally 6-OHDA lesioned rats resulted a restoration of reaction times compared to acute treatment or vehicle controls (Breysse et al., 2002). In a follow-up study, 3 week treatment with MPEP in bilaterally 6-OHDA lesioned rats was shown to totally reverse the increases in CO-I mRNA levels in the STN and GAD67 mRNA levels in the SNpr, suggesting chronic MPEP treatment may indeed normalise the basal-ganglia-motor loop (Breysse et al., 2003). MPEP treatment has also been shown to inhibit parkinsonian-like muscle rigidity when administered to haloperidol treated rats (Ossowska et al., 2001), and a more recently developed mGlu5 receptor antagonist, 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) also appears to share this ability (Ossowska et al., 2005). However, a very recent report has suggested MTEP may be of more benefit in combating L-DOPA-induced dyskinesias, rather than treating the motor symptoms of PD directly (Dekundy et al., 2006).

1.4.2.3 Group II mGlu receptors: Signal transduction mechanisms, distribution and potential targets for the treatment of PD.

In contrast to the group I mGlu receptors, group II receptors are are predominantly located pre-synaptically. Members of the group II mGlu receptor family include the mGlu2 and mGlu3 receptors, which are perisynaptically located on the pre-synaptic terminal. Their mechanism of action is believed to
be similar to that of the group III mGlu receptor family, and will be covered in detail in section 1.4.2.3. Briefly, they are negatively coupled through G\textsubscript{i} or G\textsubscript{o}-proteins, leading to an inhibition of adenylate cyclase, with subsequent reductions in intracellular cAMP and pre-synaptic membrane hyperpolarisation (Conn and Pin, 1997). It is this reduction in cAMP that is considered to be responsible for their effects on decreasing neurotransmission of both inhibitory (Doi et al., 2002) and excitatory (Bradley et al., 2000c) nature through acting as hetero- or auto-receptors and reducing neurotransmitter release.

Convergent results from in-situ hybridisation and immunohistochemical studies have suggested that group II mGlu receptors are widely localised throughout the CNS. The mGlu2 receptor is highly expressed in the cerebellar cortex, the hippocampus and the thalamic nuclei, whilst the mGlu3 receptor is highly expressed in the olfactory system and thalamic nuclei (Ohishi et al., 1993a; Ohishi et al., 1993b; Ohishi et al., 1993c; Ohishi et al., 1998a).

In-situ hybridisation studies have revealed the mGlu2 receptor to be relatively sparsely located within the basal ganglia, being primarily located to the premotor cortex and STN. This is in contrast with the wide expression of the mGlu3 receptor throughout the basal ganglia, with high levels in the reticular thalamic nuclei, the striatum and the nucleus accumbens (Messenger et al., 2002d). The sparse location of mGlu2 within the basal ganglia has similarly been suggested by binding studies using [\textsuperscript{3}H]LY354740, a specific agonist for mGlu2, showing its distribution to be limited to the caudate-putamen (Richards et al., 2005). However, a study using monoclonal antibodies directed against mGlu2 show widespread distribution within all components of the basal ganglia with highest levels of expression in the striatum (Ohishi et al., 1998b). These differences in expression studies could be accounted for by the different methodological approaches employed. In contrast to any other mGlu receptors, mGlu3 was also shown to be present on glia (Testa et al., 1994b).
Electrophysiological studies carried out within the basal ganglia have confirmed a pre-synaptic location of group II mGlu receptors, and shown inhibition of glutamatergic transmission to the STN using a group II mGlu receptor agonist, DCG-IV (Shen and Johnson, 2003). Other electrophysiological experiments have suggested activation of group II mGlu receptors can inhibit glutamatergic transmission from STN efferents within the SNpr (Bradley et al., 2000b). Moreover, behavioural studies within our laboratory have confirmed that both intranigral and intraventricular injections of DCG-IV are able to reverse reserpine-induced akinesia (Dawson et al., 2000). Whilst DCG-IV was effective, this compound exhibits some NMDA agonist actions and is not systemically active. Therefore, more specific and systemically active group II mGlu receptor agonists such as LY379268 and LY354740 have been developed and tested in animal models of PD. An electrophysiological study using LY354740 reported inhibition of EPSPs in the SNpr, following STN stimulation. This reduction in EPSP amplitude could also be inhibited by pre-treatment with the group II mGlu receptor antagonist, LY341495 (Bradley et al., 2000a), confirming group II mGlu receptor involvement in mediating these effects. Studies using LY379268 have described intraventricular efficacy in the reserpine-treated rat, although no anti-parkinsonian efficacy was seen following systemic administration in either 6-OHDA lesioned or reserpine-treated rats (Murray et al., 2002b). This may be due to dopamine-mediated alterations in function, although the simplest explanation remains that in these experiments, LY379268 failed to cross the blood-brain barrier. Furthermore, a loss of efficacy was reported following repeated (24 hr) intraventricular injection in the reserpine-treated rat, which may have implications for the use of group II mGlu receptor agonists as potential therapies for PD (Murray et al., 2002c). Especially when combined with a recent report suggesting activation of group II mGlu receptors by LY354740 leads to cognitive impairment and impaired spatial learning, which are not present in mGlu2 knock out mice (Higgins et al., 2004).
1.4.2.4 Group III mGlu receptors: Signal transduction mechanisms and their distribution in the basal ganglia.

The pre-synaptically located group III mGlu receptor family includes the mGlu4 (variants a & b), mGlu6, mGlu7 (variants a & b) and mGlu8 (variants a & b) receptors. These receptors are coupled to G\textsubscript{i} or G\textsubscript{o}, activation of which reduces adenylate cyclase activity and is believed to inhibit neurotransmitter release by acting as auto- or hetero-receptors. This inhibition of release, whilst not fully understood for group III mGlu receptors, is believed to be brought about through direct inhibition of voltage-dependent calcium channels by the G\textsubscript{ß}y subunit. Thus, following group III mGlu receptor activation, the G\textsubscript{ß}y subunits are released from the G\textsubscript{a}ßy heterotrimer and bind to the α1 subunit of calcium channels. At depolarising potentials the G\textsubscript{ß}y subunits unbind and thus relieve the inhibition (Ruiz-Velasco and Ikeda, 2000). In comparison with the perisynaptic localisation of group II mGlu receptors, group III mGlu receptors are predominantly found within the synaptic cleft at the pre-synaptic terminal, although mGlu6 is post-synaptic and is exclusively expressed in the ON bipolar cells of the retina. The relatively low affinity of these receptors for glutamate, coupled with their pre-synaptic localisation can be interpreted as an action of a low pass filter. In keeping with the idea that these receptors are activated only when glutamatergic transmission is massively increased compared to normal.

The most extensively studied member of the group III mGlu receptor family (in terms of second messenger coupling) is the mGlu7 receptor. Activation of the mGlu7 receptor leads to inhibition of cAMP levels (via inhibition of adenylate cyclase), and stimulates phospholipase C. The mGlu7 receptor also possesses a calmodulin (CaM) binding motif within its C-terminus (Dev et al., 2001). This CaM binding site appears to be important for G\textsubscript{ß}y subunit binding, since CaM inhibitors attenuate the inhibition of glutamate release by the broad-spectrum group III mGlu receptor agonist L-2-amino-4-phosphonobutyrate (L-AP4) in primary hippocampal neurons (O'Connor et al., 1999). Furthermore [\textsuperscript{35}S]GTPγS binding experiments (measuring G\textsubscript{a} activity) with mutant deletions in the CaM
binding site of mGlu7 have shown no difference between mutants and wild-type mGlu receptors (El Far O. et al., 2001). These data suggests that CaM is not necessary for G-protein activation per se, rather that CaM is important for the dissociation of the Gβγ from the mGlu7 receptor. It therefore follows that prevention of this dissociation may result in a loss of inhibition of neurotransmitter release, due to decreased Gβγ-induced inhibition of voltage-gated Ca$^{2+}$ channels. Therefore, increased pre-synaptic Ca$^{2+}$, such as would occur following depolarisation will not only lead to Ca$^{2+}$-dependent neurotransmitter release, but will subsequently lead to inhibition of Ca$^{2+}$ channels, via CaM activation and Gβγ dissociation, the main effect of group III mGlu receptor activation (Millan et al., 2002a; Millan et al., 2002d).

This CaM binding and interaction with group III mGlu receptors has been demonstrated for all members of the group. Furthermore, this CaM binding site appears to be phosphorylated by PKC, leading to inhibition of CaM binding and subsequent elimination of Gβγ retention on the C-terminus of these receptors (Airas et al., 2001). Additionally in group III mGlu receptors, this same serine residue appears to be phosphorylated by PKA, which also serves to inhibit the function of these receptors (Cai et al., 2001c). Therefore, pre-synaptic group III mGlu receptors can be modulated by mechanisms involving different pre-synaptic receptors such as the G$_s$-coupled β-adrenergic receptor, which following activation would lead to an enhancement of adenylyl cyclase and subsequent PKA activation. Indeed, Cai et al. report the β-adrenergic agonist, isoproterenol inhibits L-AP4-induced suppression of EPSPs at hippocampal synapses. This possibility of receptor “cross-talk” between group III mGlu receptors and other receptors will be examined in Chapter 5 of this thesis.

Recent studies have shown there is a direct interaction in the C-terminal end of mGlu7 with the protein interacting with C-kinase (PICK1) (Boudin et al., 2000). PICK1 has been demonstrated to lead to a reduction in PKCo-evoked phosphorylation of mGlu7a (Dev et al., 2000), although since both PICK1 and
mGlu7 are able to be phosphorylated by PKCa, this could be explained through competition for sites of phosphorylation (El Far O. and Betz, 2002). However, the presence of PICK1 does appear to be mandatory for the inhibition of P/Q-type calcium channels in neurons by the mGlu7a receptor. Following mutation of the PICK1-binding site, mutants were expressed in neurons but did not produce Ba$^{2+}$ current inhibition in response to L-AP4 compared to wild-type receptors (Perroy et al., 2002). The precise mechanisms between PICK1, PKA, PKC and group III mGlu receptor signalling have yet to be elucidated.

1.4.2.5 Group III mGlu receptor ligand functions in other brain areas and disease states.

Group III mGlu receptor-modulating compounds have been investigated for a number of different disease states, including epilepsy, depression, anxiety and pain. Of these pathological processes, research into group III mGlu agonists and antagonists has been the greatest in epilepsy. With the finding that ionotropic glutamate receptor antagonists are potential anti-epileptic agents, the role of glutamate in epilepsy has become more widely accepted (Moldrich et al., 2003a). However, as mentioned in section 1.4.1, administration of ionotropic glutamate receptor antagonists results in a wide variety of unacceptable side-effects such as hallucinations and memory loss. Therefore, ionotropic glutamate receptor antagonists have since been abandoned as sole therapeutic targets for anti-epileptic drugs. However, their success within the laboratory served to highlight the role of glutamate in epilepsy, thus mGlu receptor ligands are currently being investigated for pharmacological treatment of epilepsy. The mGlu receptors are widely expressed in the key epileptogenic regions of the brain, including the cerebral cortex, the thalamus, the amygdala and the hippocampus (Rouse et al., 2000). As would be expected, given their predominantly post-synaptic and excitatory nature, agonists of group I are uniformly convulsant when injected i.c.v. or focally into the CNS. Given these actions, it is unsurprising that group I mGlu receptor antagonists are anti-
convulsant in vivo, and that activation of both receptor subtypes (mGlu₁ and mGlu₅) are equally responsible for the convulsant actions (Moldrich et al., 2003c). Regarding group II mGlu receptor compounds, group II mGlu receptor agonists are anti-convulsant. For example, i.c.v. injection of LY379268 and LY389795 are anti-convulsant against both sound-induced clonic seizures and the group I mGlu receptor agonist, DHPG, in DBA/2 mice (Moldrich et al., 2001a). Furthermore, these effects were completely abolished by pre-injection with the group II mGlu receptor antagonist, LY341495, thus confirming the anti-convulsant effects of these compounds are due to activation of group II mGlu receptors (Moldrich et al., 2001b). Interestingly, group III mGlu receptor agonists have been shown to be both pro- and anti-convulsant. Initial reports suggested that i.c.v. L-AP4 and L-SOP were both pro-convulsant in DBA/2 mice (Ghauri et al., 1996a). However, further investigations have revealed that intra-inferior collicular injections of both L-SOP and L-AP4 produce rapid (circa 10 min) pro-convulsant effects followed by a long (up to 3 days post-injection) anti-convulsant effect in genetically epilepsy prone rats (Tang et al., 1997). The authors of this study suggest that the hetero-receptor mediated inhibition of GABA release underlies the pro-convulsant actions of both L-SOP and L-AP4, whilst the agonist-induced upregulation of mGlu7 may account for the delayed anti-convulsant activity (Yip et al., 2001a).

Elsewhere within the CNS, in-situ hybridisation together with immunocytochemical labelling studies have also demonstrated the presence of mGlu4 and mGlu7 receptors within the superficial dorsal horn, where they are located pre-synaptically on primary afferent nerves (Ohishi et al., 1995; Li et al., 1997; Azkue et al., 2001). Here, intrathecal L-AP4 dose-dependently attenuates behavioural allodynia in nerve-injured rats, but is without anti-nociceptive effects in normal animals. Furthermore, topical spinal application of L-AP4 significantly inhibited the evoked responses of ascending dorsal horn neurons in nerve-ligated, but not normal rats. Both of these anti-nociceptive effects are inhibited by pre-treatment with the group III mGlu receptor antagonist, M-AP4, indicating
these effects are brought about through activation of group III mGlu receptors (Chen and Pan, 2005).

The anxiolytic potential for group III mGlu receptor agonists has only recently been investigated. Immunohistochemical studies have shown high densities of group I mGlu receptors, low densities of group II mGlu receptors, and medium densities of group III mGlu receptors in the amygdala and hippocampus (Bradley et al., 1996; Shigemoto et al., 1997; Corti et al., 2002). The amygdala and hippocampus are key structures involved in regulating anxiety and the expression of emotional responses to stress (Davis, 1992; Davis et al., 1994). Injections of the group III mGlu receptor agonist, (1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-1) into the amygdala produced a dose-dependent decrease in anxiety behaviour, as measured by the elevated plus-maze test (Wieronska et al., 2005). Intrahippocampal injections of ACPT-1 have similarly been shown to reduce anxiety behaviour and this effect can be reversed by the group III mGlu receptor antagonist, CPPG (Palucha et al., 2004). Thus any agents acting at group III mGlu receptors are predicted to have multiple CNS effects.

1.4.2.6 Group III mGlu receptor localisation within the basal ganglia and pharmacology.

With the exception of the mGlu6 receptor, group III mGlu receptors are expressed throughout the basal ganglia. Through in-situ hybridisation studies, the mRNA for mGlu4, 7 & 8 receptors was found to be highest in the striatum and lowest in the SNpc, without significant differences between sub-types of receptor (Messenger et al., 2002c). Furthermore, binding studies using [3H]-L-AP4 in rat brain sections have described high levels of binding in the globus pallidus and the SNpr, suggesting these receptors are present on striatopallidal, striatonigral and subthalamonaligral synapses. Lesser binding levels was seen in the caudate-putamen, whilst comparatively lower levels were seen in the STN
and SNpc (Hudtloff and Thomsen, 1998b). Immunohistochemical studies have shown moderate to intense staining for mGlu7a in the striatum, globus pallidus and SNpr with similarly lower levels reported for the SNpc and STN (Kosinski et al., 1999e). A diagram of the localisation of group III mGlu receptor localisation within the basal ganglia is shown in Figure 3.

Figure 3: Localisation of group III mGlu receptors in the basal ganglia circuitry.

Key to References:
1. (Corti et al., 2002)
2. (Bradley et al., 1999c)
3. (Kosinski et al., 1999d)
4. (Thomsen and Hampson, 1999c)
5. (Messenger et al., 2002f)

Key to Symbol:
* Presence of group III mGlu receptors

Figure 3: The presence of group III mGlu receptors within the basal ganglia.
Table 1: Group III metabotropic glutamate receptor pharmacology:

Potencies of agonists (EC$_{50}$ values) and antagonists (IC$_{50}$ values) at mGlu receptor clones (expressed in μM)

<table>
<thead>
<tr>
<th>Compound</th>
<th>mGlu4</th>
<th>mGlu6</th>
<th>mGlu7</th>
<th>mGlu8</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group III agonists:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>3-38</td>
<td>5-36</td>
<td>&gt;1,000</td>
<td>3-11</td>
<td>(Cartmell and Schoepp, 2000d)</td>
</tr>
<tr>
<td>L-AP4</td>
<td>0.2-1</td>
<td>0.2-0.9</td>
<td>&gt;100</td>
<td>0.06-0.9</td>
<td>(Cartmell and Schoepp, 2000c)</td>
</tr>
<tr>
<td>L-SOP</td>
<td>1-4</td>
<td>0.4-3</td>
<td>&gt;100</td>
<td>0.3-2</td>
<td>(Cartmell and Schoepp, 2000b)</td>
</tr>
<tr>
<td>ACPT-1</td>
<td>7.2</td>
<td>-</td>
<td>-</td>
<td>8.2</td>
<td>(De Colle C. et al., 2000b)</td>
</tr>
<tr>
<td>(S)-3,4-DCPG</td>
<td>8.8</td>
<td>3.6</td>
<td>&gt;100</td>
<td>0.031</td>
<td>(Thomas et al., 2001b)</td>
</tr>
<tr>
<td><strong>Group III allosteric modulators:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHCCC</td>
<td>3.7</td>
<td>-</td>
<td>&gt;100</td>
<td>14.7</td>
<td>(Marino et al., 2003d)</td>
</tr>
<tr>
<td>(Rat)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group III antagonists:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-SOP</td>
<td>$K_D$ 51 against L-AP4-mediated depression of the fast component of the dorsal root-evoked ventral root potential</td>
<td>(Thomas et al., 1996a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-AP4</td>
<td>105</td>
<td>-</td>
<td>-</td>
<td>88.1</td>
<td>(De Colle C. et al., 2000a)</td>
</tr>
<tr>
<td>UBP1112</td>
<td>5.1 against depression of the fast component of the dorsal root-evoked ventral root potential induced by L-AP4</td>
<td>(Miller et al., 2003a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPPG</td>
<td>2.2 against L-AP4-mediated inhibition of cAMP (no mGlu receptor subtype mentioned)</td>
<td>(Toms et al., 1996a)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- = no data reported to date. No pharmacokinetic data is yet available for any group III mGlu receptor-interacting compounds. No compound (except (S)-3,4-DCPG) crosses the blood-brain barrier (Linden et al., 2003).
1.4 Aims of this project.

To date, relatively few studies have concentrated on group III mGlu receptors in Parkinson's disease. Those few published have largely investigated the ability of group III mGlu receptor agonists to inhibit excess GABA release in the globus pallidus (GPe) (Valenti et al., 2003a; Marino et al., 2003b; Marino et al., 2003e). The results from these studies have been promising, with group III mGlu receptor agonists able to reduce IPSPs in the GPe and provide restoration of movement following reserpine-induced akinesia. However, group III mGlu receptors are also present on glutamatergic subthalamonigral terminals (Bradley et al., 1999b; Corti et al., 2002), the activity of which is increased in PD. Surgical procedures aimed at reducing the activity of this pathway, (such as deep-brain stimulation or subthalamotomy) have shown efficacy in the treatment of PD (Alvarez et al., 2005a; Hamani et al., 2005b). However, such activities are expensive and remain limited to a few centres world-wide. Nonetheless, these approaches have validated targeting of STN efferents in PD. A possible alternative strategy would be targeting of inhibitory auto-receptors present on these terminals (such as group III mGlu receptors). This approach may provide a non-surgical mimic of subthalamotomy, and may provide symptomatic relief from the motor complications PD. Indeed, electrophysiological studies have suggested that activation of pre-synaptic group III mGlu receptors in the SNpr can reduce EPSPs generated following STN stimulation (Wittmann et al., 2001c). Furthermore, previous studies performed in this laboratory have shown that an intranigral injections of the group III mGlu receptor agonist, L-SOP in an animal model of akinesia (the reserpine-treated rat) can restore motor movement (MacInnes et al., 2004m). However, neither of these studies could qualify which group III mGlu receptors (mGlu4,7 or 8) were important in mediating these effects. Thus, initial studies reported in Chapter 1 aimed to demonstrate that activation of group III mGlu receptors in the rat SNpr could inhibit $[^{3}H]$-D-aspartate (an index of glutamate) release. Those studies reported in Chapter 2 investigated whether intranigral injection of other group III mGlu receptor-modulating compounds could similarly reverse reserpine-induced
akinesia. Furthermore, since the group III mGlu receptors in the SNpr are likely to comprise of more than one subset of receptors, a second aim of both these studies was to start to determine which group III mGlu receptor(s) may be responsible for mediating any effects seen. The aim of the final set of experiments, reported in Chapter 5, was to determine whether the inhibition of [³H]-D-aspartate release, or restoration of motor movement, was maintained following repeated agonist administration, as would be required therapeutically.

Thus, the overall aim of this thesis was to investigate the hypothesis that activation of group III mGlu receptors within the SNpr may provide an alternative, non-dopaminergic, therapy in the treatment of PD.
Materials & Methological considerations
2.1 Standard protocols

2.1.1 Animals

The female reproductive cycle has been shown to influence a number of parameters under investigation within this thesis. For example, estrodiol has recently been demonstrated to alter group I and II mGlu receptor-mediated responses (Boulware et al., 2005). In light of the fact that group III mGlu receptors share 40% sequence homology with these receptors, it is possible that group III mGlu receptor-mediated responses may also be affected. Furthermore, monthly alterations in oestrogen levels have been demonstrated to alter the susceptibility of nigral dopaminergic neurons to the neurotoxin 6-hydroxydopamine (6-OHDA, section 2.1.2.2). Investigators have reported increased striatal dopaminergic loss in rats at diestrus (low oestrogen levels) when compared to proestrus (high oestrogen levels), together with increased expression of the dopamine transporter (via which 6-OHDA enters dopaminergic neurons) at diestrus (Datla et al., 2003). Since studies in Chapters 4 & 5 of this thesis have employed the 6-OHDA lesioned rat model of PD, male animals were used to reduce individual variability to the extent of the lesion.

Further important considerations in the study of group III mGlu receptor-mediated responses are possible changes in receptor levels during ageing. It has been demonstrated for the mGlu7 receptor that both receptor mRNA and protein levels are decreased in cortical regions in aged rats (Simonyi et al., 2000). Additionally, the ability of the mGlu5 receptor to potentiate NMDA receptor-induced effects in the striatum is significantly reduced in aged versus young rats (Domenici et al., 2003).

Therefore, to avoid alterations in group III mGlu receptor-mediated responses, male Sprague-Dawley rats (B & K, U.K.) weighing between 270 and 320g, corresponding to 9 – 12 weeks of development, were used for all experiments.
2.1.2 Rodent models of Parkinson's disease

2.1.2.1 Reserpine-treated rat model of akinesia

Systemic administration of the plant alkaloid reserpine in rabbits was observed to lead to an akinetic state. Moreover, this akinesia was reversed by administration of L-DOPA (Carlsson et al., 1957). These discoveries lead to the major hypothesis involving the loss of dopaminergic transmission in PD. Following confirmation of this hypothesis in human subjects, the reserpine-treated rat model was developed in order to screen potential antiparkinsonian compounds. There are significant differences between this model and the 6-OHDA model of PD. The first is the manner in which dopaminergic transmission is inhibited; reserpine is an inhibitor of the vesicular monoamine transporter-1, preventing the accumulation of dopamine and other monoamines such as noradrenaline and 5-HT within vesicles. The second difference is that reserpine administration does not require stereotaxic administration (unlike 6-OHDA), it being able to cross the blood-brain barrier. Thus peripheral (subcutaneous) injection will not only deplete CNS catecholamines, but also those in the periphery. It is this loss of dopaminergic tone in the gastrointestinal tract that underlies the severe side-effect of diarrhoea seen following reserpine treatment. The final difference between reserpine and 6-OHDA treatments is the lack of neurodegeneration in the former. Following reserpine injection (at the dose used in studies described in Chapters 4 & 5 of 5 mg kg\(^{-1}\)), striatal dopamine levels are reduced to 1-2% of pre-treatment levels for between 4 and 48 hours following reserpine-treatment, as demonstrated using HPLC coupled to electrochemical detection (Trugman and James, 1992b). Approximately 18 hours following reserpine-treatment rats display a hunched, akinetic form with loss of normal exploratory behaviour. Additionally, unlike 6-OHDA, reserpine-treatment is temporary and reversible, with rats recovering from reserpine-induced akinesia after a few days following drug washout. As there is no neurodegeneration there are no morphological changes in the dopaminergic neurons of the SNpc (Betarbet et al., 2002a). This lack of morphological changes, coupled with the
fact that reserpine is short-acting and is non-specific for dopaminergic terminals has lead to some criticisms of this as an animal model for PD. However, since it is increasingly known that parkinsonism is not solely restricted to the degeneration of dopaminergic neurons, but involves additional alterations in other brain monoamine transmission, this may result in a resurgence of interest in this model. Furthermore, practically reserpine treatment is quick, cheap and allows a number of antiparkinsonian compounds to be screened with relative ease. It is therefore considered a very worthwhile neurochemical and behavioural model of PD in the early stages of experimentation with new symptomatic treatment approaches.

2.1.2.2 6-hydroxydopamine model of PD

6-Hydroxydopamine (6-OHDA) is a hydroxylated analogue of the neurotransmitter dopamine. Its first biological effects were reported over forty years ago, it being noticed to induce noradrenaline depletion in the autonomic nervous system of the heart (PORTER et al., 1963). Shortly afterwards, it was demonstrated to be able to destroy nerve cell endings of sympathetic neurons (Ungerstedt, 1968). Today, 6-OHDA is widely used as a rodent model for PD, allowing lesioning of various sites within the CNS (Deumens et al., 2002b). As discussed in section 2.1.2.1 6-OHDA does not readily cross the blood-brain barrier and therefore administration necessitates the use of stereotaxic surgery. Within the CNS three surgical targets are used to target destruction of nigrostriatal dopaminergic neurotransmission. These are the striatum, the SNpc and the median forebrain bundle. Direct injection of 6-OHDA into the SNpc causes rapid cell loss (within 24hr) within the lateral borders of this nucleus (Deumens et al., 2002a), although the small size of this nucleus can lead to inaccurate toxin placement, thus necessitating the use of larger sample sizes. Conversely, striatal injection of 6-OHDA leads to a more protracted retrograde degeneration of the dopaminergic neurons within the SNpc, taking between one to three weeks for a stable lesion to develop (Przedborski et al., 1995). The site
of injection used in the studies within this thesis was the median forebrain bundle (MFB), which contains the densely packed ascending neurons of the nigrostriatal pathway (Parent and Hazrati, 1995a). Lesion of the MFB can cause a total destruction of A9 (SNpc) and A10 (VTA) dopaminergic areas, reflecting the degeneration in idiopathic PD (Perese et al., 1989). These surgical techniques are commonly only performed on one hemisphere, producing a unilateral 6-OHDA lesion. Bilateral lesions are possible, and may more accurately reflect changes in basal ganglia transmission. However, bilateral MFB lesions have been reported to manifest as extreme akinesia with aphagia and adipsia, necessitating gavage-feeding, and are associated with a high level of mortality (Deumens et al., 2002c). In any event use of the unilateral model allows for intact hemisphere control for neurochemical and histological analysis, and for drug-induced rotations as behavioural indicators of lesion size where such information is required.

As 6-OHDA and dopamine are structurally similar, 6-OHDA enters neurons (noradrenergic and dopaminergic) via the high affinity dopamine or noradrenaline transporter. Retrograde transport then conveys the toxin to the cell body, leading to the accumulation of 6-OHDA within CNS neurons and induction of cell death in the absence of apoptotic markers (Jeon et al., 1995). The mechanisms by which 6-OHDA induces cell death include oxidative stress and mitochondrial inhibition, since 6-OHDA undergoes non-enzymatic auto-oxidation (Blum et al., 2001), producing hydrogen peroxide and hydroxyl radicals in the presence of iron (Kumar et al., 1995). Furthermore 6-OHDA induces a reduction of striatal glutathione and superoxide dismutase enzyme activities, together with increased levels of malondialdehyde (Betarbet et al., 2002b). All of these mechanisms are involved in oxidative stress, since accumulation of hydroxyl radicals and H$_2$O$_2$, coupled with a reduction in the antioxidants glutathione will lead to neuronal membrane damage, loss of mitochondrial membrane potential and subsequent cellular integrity. Furthermore, 6-OHDA has been shown to inhibit mitochondrial complex I,
resulting in increased free radical formation together with a reduction in cellular ATP. The involvement of oxidative stress in the neurotoxicity of 6-OHDA can be exemplified by the prevention of 6-OHDA toxicity by pre-treatment with antioxidants such as catalase (Blum et al., 2000). Since 6-OHDA can also enter noradrenergic neurons present within the MFB, the uptake-1 inhibitor, desipramine is commonly given as a pre-lesion treatment to prevent this uptake, and maintain specific destruction of dopaminergic neurons. Additionally, to enhance the local 6-OHDA concentration, the monoamine oxidase type-B inhibitor, pargyline is given pre-6-OHDA to inhibit 6-OHDA degradation, and enhance lesion efficacy.

As alluded to above, unilateral 6-OHDA injection leads to an asymmetric and quantifiable motor behaviour which can be induced by either dopamine receptor agonists (e.g. apomorphine) or dopamine releasing agents (e.g. amphetamine). Following MFB lesion, postsynaptic striatal dopamine receptors on striatofugal pathways on the same side (ipsilateral) to the lesioned site become supersensitive (Schober, 2004b). Since apomorphine is a mixed D₁ / D₂ receptor agonist, injection of apomorphine therefore stimulates the supersensitive dopamine receptors in the lesioned hemisphere, resulting in a pronounced turning response away from the lesion (contraversive turning). Conversely injection with the sympathomimetic agent amphetamine will result in release of vesicular dopamine from the non-lesioned hemisphere only, resulting in rotational behaviour towards the lesion site (ipsiversive turning).

A measure of pre-mortem lesion extent is the number of rotations induced by either amphetamine or apomorphine. Only when greater than 90 % or more of the nigrostriatal cells have been lost will a 1 mg kg⁻¹ dose of apomorphine induce rotational behaviour of greater than 4 turns min⁻¹ (Hefti et al., 1980c). In contrast a 5 mg kg⁻¹ dose of amphetamine will induce rotational behaviour once only 50 % of the nigrostriatal cells are lost, and is therefore less stringent than apomorphine, although amphetamine is good for monitoring partial lesions,
where these are appropriate (Hefti et al., 1980b). Studies in chapters 4 & 5 of this thesis investigated the effects of a full dopaminergic lesion, therefore apomorphine, rather than amphetamine was used pre-mortem to screen the effectiveness of 6-OHDA-induced lesions.

The relative selectivity of 6-OHDA for dopaminergic neurons when given with the pre-treatment previously mentioned means this model does not fully mimic the changes seen in PD. Structures other than the nigrostriatal tract degenerate in idiopathic PD, for example the locus coeruleus and anterior olfactory structures (Schober, 2004a). Furthermore, intracytoplasmic inclusions such as Lewy bodies are rarely seen post-mortem following 6-OHDA, and the acute nature of nigral damage does not mimic the prolonged degeneration seen in PD (Betarbet et al., 2002c). Nevertheless, this model has been used to evaluate the efficacy of antiparkinsonian compounds (such as investigated in this thesis), along with gene and stem cell therapies (Dauer and Przedborski, 2003b) as a pre-clinical step before their administration to the MPTP-treated primates, a further model of PD. This latter model does not feature in the present thesis so will not be described here further.

2.1.3 Measurement of neurotransmitter release

Chapter 3 of this thesis describes the study of glutamate release from the rodent substantia nigra, together with the ability of group III mGlu receptors to alter this release.

Neurotransmitter release within the basal ganglia has been investigated using a wide variety of techniques. These include in vivo microdialysis, in vitro electrophysiology, and release studies from brain slices or synaptosomes. In electrophysiological techniques, iontophoretic application of drugs or electrical stimulation of pre-synaptic neurons may result in changes in EPSPs or IPSPs recorded from the post-synaptic neuron. However, this approach does not
measure neurotransmitter release directly, rather changes in extracellular neurotransmitter levels and their ability to elicit a post-synaptic response are recorded. Moreover, this technique requires sophisticated equipment which was not available in our laboratory. Similarly, microdialysis also measures changes in extracellular neurotransmitter levels, rather than directly measuring the pre-synaptic transmitter release. Microdialysis involved surgical placement of probes into target regions that record alterations in neurotransmitter levels following stimulation, compared to basal levels. However, in addition to not measuring release directly, microdialysis is a labour intensive technique, requiring extensive training and equipment which was not available at the start of these studies, and necessitates the use of large numbers of animals. One way in which these potential problems can be overcome is the use of in vitro techniques.

In vitro neurotransmitter release can be measured either by tissue slice preparations, or through the use of synaptosomes. Synaptosomes are sealed presynaptic nerve terminals obtained by homogenising brain tissue under iso-osmotic conditions. Since synaptosomes are devoid of normal tissue architecture, including glia and their associated synapses they can be used to identify neurotransmitter release in a more idealised fashion when compared to tissue slice use (Garcia-Sanz et al., 2001). Conversely, tissue slices retain cell bodies and supporting glial components of synapses, therefore more closely mimicking physiological conditions. Synaptosomes are a valuable approach in elucidating the pre-synaptic mechanism by which neurotransmitters are released, since any change in release measured should be the effect of experimental manipulation, whereas tissue slices are of greater value when manipulating physiological or pathophysiological neurotransmitter release. Thus both experimental approaches have advantages and disadvantages which need to be carefully considered before experimental work is carried out.
Measurements of neurotransmitter release from tissue slices, as carried out in this thesis, can be achieved in a number of different experimental paradigms. Generally tissue is held in Perspex chambers with a temperature-controlled physiological buffer solution being constantly superfused over the tissue. Tissue eluent can then be removed in timed fractioned samples and assayed for transmitter content. Neurotransmitters can be assayed either by the use of high-performance liquid chromatography (HPLC) for endogenous transmitters, or may involve scintillation counting of pre-radioactively labelled ([³H] or [¹⁴C]) transmitter compounds. Radiolabelled amino acid markers have the advantages of greater sensitivity, less expense and methodological ease when compared to HPLC. Since glutamate is involved in a wide variety of intracellular metabolic pathways including the production of peptides and protein synthesis, fatty acid synthesis and GABA synthesis, there is difficulty in separating radiolabelled glutamate for use in metabolism compared with glutamate used as a transmitter. For this reason, D-aspartate, a non-metabolisable analogue of L-glutamate is used in these release studies. D-aspartate shares the same neuronal transporter as endogenous glutamate, and is released in an identical fashion (Ferkany and Coyle, 1983; Savage et al., 2001), but is not metabolised.

As uptake of transmitters released following depolarisation can readily occur, overall neurotransmitter release therefore measures the balance between uptake and release. Thus, net efflux of transmitter would be expected to be higher with a rapid removal of eluent by higher flow rates. Experimentally, this appears to be the case, with slow (0.5 ml min⁻¹) perfusion rates yielding lesser efflux of D-[³H]-aspartate when compared with medium (1.0 ml min⁻¹) and high (1.6 ml min⁻¹) perfusion rates; although no further increase in efflux was observed between medium and high perfusion rates (Palmer and Reiter, 1994b). Accordingly, release studies described in this thesis used 1.0 ml min⁻¹ flow rate.
The nature of the depolarising stimulus, used to mimic the physiological effects of action potential-evoked transmitter release, is another factor for consideration in release studies. The use of depolarising stimuli permits further differentiation between vesicular release and free amino acids present in the extracellular environment, which may otherwise be included in basal release rates. A variety of mechanisms can be employed to induce neuronal depolarisation including: elevated extracellular K⁺ (KCl), veratridine, Na⁺ / K⁺-ATPase inhibitors and potassium channel blockers, all of which have been shown to induce depolarisation (Bernath, 1992). Elevated KCl leads to depolarisation through decreasing the outward potassium gradient from neurons. This therefore increases the resting membrane potential beyond threshold and allows voltage-gated sodium channels to open, calcium influx and subsequent exocytotic neurotransmitter release. Furthermore, elevated KCl inhibits uptake of transmitter substances through a variety of mechanisms including a reduction in Na⁺ concentration gradient and inhibition of binding of Na⁺ to ionic transporters (Bernath, 1992). In contrast, veratradine directly interacts with tetrodotoxin-sensitive ion channels, leading to rapid Na⁺ influx, associated Ca²⁺ influx and transmitter release whilst ouabain inhibits the Na⁺ / K⁺-ATP carrier protein, therefore leading to depolarisation if Na⁺ is present externally. As opening of potassium channels tend to hyperpolarise the cell membrane potential towards the equilibrium potential for potassium, blockade of these channels by 4-aminopyrididine prolongs depolarisation and is required for 4-aminopyridine-induced transmitter release. Studies in Chapter 3 of this thesis have used elevated KCl as a depolarising stimulus. Elevated KCl is the most frequently used method for inducing depolarisation, since potassium ions are already present in the extracellular environment. Changes in endogenous ion concentrations may be of greater physiological relevance compared to synthetic compounds whose side-effects may be difficult to gauge. The concentration of KCl used to evoke release is also worthy of consideration. Free potassium ions are present in the extracellular fluid at approximately 5mM, a factor which is mimicked in normal Krebs’ solution. Whilst effective transmitter release can be
induced by 25mM KCl, concentrations as high as 50mM have also been used (Maneuf et al., 1995c). There appears to be a linear relationship between the concentration of KCl used and the amount of evoked transmitter release (Mangano et al., 1991; Martire et al., 2000). Nevertheless the intense release initiated by 50mM KCl has been shown to release amino acids from glia in a manner that is largely calcium-independent, together with swelling of cells indicative of an osmotic imbalance (Bernath, 1992). The lower concentration of 25mM KCl has already been established to induce neurotransmitter release in this laboratory (Chadha et al., 2000b) and was therefore used for studies described in this thesis. Furthermore, this lower concentration of 25mM KCl has previously been described to have little (if any) effect on depolarising glial cells, which may complicate the interpretation of any results obtained (Bernath, 1992).

A fuller methodology of all the methods used in this thesis is given in the relevant chapters.
2.2 Materials

2.2.1 Standard solutions

Krebs' solution 134 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂
1 mM MgSO₄, 25 mM NaHCO₃
1.25 mM KH₂PO₄, 10 mM glucose

2.2.2 Standard reagents and consumables

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid, glacial</td>
<td>Sigma</td>
</tr>
<tr>
<td>Apomorphine</td>
<td>Sigma</td>
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<tr>
<td>Ascorbic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>[³H]-D-aspartate</td>
<td>Amersham</td>
</tr>
<tr>
<td>(R)-4-Amino-3-(4-chlorophenyl) butanoic acid</td>
<td>Tocris Cookson</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td>Tocris Cookson</td>
</tr>
<tr>
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<td>Sigma</td>
</tr>
<tr>
<td>Cannulae tubing</td>
<td>Coopers</td>
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<tr>
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</tr>
<tr>
<td>(RS)-alpha-cyclopropyl-4-phosphonophenylglycine (CPPG)</td>
<td>Tocris Cookson</td>
</tr>
<tr>
<td>Cresyl fast violet</td>
<td>Sigma</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>Supplier</td>
</tr>
<tr>
<td>---------------</td>
<td>----------</td>
</tr>
<tr>
<td>(S)-3,4-dicarboxyphenylglycine</td>
<td>Tocris Cookson</td>
</tr>
<tr>
<td>Dental cement</td>
<td>De Trey</td>
</tr>
<tr>
<td>Desipramine</td>
<td>Sigma</td>
</tr>
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<td>Dimethyl sulfoxide</td>
<td>Sigma</td>
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<tr>
<td>D-glucose</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>6-hydroxydopamine</td>
<td>Sigma</td>
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<tr>
<td>Isopentane</td>
<td>BDH</td>
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<tr>
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<td>Tocris Cookson</td>
</tr>
<tr>
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<td>Packard</td>
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<td>Sigma</td>
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<tr>
<td>N-phenyl-7-(hydroxyminocyclopropa[b]chromen-1a-carboxamide)</td>
<td>Tocris Cookson</td>
</tr>
<tr>
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<td>Sigma</td>
</tr>
<tr>
<td>Pipette tips</td>
<td>BDH</td>
</tr>
</tbody>
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Portex tubing  Portex
Potassium chloride  Sigma
Potassium hydrogen phosphate  Sigma
Reserpine  Sigma
Scintillation vials  BDH
Sodium chloride  Sigma
Sodium hydrogen carbonate  Sigma
(RS)-alpha-methyl-3-methyl-4-phosphonophenylglycine (UBP1112)  Tocris Cookson

2.2.3  Supplier contacts

Amersham  Amersham Pharmacia Biotech UK Ltd, Bucks, UK
BDH  BDH Laboratory Supplies, Poole, Dorset, UK
Bright  Bright Instruments Company Ltd, Cambs, UK
Clerkenwell Screws  Clerkenwell Screws, Farringdon, London, UK
Coopers  Coopers Needleworks, Birmingham, UK
De Trey  Dentsply Ltd, Weybridge, Surrey, UK
Packard  Packard Bioscience Ltd, Pangbourne, Berkshire, UK
Portex  Promega, Southampton, Hants, UK
Sigma  Sigma Chemical Company Ltd, Poole, Dorset, UK
Tocris Cookson  Tocris Cookson Ltd, Avonmouth, Bristol, UK
Chapter 3: Investigating the ability of group III mGlu receptor agonists to inhibit glutamate release within the rodent substantia nigra.
3.1. Introduction

In Parkinson's disease (PD) the loss of striatal dopamine following degeneration of the substantia nigra pars compacta initiates a number of downstream changes within the basal ganglia. One of these changes is over-activity of the glutamatergic subthalamic nucleus (STN). This over-activity of the STN subsequently leads to increases in activity levels of the output nuclei of the basal ganglia, the substantia nigra pars reticulata (SNpr) and the internal globus pallidus (GPI). This increased activity of the output regions in turn leads to inhibition of thalamocortical feedback. It is this inhibition of cortical feedback which is believed to underlie the poverty of movement and postural instability seen in PD (Obeso et al., 2000b).

As detailed in Chapter 1, current therapies for PD primarily focus on the restoration of the lost striatal dopamine, either through administration of the dopamine precursor L-DOPA or dopamine agonists. Whilst these therapies are initially effective, with long-term use disabling side-effects such as dyskinesias appear, limiting their continued use (Lozano et al., 1998a; Nutt, 2001b). Thus novel therapeutic agents which are devoid of the side-effects associated with L-DOPA therapy, whilst still maintaining therapeutic efficacy, are keenly sought. Current surgical procedures have been shown to be remarkably effective at reducing the symptoms of PD, with the main surgical target being the STN. Inactivation of the STN by subthalamotomy, or more commonly deep-brain stimulation (DBS) is, however, expensive and only available in a few centres worldwide. This procedure also carries increased surgical risks since it is performed on a predominantly elderly population (Lozano, 2003). Clearly therefore a pharmacological mimic of subthalamotomy may maintain the beneficial effects of the procedure, whilst minimising the risks associated with surgery and be more available to patients.
One pharmacological approach aiming at reducing glutamatergic transmission at the subthalamonigral synapse has been the use of ionotropic glutamate (iGlu) receptor antagonists. Administration of these antagonists would be expected to inhibit the post-synaptic actions of glutamate at this synapse and theoretically relieve the motor dysfunctions seen in PD. Indeed, administration of selective iGlu receptor antagonists has been shown to alleviate parkinsonian symptoms in animal models of PD (Nash et al., 2000; Nash and Brotchie, 2002a). However due to the widespread distribution of iGlu receptors within the CNS, coupled with their pivotal role in mediating fast excitatory transmission, iGlu receptor antagonists are currently plagued with side effects that prevent their use as therapeutic agents for PD. For example, administration of selective NMDA antagonists can lead to learning and memory problems (Starr, 1995).

Other approaches to inhibiting the actions of the overactive STN could involve reduced activation of the STN, brought about by inhibition of excitatory afferents (or activation of inhibitory afferents) to the STN. One problem with this approach is the wealth of afferents to the STN (discussed in detail in Chapter 1, section 1.2.4.2.) which include corticosubthalamic, pallidosubthalamic, thalamosubthalamic, pedunculopontine-subthalamic and nigrosubthalamic projections. At present the roles of these various afferents in the generation of over-activity within the STN are poorly understood (discussed in Chapter 1, section 1.3.2.2). Until the different contributions of each pathway to STN over-activity are further elucidated, interference with those brain nuclei projecting to the STN is hard to rationalise and may, at best, provide limited therapeutic efficacy. Indeed, animal studies examining inhibition of the STN by direct micro-injection of the GABA-A receptor agonist, muscimol into the STN of rats, report the appearance of orofacial dyskinesias and postural asymmetry (Dybdal and Gale, 2000a; Mehta et al., 2005). The appearance of such unwanted side-effects following STN inhibition may also prove difficult to manage therapeutically.
Having discounted any interference at this stage with afferents to the STN, one remaining possibility is to bring about a normalisation of STN activity within basal ganglia outputs is to reduce glutamate release from these neurons. One way in which this might be achieved is through activation of pre-synaptic auto-receptors present on subthalamonicgrial terminals. In this respect, the group III metabotropic glutamate (mGlu) receptors may provide an interesting target worthy of consideration.

Group III mGlu receptors are pre-synaptic G\textsubscript{i} / G\textsubscript{o} coupled receptors whose activation in other brain regions inhibits transmitter release in a hetero- or auto-receptor fashion. The pre-synaptic inhibition of glutamatergic transmission in particular has been well documented in several brain regions including the thalamus (Turner and Salt, 1999) and superior colliculus (Pothecary et al., 2002). Therefore, if group III mGlu receptors are present within the basal ganglia, they may be expected to inhibit glutamatergic transmission in a manner similar to that seen in other brain nuclei.

Fortunately, the presence of pre-synaptic group III mGlu receptors in the rodent basal ganglia, and of special relevance here in the SNpr, has recently been confirmed using a variety of techniques. As described in chapter 1, group III mGlu receptors at least four distinct receptors, mGlu 4, 6, 7 & 8; of which mGlu 4, 7 & 8 are found within the brain, whilst mGlu6 is largely restricted to the retina. In-situ hybridisation studies have shown the presence of mGlu 4,7 & 8 mRNA within basal ganglia regions including the STN (Testa et al., 1994c; Messenger et al., 2002b). Therefore receptor expression on STN terminals within the SNpr is highly likely. Radioligand binding assays using [\textsuperscript{3}H]-L-AP4, a broad-spectrum group III mGlu receptor agonist, have shown high expression of group III binding sites within the striatum, globus pallidus and SNpr of the rat brain (Hudtlof and Thomsen, 1998a). However, the concentration of L-AP4 used in this study (30 nM) was specifically designed to investigate high affinity
receptors and is therefore unlikely to pick out mGlu7, due to the relatively low affinity of the mGlu7 receptor for this agonist (Cartmell and Schoepp, 2000a).

More specifically within the SNpr, current evidence suggests that more than one subtype of group III receptor is present. Thus, confocal microscopy using antibodies raised against the mGlu7a receptor and the pre-synaptic terminal marker synaptophysin have demonstrated their pre-synaptic co-localisation on (anatomically unidentified) afferents in the SNpr. However, intrastriatal injection of quinolinic acid, which preferentially destroys striatal projection neurons, markedly reduced (by approximately 40%) the immunoreactivity for mGlu7a in the SNpr (Kosinski et al., 1999b). Whilst these data suggest a large component of the total mGlu7a immunoreactivity within the SNpr may arise from striatal GABAergic efferents, the source of the remaining immunoreactivity is as yet unknown. Given that the STN makes up a large proportion of the remaining SNpr afferent innervation, it is reasonable to assume that at least some of this immunoreactivity represents mGlu7a receptors present on STN terminals.

A similar pattern of immunoreactivity has also been shown for mGlu4a in the SNpr. Double labelling of mGlu4a antibodies and synaptic vesicle protein-2 suggest a similar pre-synaptic localisation of mGlu4 receptors (Bradley et al., 1999d). Electron microscopy studies using antibodies directed against the mGlu4 receptor have furthermore shown their presence on pre-synaptic asymmetric terminals in the SNpr (Corti et al., 2002) suggesting that at least some of this labelling probably reflects glutamatergic neurons of STN origin.

To date no immunohistochemical studies have investigated the distribution of mGlu8 within the basal ganglia. However, comparing [$^3$H]-L-AP4 binding in wild-type and mGlu4 receptor knock-out mice reveals 20% specific binding remaining in the SNpr of knock-out mice (Thomsen and Hampson, 1999b). These results indicate a major involvement of mGlu4 in the binding of L-AP4 in this region. The remaining high-affinity specific binding is most likely due to
mGlu8. However, similar studies have not been performed in mGlu8 or mGlu7 knock-out mice to make a direct comparison of the relative involvement of the different group III receptor subtypes. Future immunohistochemical studies will be needed to confirm this hypothesis and to determine whether mGlu8 is indeed present in the rodent SNpr.

In summary therefore, mGlu4 is likely to be a key player, whilst mGlu7 and mGlu8 remain potential candidates. Since group III receptors have been shown to be present on pre-synaptic terminals within the SNpr, it is not surprising that the functional role of these receptors has already been examined to some extent.

To date only one study has examined the group III mGlu receptor-mediated modulation of glutamatergic transmission within the SNpr. In this study, a slice preparation of rat brain was generated containing the STN and SNpr. EPSPs were generated by stimulating the STN and recorded in the SNpr. Since these EPSPs were completely abolished following application of 10 µM CNQX (an AMPA receptor antagonist), these EPSPs were characterised as monosynaptic glutamatergic responses. Bath application of both broad-spectrum group III mGlu receptor agonists, L-AP4 (500 µM) and L-SOP (1 mM) produced a reversible depression of these EPSPs. This L-AP4-mediated depression of EPSPs was reversed by prior application of the group III mGlu receptor antagonist, CPPG, confirming the effects of both L-SOP and L-AP4 were mediated by group III mGlu receptors. This same study investigated whether this inhibition of EPSPs observed following L-AP4 application resulted from a pre-synaptic mechanism. Application of the AMPA / kainate receptor agonist, kainate (in the presence of the sodium channel inhibitor, tetrodotoxin) into the slice elicited an inward current in the SNpr. Application of 500 µM L-AP4 had no effect on this inward current suggesting L-AP4 does not modulate post-synaptic AMPA / kainate-activated channels in the SNpr (Wittmann et al., 2001b). Current limited evidence therefore favours an autoreceptor role for group III
mGlu receptors in modulating glutamate release from STN terminals within the SNpr.

3.1.2 Aims

Whilst the ability of group III metabotropic glutamate receptor agonists to modulate glutamatergic transmission in the SNpr has been supported using electrophysiological techniques, the potential auto-receptor role of group III mGlu receptors within the SNpr has not been fully investigated. The initial aim of these studies was therefore to investigate the effect of group III mGlu receptor modulation on glutamate release in the SNpr. Furthermore, since the group III mGlu receptors present in this region are likely to be comprised of more than one subtype, a second aim was to start to determine which group III mGlu receptor may be responsible for mediating these effects.
3.2. Materials and methods

3.2.1. Animals

Male Sprague-Dawley rats (270-320g) were housed in a temperature- and humidity-controlled environment with a 12h light/dark cycle with access to food and water ad libitum.

3.2.2. $[^3H]$-D-aspartate release studies

Animals were killed by stunning and decapitation, and their brains rapidly removed. Brains were placed in ice cold Krebs' buffer (composition in mM: NaCl, 134; MgSO$_4$, 1; NaHCO$_3$, 25; KCl, 5; KH$_2$PO$_4$, 1.25; glucose, 10) at pH7.4, and the substantia nigra (SNpr) was dissected out. The temporary removal of the calcium component from Krebs' buffer was due to the propensity for calcium to precipitate out of solution at low temperatures. The SNpr was dissected by placing the brain in a brain mould and cutting away the cerebellum and caudal 3mm of the cerebrum with a single-edged razor blade to expose the SNpr, which was then dissected using fine forceps. For any given experiment, the nigras of 3 animals were then pooled together for further processing. Tissue was then further sliced, using a McIlwain tissue chopper, set at 350 µm. Sliced tissue was then rotated through 45 degrees and further chopped. This stage was repeated twice. Chopped tissues were then placed in aerated (95% O$_2$ / 5% CO$_2$) calcium-containing Krebs' buffer (composition in mM: NaCl, 134; CaCl$_2$ 1.3; MgSO$_4$, 1; NaHCO$_3$, 25; KCl, 5; KH$_2$PO$_4$, 1.25; glucose, 10) for 45 min in a water bath at 37°C. All Krebs' buffer from this stage onwards included CaCl$_2$. Following this equilibration period, Krebs' buffer was removed and replaced with 5 ml fresh Krebs' to which was added $[^3H]$-D-aspartate, to permit loading of the tissue with this radiolabelled non-metabolisable analogue of glutamate (6 µl of
Specific activity 9.25 MBq/ml to give a total experimental activity of 0.222 MBq) for 45 min at 37°C. Supernatant was then removed and the tissue washed 3 times for 10 min in 5 ml fresh Krebs' buffer to remove any [3H]-D-aspartate not incorporated into the tissue. On the final wash stage, Krebs' buffer was removed and replaced with 2 ml fresh Krebs'. The tissue suspension was then transferred to a 12 chamber (100 µl per chamber) superfusion system (Brandel, USA) through use of a 200 µl plastic pipette with the sharp end removed. For each superfusion chamber, tissue was held in place by 5 mm diameter Whatmann GF/B filter discs placed above and below the tissue.

Following a 1h equilibration period where gassed Krebs' buffer was superfused at 1.0 ml min⁻¹ and the eluent discarded, collection of 2 min release fractions commenced. Superfused eluent was collected into 5 ml scintillation vials, each containing 3 ml of Optiphase HiSafe scintillation fluid, set underneath the apparatus. Basal [3H]-D-aspartate release was determined over the first 4 fractions (8 min). This was followed by an initial 2 min stimulation (S1) with Krebs' buffer containing 25mM KCl. The 25mM KCl solution was prepared by equimolar substitution with NaCl, to maintain osmolarity (composition in mM: NaCl, 109; CaCl₂ 1.3; MgSO₄, 1; NaHCO₃, 25; KCl, 25; KH₂PO₄, 1.25; glucose, 10, pH 7.4). An 18 min washout period in Krebs' buffer was then followed by a second 25mM KCl stimulation for 2 min (S2) and a further 10 min in normal Krebs' buffer to the end of the experiment. Where agonists or allosteric modulators were used, these were included in the Krebs' buffer at the start of fraction 10 (20 min after the start of the experiment), until the final 10 min was in normal Krebs' buffer. For studies using antagonists, these were included in the Krebs' buffer at fraction 8 (16 min after the start of the experiment) and thereafter until the final 10 min wash in normal Krebs' buffer. In all cases vehicle was Krebs' buffer alone, except for PHCCC which was 1% DMSO in Krebs' buffer. At the end of the experiment all collection vials were tightly capped and inverted a number of times to thoroughly mix the eluent and scintillation fluid. Finally the tissue suspension and filter paper discs from the superfusion
chamber were added to vials containing 3 ml scintillation fluid with 950 µl Krebs’ buffer, capped and mixed. This resulted in 252 scintillation vials, comprising 20 fractions per tissue chamber and 12 tissues. These scintillation vials were then analysed for [3H]-D-aspartate content by liquid scintillation spectroscopy to provide counts per minute (cpm) per fraction.

3.2.2.1. Data handling

Release rate graphs were generated describing the amount of [3H]-D-aspartate released from the tissue in each 2 min fraction as a percentage of the total [3H]-D-aspartate present in the tissue at the start of that collection fraction. This amount was derived from the following steps.

Step 1: Determining the total amount of [3H]-D-aspartate in tissue at the start of each collection fraction.

To obtain the total amount (t) of [3H]-D-aspartate present in the tissue at the start of each experimental fraction (tF), the [3H] counts (c) from each sample (e.g. cF1) were added together, in reverse order of collection. For example, starting at the last sample (F20);

\[
\begin{align*}
\text{tF20} &= c \text{ (tissue)} + cF20 \\
\text{tF19} &= c \text{ (tissue)} + cF20 + cF19 \\
\text{tF18} &= c \text{ (tissue)} + cF20 + cF19 + cF18 \\
\end{align*}
\]

… etc until

\[
(c\text{ (tissue)}) = \text{amount of [3H] in the tissue at the end of the experiment}).
\]
Step 2: Determining the percentage $[^{3}H]$-D-aspartate released per fraction.

Secondly, the amount of $[^{3}H]$-D-aspartate released in each 2 min fraction was calculated as a percentage of the total in the tissue at the start of each fraction. This was assessed in the following way, by calculating the $[^{3}H]$ counts (c) from each sample (e.g. cF1) as a percentage of the total $[^{3}H]$ present (tF1) at the start of that fraction (e.g. tF1, obtained from step 1). For example, the percentage $[^{3}H]$-D-aspartate release for fraction 1 = cumulative total of fraction 2 (tF2) / cumulative total of fraction 1 (tF1) (x 100). An example of the data handling performed in Excel is shown below.

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<th>Column B</th>
<th>Column C</th>
<th>Column D</th>
<th>Column E</th>
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The fractional $[^{3}H]$ release thus determined for each 2 min fraction was then plotted as a time versus fractional release rate curve of the $[^{3}H]$ released from each sample in an experimental run. The total $[^{3}H]$ content in S1 or S2 from each chamber was calculated automatically by use of Origin 7 Peak-fitting.
software where the integrated area under the curve was measured (Aston Scientific, UK), comprising the period following KCl stimulation until $[^3\text{H}]$-D-aspartate levels returned to baseline (approximately 4 fractions), for each sample.

Each experiment was conducted using the tissue taken from 3 animals and repeated 2 or 3 times on different days. Within any given experiment, each condition was performed in 3 chambers, giving a total number of replications of 6 or 9, taken from 6 or 9 rats for any given condition. Where depicted n represents the number of animals used.

Mean fractional release (mean ± s.e.m.) was then calculated for each experimental condition pooling data from all experimental runs. Using the dual stimulation release protocol, the total mean fractional release of S2 / S1 gave the ratio of neurotransmitter release for each chamber. This approach thereby allowed the effects of experimental manipulation on release to be quantified.

A typical percentage release rate curve is shown in figure 3.1. overleaf.

3.2.2.2 Positive controls and calcium dependency in the SNpr

Initial experiments were performed to (a) confirm the sensitivity of this protocol for assessing $[^3\text{H}]$-D-aspartate release and (b) to establish how much $[^3\text{H}]$-D-aspartate release was calcium dependent and therefore likely to be neuronal in origin.
(a) The κ-opioid receptor agonist, BRL 53537 an acknowledged inhibitor of glutamate release in the SNpr (Maneuf et al., 1995a), or vehicle (Krebs' buffer) was included in the superfusate 10 min before, during and after S2 in order to examine its effects on release.

(b) Calcium dependency of the evoked \( ^{3}\text{H}\)-D-aspartic acid release was assessed by replacing the superfusate with Ca\(^{2+}\) free Krebs' buffer containing 1mM EDTA 10 min prior to (and for the duration of) S2 (now 25mM KCl in Ca\(^{2+}\) free Krebs' buffer).

**Figure 3.1:** Percentage release rate curve of \( ^{3}\text{H}\)-D-aspartate from rat SNpr

![](image)

**Figure 3.1:** Percentage release rate of \( ^{3}\text{H}\)-D-aspartate from slices of rodent substantia nigra pars reticulata evoked by dual 25 mM KCl stimulation (S1 at 8 min and S2 at 28 min). \( n = 8 \) animals.
3.2.3. Data Analyses

Differences between the various concentrations of a given drug, e.g. BRL 52537; group III mGlu receptor agonists or allosteric modulators; group III mGlu receptor antagonists were analysed using a one-way Analysis of Variance (ANOVA) and a Student-Newman-Keuls post-hoc test. A one-way Analysis of Variance (ANOVA) and a Student-Newman-Keuls post-hoc test were also used to examine the effects of group III mGlu receptor antagonists against the effects of group III mGlu receptor agonists. Differences between calcium absence or presence within Krebs' solution were analysed using a two-tailed, unpaired Students t-test. In all cases, \( P < 0.05 \) was taken to represent statistical difference.
3.3. Results


Representative percentage release rate curves for 25mM KCl-evoked release of $[^3H]$-D-aspartate from rodent SNr tissue in the presence and absence of calcium in the perfusing Krebs' are shown in figure 3.2 (a) overleaf. Under control conditions basal $[^3H]$-D-aspartate release was approximately 1% per 2 min fraction. $[^3H]$-D-aspartate release increased approximately 2.5 fold over basal levels following the first 25mM KCl stimulus and increased the same amount following the second 25mM KCl stimulus. In contrast, a clear reduction in the release evoked by the second stimulus could be seen under calcium-free conditions. Removal of calcium did not affect the basal level of release. The resultant mean S2 / S1 ratio in control slices was 0.99 ± 0.05 (mean ± s.e.m., n = 9). 25mM KCl-evoked $[^3H]$-D-aspartate release was significantly (P < 0.001) reduced in Ca$^{2+}$-free conditions by 61.71 ± 4.3% (mean ± s.e.m., n = 9) compared to control conditions, show in figure 3.2 (b).

3.3.2. Effect of the K-opioid agonist, BRL 52537 on KCl-evoked $[^3H]$-D-aspartate release

BRL 52537 (200µM) had no effect on basal $[^3H]$-D-aspartate release (P<0.01; n=9), but produced an approximate 80% reduction in KCl-evoked $[^3H]$-D-aspartate release at S2, when compared to vehicle (figure 3.3 (a)) . The effects of increasing concentrations of BRL 52537 on $[^3H]$-D-aspartate release ratios (S2 / S1) are shown in figure 3.3 (b) over the full concentration range tested. BRL 52537 (100 and 200 µM) produced a significant concentration-dependent inhibition of 25mM KCl-evoked $[^3H]$-D-aspartate release reaching a maximum inhibition of 77 ± 2.4 % (mean ± s.e.m., n = 9) with the highest concentration tested (200 µM). Concentrations of BRL 52537 below 100 µM failed to inhibit the release of $[^3H]$-D-aspartate.
Figure 3.2: Calcium dependency of 25mM KCl-evoked [3H]-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation in the presence (black) and absence (blue) of 1.3mM calcium in the perfusing Krebs' solution where indicated (n = 9).

(b) Integrated mean S2 / S1 ratio data showing effect of calcium removal (mean ± s.e.m., n = 9). *** = significant difference (P < 0.001) compared to vehicle (two-way unpaired t-test).
Figure 3.3: Effects of the κ-opioid agonist, BRL 52537 on 25mM KCl-evoked
[^3H]-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation
showing the effects of BRL 52537 on S2 compared to vehicle. (b) Integrated mean S2 / S1 ratio
data showing effects of increasing concentration of BRL 52537 on[^3H]-D-aspartate release.
Values represent mean ± s.e.m. (n = 9). *** (P < 0.001) and ** (P < 0.01) indicate significant
difference compared to vehicle; # indicates a significant difference to 100µM BRL 52537 (1-way
ANOVA, P < 0.05)
3.3.3. Effect of the broad-spectrum group III mGlu receptor agonist, L-SOP on KCl-evoked [³H]-D-aspartate release

L-SOP (300µM) had no effect on basal [³H]-D-aspartate release (P<0.01; n=9), but produced an approximate 30% reduction in KCl-evoked [³H]-D-aspartate release at S2, when compared to vehicle (figure 3.4 (a)). The effects of increasing concentrations of L-SOP on [³H]-D-aspartate release ratios (S2 / S1) are shown in figure 3.4 (b). L-SOP (100 and 300 µM) produced a significant concentration-dependent inhibition of 25mM KCl-evoked [³H]-D-aspartate release, reaching a maximum inhibition of 32 ± 5.4 % (mean ± s.e.m., n = 9) with 300 µM L-SOP. Concentrations of L-SOP below 100 µM and above 300 µM failed to inhibit the release of [³H]-D-aspartate.

3.3.4. Effect of the broad-spectrum group III mGlu receptor agonist, L-AP4 on KCl-evoked [³H]-D-aspartate release

L-AP4 (30µM) had no effect on basal [³H]-D-aspartate release (P<0.01; n=6), but produced an approximate 40% reduction in KCl-evoked [³H]-D-aspartate release at S2, when compared to vehicle (figure 3.5 (a)). The effects of increasing concentrations of L-AP4 on [³H]-D-aspartate release ratios (S2 / S1) are shown in figure 3.5 (b) L-AP4 (10, 30 and 300 µM) produced a significant inhibition of 25mM KCl-evoked [³H]-D-aspartate release, reaching a maximum inhibition of 52 ± 8.1 % (mean ± s.e.m. , n = 6) with 10 µM L-AP4. L-AP4 exhibited a biphasic concentration-response curve, with concentrations of L-AP4 below 10 µM failing to significantly inhibit the release of [³H]-D-aspartate. Furthermore, whilst 100 µM L-AP4 did not inhibit aspartate release, the higher concentration of 300 µM did significantly reduce the release of [³H]-D-aspartate. At 600 µM basal release was significantly increased (p<0.001; n=6), resulting in loss of peak definition resulting in inability to measure S2 / S1 release ratios (data not shown).
Figure 3.4: Effects of the broad-spectrum group III mGlu receptor agonist, L-SOP on 25mM KCl-evoked [3H]-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation showing the effects of L-SOP (300 µM) on S2 compared to vehicle. (b) Integrated mean S2 / S1 ratio data showing effect of increasing concentrations of L-SOP on [3H]-D-aspartate release. Values represent mean ± s.e.m. (n = 9). * (P < 0.05) indicates significant difference compared to vehicle (1-way ANOVA, P < 0.05).
Figure 3.5: Effects of the broad-spectrum group III mGlu receptor agonist, L-AP4 on 25mM KCl-evoked \[^{3}H\]-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation showing the effects of L-AP4 (30 µM) on S2 compared to vehicle. (b) Integrated mean S2 / S1 ratio data showing effect of increasing concentrations of L-AP4 on \[^{3}H\]-D-aspartate release. Values represent mean ± s.e.m. (n = 6). *** (P < 0.001) and ** (P < 0.01) indicates significant difference compared to vehicle; ### (P < 0.001) indicate a significant difference compared to 100 µM L-AP4 and ~ (P < 0.05) indicates a significant difference compared to 3 µM L-AP4 (1-way ANOVA, P < 0.001).

Figure 3.5: (a) Representative percentage release rate curves for dual 25mM KCl stimulation showing the effects of L-AP4 (30 µM) on S2 compared to vehicle. (b) Integrated mean S2 / S1 ratio data showing effect of increasing concentrations of L-AP4 on \[^{3}H\]-D-aspartate release. Values represent mean ± s.e.m. (n = 6). *** (P < 0.001) and ** (P < 0.01) indicates significant difference compared to vehicle; ### (P < 0.001) indicate a significant difference compared to 100 µM L-AP4 and ~ (P < 0.05) indicates a significant difference compared to 3 µM L-AP4 (1-way ANOVA, P < 0.001).
3.3.5. Effect of the broad-spectrum group III mGlu receptor antagonist, CPPG on L-SOP-mediated decreases in KCl-evoked $[^3]$H-D-aspartate release

Figure 3.6 (a) shows the representative release rate curves for L-SOP in the presence of vehicle or CPPG. CPPG was perfused for 4 min (2 fractions) prior to the addition of L-SOP. CPPG (100 µM) alone had no effect on basal $[^3]$H-D-aspartate release (P<0.01; n=6), but abolished the inhibition of $[^3]$H-D-aspartate seen following perfusion with 100 µM L-SOP, returning $[^3]$H-D-aspartate release levels to vehicle values. Addition of CPPG (100 µM) abolished the effect of 100 µM L-SOP on $[^3]$H-D-aspartate release shown in Figure 3.6 (b). Thus, whilst 100 µM L-SOP alone gave an approximate 50% reduction in $[^3]$H-D-aspartate release, pre-treatment with CPPG negated this effect and returned $[^3]$H-D-aspartate release levels to those of vehicle.

3.3.6. Effect of the broad-spectrum group III mGlu receptor antagonist, CPPG on L-AP4-mediated decreases in KCl-evoked $[^3]$H-D-aspartate release

Figure 3.7 (a) shows the representative release rate curves for vehicle and CPPG together with L-AP4. CPPG was perfused for 4 min (2 fractions) prior to the addition of L-AP4. CPPG (100 µM) alone had no effect on basal $[^3]$H-D-aspartate release (P<0.01; n=6), but abolished the inhibition of $[^3]$H-D-aspartate seen following perfusion with 30 µM L-AP4, returning $[^3]$H-D-aspartate release levels to vehicle values. Addition of CPPG (100 µM) abolished the effect of 30 µM L-AP4 on $[^3]$H-D-aspartate release shown in Figure 3.7 (b). Thus, whilst 30 µM L-AP4 alone gave an approximate 50% reduction in $[^3]$H-D-aspartate release, pre-treatment with CPPG negated this effect and returned $[^3]$H-D-aspartate release levels to those of vehicle.
Figure 3.6: Effects of the group III mGlu receptor antagonist, CPPG on 25mM KCl-evoked [3H]-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation showing the effects of L-SOP & Vehicle (red), compared to Vehicle alone (black) and L-SOP & CPPG (blue). (b) Integrated mean S2 / S1 ratio data showing effects of L-SOP and CPPG alone and together in combination (all concentrations are 100µM) on [3H]-D-aspartate release. Values represent mean ± s.e.m. (n = 6). *** (P < 0.001) and indicates significant difference compared to vehicle, 100 µM CPPG and the combination of 100µM L-SOP and 100µM CPPG (1-way ANOVA, P < 0.001).
Figure 3.7: Effects of the group III mGlu receptor antagonist, CPPG on 25mM KCl-evoked [3H]-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation showing the effects of L-AP4 & Vehicle (red), compared to Vehicle alone (black) and L-AP4 & CPPG (blue).

(b) Integrated mean S2 / S1 ratio data showing effects of L-AP4 (30µM) and CPPG (100µM) alone and together in combination on [3H]-D-aspartate release. Values represent mean ± s.e.m. (n = 6). ***(P < 0.001) and indicates significant difference compared to vehicle, 100 µM CPPG and the combination of 30µM L-AP4 and 100µM CPPG (1-way ANOVA, P < 0.001).
3.3.7. Effect of the broad-spectrum group III mGlu receptor antagonist, M-SOP on L-SOP-mediated decreases in KCl-evoked $[^3]$H-D-aspartate release

M-SOP (50 µM) had no effect on basal $[^3]$H-D-aspartate release (P<0.01; n=6), but surprisingly produced a significant reduction of approximately 20% in KCl-evoked $[^3]$H-D-aspartate release at S2, when compared to vehicle. Pre-treatment with M-SOP (50 µM) similarly did not reverse the inhibition of $[^3]$H-D-aspartate release following L-SOP (100 µM) incubation. Interestingly, the combination of M-SOP (50 µM) and L-SOP (100 µM) lead to a synergistic reduction in release levels, with the combination of antagonist and agonist enhancing the reduction in $[^3]$H-D-aspartate levels compared to either antagonist or agonist alone. The combination of M-SOP and L-SOP had no effect on basal release (P < 0.05, n = 6) but lead to an approximate 43% reduction in KCl-evoked $[^3]$H-D-aspartate release at S2, when compared to vehicle shown in figure 3.8 (a). The effects of vehicle, 100 µM L-SOP alone, 50 µM M-SOP alone and the combination of the two agents (at above concentrations) on $[^3]$H-D-aspartate release ratios (S2 / S1) are shown in figure 3.8 (b) overleaf. M-SOP was perfused for 4 min (2 fractions) prior to the addition of L-SOP for co-perfusion studies.
Figure 3.8: Effects of the group III mGlu receptor antagonist, M-SOP on 25mM KCl-evoked $[^3]$H-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation showing the effects of M-SOP & L-SOP (red), compared to Vehicle alone (black) and L-SOP & Vehicle (blue). (b) Integrated mean S2 / S1 ratio data showing effects of M-SOP (50µM) and L-SOP (100µM) alone and together in combination on $[^3]$H-D-aspartate release. Values represent mean ± s.e.m. (n = 6). *** (P < 0.001), ** (P < 0.01) and * (P < 0.05) indicates significant difference compared to vehicle (1-way ANOVA, P<0.001 and # (P < 0.05) indicates a significant difference compared to M-SOP alone (1-way ANOVA, P < 0.001).
3.3.8. Effect of the mGlu4 positive allosteric modulator, PHCCC on KCl-evoked \([^{3}\text{H}]-\text{D-aspartate release}

PHCCC (30 µM) alone had no effect on basal \([^{3}\text{H}]-\text{D-aspartate release} \) (P<0.01; n=6), and produced no significant alterations in KCl-evoked \([^{3}\text{H}]-\text{D-aspartate release} \) at S2, when compared to vehicle (1% DMSO in Krebs' buffer, figure 3.9a). However, when given simultaneously with a sub-threshold concentration of L-SOP (30 µM), the combination of L-SOP and PHCCC resulted in a significant reduction in release of 45% at S2, compared with S1; shown in figure 3.9 (b). The effects of vehicle, 30 µM L-SOP alone, 30 µM PHCCC alone and the combination of the two agents (both 30 µM) \([^{3}\text{H}]-\text{D-aspartate release ratios} \) (S2 / S1) are shown in figure 3.9 (c). The combination of L-SOP and PHCCC produced a significant inhibition of 25mM KCl-evoked \([^{3}\text{H}]-\text{D-aspartate release} \), with an inhibition of 45 ± 2.2% (mean ± s.e.m., n = 6).
Figure 3.9: Effects of the mGlu4 positive allosteric modulator, PHCCC on 25mM KCl-evoked \([\text{^3H}]\)-D-aspartate release from slices of rodent substantia nigra.

(a) 

(b)
Figure 3.9: Representative percentage release rate curves for dual 25mM KCl stimulation showing the effects of (a) Vehicle (black) and 30 µM PHCCC (blue) on S2 compared to vehicle and (b) 30µM L-SOP and vehicle (red) and the combination of 30µM L-SOP and PHCCC (green). (b) Integrated mean S2 / S1 ratio data showing effects of L-SOP and PHCCC alone and together in combination (all concentrations are 30µM) on [3H]-D-aspartate release. Values represent mean ± s.e.m. (n = 6). *** (P < 0.001) and indicates significant difference compared to vehicle (1-way ANOVA, P < 0.001).
This study examined the hypothesis that activation of group III mGlu receptors may reduce glutamate release in the SNpr. The data presented here indicate that both of the broad-spectrum group III mGlu receptor agonists examined, L-SOP and L-AP4 inhibit the release of \(^{[3]}\text{H}\)-D-aspartate in a concentration-dependent manner with no detectable alterations in basal release levels at the concentrations reported. These effects of both L-SOP and L-AP4 were antagonised by the competitive antagonist for group III mGlu receptors, CPPG. Furthermore, whilst the mGlu4 positive allosteric modulator, PHCCC alone did not affect depolarisation-induced \(^{[3]}\text{H}\)-D-aspartate, when given in conjunction with a sub-threshold concentration of L-SOP, a significant reduction in \(^{[3]}\text{H}\)-D-aspartate release was observed. Taken together these data suggest that activation of group III mGlu receptors in the SNpr may prove useful in the search for novel pharmacological approaches to the treatment of PD, by reducing release of glutamate (potentially from STN terminals) in the SNpr. Furthermore this inhibition of glutamate release is likely mediated at least in part by mGlu4.

Basal release of \(^{[3]}\text{H}\)-D-aspartate from the SNpr of approximately 1% was in keeping with that reported in studies using \(^{[3]}\text{H}\)-D-aspartate as an index of glutamate release in other brain regions, such as the cortex (Meldrum et al., 1992). The amount of calcium dependency reported here (circa 70%) was broadly in agreement with other published research, suggesting circa 80% of KCl-evoked \(^{[3]}\text{H}\)-D-aspartate release is calcium dependent (Palmer and Reiter, 1994a). This indicates that the majority of the \(^{[3]}\text{H}\)-D-aspartate released during the present studies was likely to be neuronal in origin. Since the STN efferents comprise the major glutamatergic input to the SNpr (Parent and Hazrati, 1995h) these neurons likely represent the source of a large proportion of the evoked \(^{[3]}\text{H}\)-D-aspartate release in the present study. However, since the STN is not the sole source of excitatory inputs to the SNpr, some contribution of other glutamatergic synapses cannot be excluded. These include glutamatergic
efferents from the pedunculopontine nucleus and cortex. The source of the remaining (~30%) Ca\(^{2+}\)-independent fraction of \([^{3}H]\)-D-aspartate is unknown though a number of possibilities exist. Once such possibility is that a component of these Ca\(^{2+}\)-independent responses arises from the reversal of glutamate transporters driving glutamate extrusion. Whilst this possibility cannot be entirely eliminated, earlier studies have suggested a minimal impact of transporter reversal on \([^{3}H]\)-D-aspartate release under calcium-free conditions (Palmer and Reiter, 1994c). One further possibility for calcium-independent release is depolarisation of glia, leading to transmitter efflux. However previous studies have reported external K\(^{+}\) concentrations of 50-55 mM to be required for glial amino acid transmitter release, rather than 25 mM used in this study (Bernath, 1992), so again this is not a convincing source of Ca\(^{2+}\)-independent release.

The endogenous opioid agonist dynorphin is used as a transmitter by striatal medium spiny neurons projecting to the SNpr and GPi. Additionally pre-synaptic kappa opioid receptors are found in high concentrations within the SNpr (McCormick and Stoessl, 2002). Activation of pre-synaptic G\(_{i}\) / G\(_{o}\)-coupled kappa opioid receptors by the specific agonist, enadoline had previously been reported to reduce \([^{3}H]\)-glutamate release from the SNpr, with a maximum effect of 78% inhibition at 200 \(\mu\)M ( Maneuf et al., 1995b). The effects of a kappa opioid agonist, in this case BRL 52537, were therefore examined here initially to serve as a positive control. Superfusion with the kappa opioid agonist, BRL 52537 (100 & 200 \(\mu\)M) resulted in a concentration-dependent inhibition of depolarisation-evoked \([^{3}H]\)-D-aspartate from the SNpr. These data (inhibition of 77%) correspond well with the level of inhibition of \([^{3}H]\)-D-aspartate reported in the study of Maneuf et al., and thus validate the use of BRL 52537 as a positive control for transmitter release studies in the SNpr. More importantly, this study served to confirm that the experimental protocol employed here was sensitive enough to detect changes in release should they occur under future experimental manipulations.
Superfusion with the group III mGlu receptor agonist, L-SOP (1 – 1000 µM) resulted in inhibition of depolarisation-induced [3H]-D-aspartate release from the SNpr at 100 and 300 µM, but not at the highest concentration tested. Since the EC_{50} values for L-SOP can be broadly categorised into two groups (mGlu4 & 8: 0.3– 4 µM and mGlu7: < 100 µM, (Cartmell and Schoepp, 2000g)) the results presented here would suggest the involvement of mGlu7 in mediating these inhibitory effects. These findings are consistent with anatomical data whereby the presence of mGlu7 has been localised to pre-synaptic sites within the SNpr (Kosinski et al., 1999a). At the highest concentration used, L-SOP may no longer be fully selective for group III mGlu receptors, but may also be expected to start to act on G_{q/11} group I receptors (EC_{50} for these receptors < 1000 µM (Cartmell and Schoepp, 2000f)) which are also present on pre-synaptic terminals of excitatory neurons within the SNpr (Wittmann et al., 2001a). Therefore the loss of efficacy seen with 1 mM L-SOP may reflect a balance of group III mGlu receptor-mediated inhibition and group I mGlu receptor-mediated elevation of transmitter release. Activation of group I mGlu receptors has certainly been demonstrated to increase potassium-evoked [3H]-D-aspartate release from excitatory neurons in the rat solitary nucleus (Jones et al., 1998). However, in the SNpr, activation of pre-synaptic group I mGlu receptors has been reported to inhibit EPSPs generated following stimulation of the STN, possibly through the release of a retrograde messenger acting at pre-synaptic sites (Wittmann et al., 2001a), although this is an isolated report. Another possibility for the loss of efficacy observed is that at such high concentrations, L-SOP is no longer selective for mGlu receptors. L-SOP may then activate other, as yet unidentified, receptors opposing the inhibitory influence seen following activation of group III mGlu receptors. One further possibility is that desensitisation or uncoupling of group III mGlu receptors may occur following 1000 µM L-SOP application, thus masking any inhibitory effects seen. This possibility will be further examined in Chapter 5 of this thesis.
Superfusion with L-AP4 (10, 30 & 300 µM) similarly reduced [3H]-D-aspartate release from nigral sections, although 100 µM failed to inhibit transmitter release. The EC$_{50}$ values for L-AP4 can likewise be broadly categorised into two groups (mGlu4 & 8: 0.06 – 1 µM and mGlu7: >100 µM (Cartmell and Schoepp, 2000e)). Therefore it appears that at the lower effective concentrations, recruitment of mGlu4 and mGlu8 mediates the inhibition of [3H]-D-aspartate release, whilst at the higher concentration of 300 µM, activation of mGlu7 may lead to additional inhibition of transmitter release. Why the concentration-release curve for L-AP4 appears biphasic, whilst that of L-SOP does not warrants further investigation, since both agonists have similar EC$_{50}$ values at all group III mGlu receptors and are almost identical in steric structure (Thoreson and Ulphani, 1995). However a similar biphasic response to L-AP4 has been reported in electrophysiological studies investigating the effects of L-AP4 on IPSP inhibition at the striatopallidal synapse (Valenti et al., 2003d). These authors suggest desensitisation of effects at higher doses, which may account for the decrease seen at 100 µM L-AP4. However experimental evidence supporting this hypothesis has yet to be reported and this explanation still seems incompatible with the efficacy returning at 300 µM L-AP4. It is interesting to note that despite similar EC$_{50}$ values at group III mGlu receptors for both L-SOP and L-AP4, the concentration of L-SOP required to achieve inhibition of EPSPs in the SNpr was twice that (1 mM) required by L-SOP (500 µM) to achieve a similar level of inhibition, suggesting in the SNpr at least, L-AP4 is the more potent of the two agonists (Wittmann et al., 2001a). The studies reported here are in agreement with these findings, although lower concentrations of both agonists were required to inhibit transmitter release than those required for inhibition of EPSPs. One further difference between the two agonists is that whilst L-AP4 does not exist in the brain, L-SOP has been found in the mammalian central nervous system at micromolar concentrations (Klunk et al., 1991). However it is unclear how the presence of endogenous L-SOP could reduce the efficacy of exogenous L-SOP without affecting that of L-AP4, since both agonists act at the same group III mGlu receptors. The possibility exists
that the loss of efficacy at 100 µM is anomalous. However this was seen on a number of occasions – not just with one group of animals, so that this is an artefact is unlikely.

The receptor specificity of both L-SOP and L-AP4 was examined using the group III mGlu receptor antagonist, CPPG. Pre-incubation with CPPG served to reverse the inhibition of evoked [³H]-D-aspartate release seen following L-SOP and L-AP4 application. Since superfusion of CPPG alone produced no effects on [³H]-D-aspartate release, this suggests group III mGlu receptors are not activated by endogenous glutamate under basal or release conditions within this slice preparation. CPPG shows an approximate 20 fold higher selectivity at group III mGlu (IC₅₀ = 2.2) versus group II mGlu receptors (IC₅₀ = 46.2 nM) (Toms et al., 1996c). Whilst the concentration of CPPG employed here (100 µM) would therefore be able to inhibit both group II and group III mediated effects, the agonists investigated are specific for activation of group III. Furthermore, the concentration of CPPG used in these studies has also been shown to be without effect on basal transmission at the striatopallidal synapse, whilst inhibiting the actions of L-AP4 at the same synapse in electrophysiological studies (Valenti et al., 2003g). Taken together, these data suggest the observed inhibitory effects of CPPG are believed to represent inhibition of the group III mGlu receptor-mediated effects of L-SOP and L-AP4.

In a bid to start to identify which group III mGlu receptor subtype mediates the observed group III mGlu receptor-mediated inhibitions in [³H]-D-aspartate release, PHCCC, a positive allosteric modulator for mGlu4, was utilised. The concentration of PHCCC used in these studies was chosen on the basis of it previously been reported to have mGlu4 positive modulatory capacity (Maj et al., 2003; Marino et al., 2003j). Superfusion of PHCCC alone had no effect on basal or evoked [³H]-D-aspartate release. This finding is in agreement with the role of PHCCC as a modulatory agent, rather than possessing any intrinsic agonist properties. When given in conjunction with a sub-threshold
concentration of L-SOP a significant reduction in evoked $[^3$H]-D-aspartate release was observed. This provides strong evidence in favour of an involvement of mGlu4 in underlying, at least in part, group III mGlu receptor-mediated inhibition of $[^3$H]-D-aspartate release in the rodent SNpr. This result is in agreement with a previous study reporting potentiation of the effects of L-AP4 by PHCCC on the inhibition of IPSPs at the GABAergic striatopallidal synapse (Marino et al., 2003k). In this previous study, bath application of L-AP4 alone was found to inhibit striatopallidal IPSPs, again through an electrophysiologically defined pre-synaptic mechanism of action. That the L-AP4-mediated inhibition is not seen in mGlu4 knock-out mice, was taken to suggest the group III mGlu receptor modulation of transmission at the striatopallidal synapse is due exclusively to the actions on the mGlu4 receptor (Valenti et al., 2003f). Parallel knock-out studies have not been performed in the present studies, so it is not possible to determine the extent to which mGlu4 contributes to the overall L-SOP or L-AP4 responses. Suffice to say that it clearly mediates at least a part of their effectiveness. However contributions of both mGlu7 and mGlu8 still remain possibilities.

Whilst PHCCC is a highly selective potentiator of mGlu4, this compound does display antagonist activity at mGlu1b, and has recently been reported to antagonise the effects following activation of mGlu2, mGlu5 and mGlu8 (Marino et al., 2003i). However, blockade of either of these receptors is unlikely to produce inhibition of $[^3$H]-D-aspartate release, since both mGlu2 and mGlu8 are negatively coupled pre-synaptic receptors, whose activation would lead to a reduction in transmitter release (Cartmell and Schoepp, 2000h). Additionally, blockade of group I mGlu receptors would be expected to increase $[^3$H]-D-aspartate release, since agonists at these receptors have been shown to inhibit EPSPs in the SNpr generated following STN stimulation (Wittmann et al., 2001a). Therefore it is most likely that the response to PHCCC observed in the present studies reflects the mGlu4 potentiator actions.
Taken together with the aforementioned concentrations of L-AP4 able to produce inhibition of $[^3H] \text{-D-aspartate}$ being within the range to stimulate mGlu4, these data suggest that group III modulation of synaptic transmission within the rat SNpr is mediated at least in part, by activation of mGlu4 on STN efferents.

In initial studies attempting to establish receptor specificity, the effects of another group III mGlu receptor antagonist, M-SOP were investigated. M-SOP is a highly selective group III antagonist, reported to antagonise the inhibition caused by L-AP4 in the rat spinal cord with a $K_D$ of 51 µM (Thomas et al., 1996b). However, no antagonism of L-SOP-induced reduction of $[^3H] \text{-D-aspartate}$ release was observed. Indeed, M-SOP alone inhibited $[^3H] \text{-D-aspartate}$ release and the combination of L-SOP and M-SOP inhibited release to a greater extent than M-SOP alone. These findings suggest that there is a difference in sensitivity to M-SOP in the rat SNpr compared with the rat spinal cord. Indeed the group III mGlu receptor believed to be underlying the response in the spinal cord appears to be almost exclusively mGlu7 (Jane et al., 1996). Whilst mGlu7 may contribute to responses within the SNpr, it is most likely that mGlu4 and possibly mGlu8 also mediate at least some component of the response seen in the SNpr. Although there appear to be no previous reports in the literature that M-SOP can act in an agonist-like manner, there are reports that a second related antagonist, M-AP4 can do so. Thus in 4-aminopyridine stimulated striatal synaptosomes, 30 µM M-AP4 alone inhibited endogenous glutamate release by approximately 45% (East et al., 1995b). In the same study both 3 and 30 µM M-AP4 failed to antagonise the effects of L-AP4. In a second study showing M-AP4 can act in an agonist-like fashion, application of M-AP4 reduced forskolin-stimulated cAMP production (East et al., 1995a; Kemp et al., 1996). That M-SOP was able to further inhibit $[^3H] \text{-D-aspartate}$ release when given in conjunction with L-SOP suggests M-SOP may act as an agonist at mGlu4 or mGlu8. However, it may also be possible that the actions of M-SOP are by acting as an antagonist at mGlu7 receptors thus leading to an enhancement of GABA release (hetero-receptor function), possibly from striatal
terminals within the SNpr. This increased GABA release may lead to a reduction in $[^3H]$-D-aspartate release through activation of pre-synaptic GABA$_B$ receptors, as discussed in Chapter 5 (Figure 5.3.8), and expanded on below.

Within the SNpr there are both glutamatergic terminals (from the STN) and GABAergic terminals (from the striatum and globus pallidus) (Parent and Hazrati, 1995m). Thus, using this experimental set-up, following 25mM KCl application, it is likely that GABA and $[^3H]$-D-aspartate will subsequently be released from these terminals within the SNpr. This dual effect on transmitter release has potentially complex effects on overall transmitter levels within the SNpr. The released GABA may then activate pre-synaptic GABA$_B$ receptors present on striatonigral and subthalamonigral terminals and serve to reduce further GABA or $[^3H]$-D-aspartate release (Shen and Johnson, 1997b). The situation becomes slightly more complex when group III mGlu receptors are involved, since activation of these receptors has been shown to reduce both IPSPs and EPSPs within the SNpr (Wittmann, 2001). Furthermore, GABA$_B$ receptor agonists, such as baclofen, have been shown to reduce $[^3H]$-D-aspartate release (figure 5.3.8, chapter 5 of this thesis). Thus, in interpreting the actions of group III mGlu receptor agonists within the SNpr, the release of GABA by striatal terminals and the subsequent binding to and activation of GABA$_B$ receptors located on the STN terminals (by the released GABA) may mask the underlying actions of the group III mGlu receptor agonists. If this were the case, this would lead to an underestimation of the effects of group III mGlu receptor agonists and therefore inclusion of a GABA$_B$ receptor antagonist in the experimental paradigm should reveal a greater effect of group III mGlu receptor agonists. However, superfusion of the selective GABA$_B$ receptor antagonist, CGP46831 resulted in no changes to either basal or elevated KCI-evoked release in the SNpr (figure 5.3.8, chapter 5). These results indicate that activation of GABA$_B$ receptors do not play a role in determining the amount of $[^3H]$-D-aspartate released from terminals in the SNpr. It is therefore unlikely that the effects of group III mGlu receptor agonists are being underestimated in this case.
In conclusion, studies presented in this chapter serve to demonstrate that activation of group III mGlu receptors can lead to a reduction of glutamate release within the SNpr, and that the mGlu4 receptor, at least, is likely to be involved in mediating these effects, whilst the possibility remains that both the mGlu7 and mGlu8 receptors may also contribute. These receptors may therefore provide new targets for the development of pharmacological treatments for Parkinson's disease, and warrant further investigation \textit{in vivo} in rodent models of this condition.
Chapter 4: Investigating the \textit{in vivo} effects of group III mGlu receptor modulation in the parkinsonian rat substantia nigra pars reticulata.
4.1 Introduction

As discussed in chapter 3 of this thesis, activation of group III metabotropic glutamate receptors within the substantia nigra pars reticulata (SNpr) leads to a reduction in glutamate (as measured using the analogue aspartate) release from neuronal terminals within this region. Since the majority of glutamatergic terminals within the SNpr arise from subthalamic nucleus (STN) efferents (Parent and Hazrati, 1995n), activation of pre-synaptic group III mGlu receptors in the SNpr would be predicted to decrease glutamate release from these STN efferents and thereby reduce subsequent excitation of the SNpr. In Parkinson’s disease (PD) the STN is over-active (discussed in Chapter 1, section 1.2.3.2.11). One consequence of this over-activity of the STN is excessive activation of the output nuclei of the basal ganglia, the SNpr and the internal segment of the globus pallidus. This over-activity of the output nuclei leads to inhibition of thalamo-cortical feedback, resulting in motor dysfunctions such as akinesia and bradykinesia seen in PD patients (Obeso et al., 2000c). Inhibition of excessive glutamate release from STN terminals in the SNpr would therefore be hypothesised to reduce excitation in at least one of the output regions of the basal ganglia. This reduction in activity of the output area would then be expected to restore thalamo-cortical feedback which, in turn, should restore normal motor functions, alleviating some primary symptoms of PD. Such an effect might be witnessed experimentally as relief from akinesia in animal models of PD.

Previous in vivo studies have investigated the effects of mGlu receptor modulation in animal models of PD. However, to date only a handful of studies have investigated the role of group III mGlu receptor activation and of these studies only one has investigated the role of group III mGlu receptor activation specifically in the SNpr. One possible reason for the paucity of in vivo experiments investigating the effects of group III mGlu receptor modulation is that most currently available agents do not readily cross the blood-brain barrier, necessitating direct intracerebral stereotaxic administration.
Relatively diffuse activation of receptors throughout the mammalian CNS can be brought about through injection of drugs into the cerebral ventricles. Thus diffusion and net cerebrospinal fluid flow will allow receptors expressed in different brain regions to be modulated, reflecting a state which may more closely mimic that seen with blood-brain barrier penetrant agents. Such routes of administration have been used in group III mGlu receptor modulation studies. Thus in animals rendered akinetic by the monoamine depleting agent reserpine, intracerebroventricular (i.c.v.) injection of L-AP4 resulted in a significant increase in motor activity compared to vehicle (Valenti et al., 2003b). In the same study, i.c.v. L-AP4 was also found to reduce haloperidol-induced catalepsy scores, compared with vehicle in a different model of acute dopamine-depletion. In a separate study from this laboratory, L-AP4 together with a second broad-spectrum group III agonist L-SOP, was similarly found to reduce reserpine-induced akinesia following i.c.v. injection compared to vehicle (MacInnes et al., 2004). To date only one study investigating the pharmacology more closely has been carried out. In this study a similar reversal of akinesia was observed following injection of PHCCC, the mGlu4 positive allosteric modulator (Marino et al., 2003). These results suggest a possible role for this receptor in mediating the in vivo efficacy of the broad-spectrum group III mGlu receptor agonists.

Since group III mGlu receptors are present within the striatum, globus pallidus and substantia nigra (Thomsen and Hampson, 1999a; reviewed in Chapter 1 section 1.4.3.3), site-directed injections have been performed in vivo to further investigate the location of group III mGlu receptors likely to be underlying this possible therapeutic effect seen following i.c.v. injections.

Injection of L-AP4 into the striatum of 6-OHDA-lesioned rats induced contraversive rotations. This induction of rotational behaviour is indicative of an anti-parkinsonian response (Kearney et al., 1998). However, in the same study, in rats treated with the monoamine-depleting agent reserpine, striatal injection of
the same dose of L-AP4 failed to reverse the reserpine-induced akinesia. These data suggest that, in acutely dopamine-depleted rats (unlike chronically lesioned animals) group III mGlu receptors present within the striatum do not mediate an anti-akinetic response. The reasons being this phenomenon are not yet clear, however this is an isolated study and these findings remain to be corroborated.

Intrapallidal (GPe) injections of L-SOP have similarly been shown to reverse reserpine-induced akinesia in the rat, presumably through inhibition of GABA release from striatopallidal fibres (MacInnes et al., 2004k). This inhibition of GABA release from an overactive striatopallidal pathway would be expected to indirectly increase GP activity which would, in turn decrease excitation of the STN via increases in GABAergic innervation from the GPe. Therefore rotational behaviour observed following intrapallidal injection of group III mGlu receptor agonists may result from decreased STN activity.

L-AP4 injection directly into the STN in naïve non-dopamine-depleted rats also resulted in contraversive rotations (Keamey and Albin, 2000). Whilst the origin of this motor behaviour is as present unknown, following intrasubthalamic L-AP4 injections a decrease in relative regional glucose utilisation was seen in the striatum, substantia nigra and areas of the ventrolateral thalamus. Since these areas are all brain regions which receive glutamatergic STN efferents, it may be hypothesised that the rotational responses are driven by inhibition of the STN, as a result of this nucleus receiving less glutamatergic innervation. Certainly such an effect could theoretically lead to a state of increased motor activity on the injected side of the animal, thereby explaining its propensity to circle in a contraversive (i.e. away from the side of injection) manner. No studies have examined how this direct manipulation of the STN would fare in a parkinsonian animal however.

It is important to note however, that administration of both L-SOP and L-AP4 are without any effect in normal (non-reserpinised) animals (MacInnes et al., 2004).
As noted in Chapter 3 however, some behavioural experiments aimed at effecting a reduction in STN activity by reducing excitatory inputs to the STN have resulted in the production of dyskinesias in rodents (Dybdal and Gale, 2000b). Thus perhaps therapeutic strategies aimed at normalising the output of the STN in such target areas as the SNpr, rather than inhibiting its firing may meet with more success. Interestingly, only one paper from work carried out in this laboratory has so far investigated the effect of group III mGlu receptor modulation in the SNpr (MacInnes et al., 2004i). Intranigral injection of L-SOP to this STN target produced a rapid reversal of reserpine-induced akinesia. The authors presumed that this reversal was brought about in the SNpr through inhibition of glutamate release. Data presented here in Chapter 3 now back up this suggestion. However, much remains to be clarified regarding this intranigral efficacy of group III mGlu receptor agonists.
4.1.2. Aims

The aim of the studies outlined in Chapter 4 was to examine whether intranigral injection of another broad-spectrum group III mGlu receptor agonist, L-AP4 was also able to reverse reserpine-induced akinesia. Furthermore, given the results of Chapter 3, it is likely that the receptor population underlying any anti-akinetic effects is likely to be comprised of more than one receptor subtype in the SNpr. A second aim was therefore to characterise which group III mGlu receptors may be responsible for mediating the anti-akinetic effects in this brain region. Additionally, no study has yet investigated the role of group III mGlu receptor modulation in the SNpr in a more chronic model of PD, the 6-OHDA-lesioned rat. Therefore, the effects of intranigral L-SOP injection on rats bearing a stable 6-OHDA lesion were also investigated, to ascertain that the anti-parkinsonian efficacy was not only evident in acute neurochemical models of this disease.
4.2 Methods

4.2.1 Animals

Male Sprague-Dawley rats (280-320g) were housed in a temperature- and humidity-controlled environment with a 12h light/dark cycle and access to food and water *ad libitum*. All procedures were performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986, and all efforts were made to minimise animal suffering and the number of animals used.

4.2.2 Studies in the reserpine-treated rat model of akinesia

4.2.2.1 Intranigral bilateral cannulation

Bilateral cannulation was performed so that in the event of blockage of one cannula, the animal could still be used for behavioural testing. Two 12mm long, 23 gauge stainless steel guide cannulae (Coopers, UK) spaced 4 mm apart were attached by means of a cannula holder connected to a stereotaxic micromanipulator (Kopf). Due to the extreme ventral location of the SNpr, co-ordinates were taken using the interaural line, with cannulae being placed 2 mm above the SNpr (co-ordinates from interaural line AP + 0.32, DV + 0.36 and ML ± 0.20 cm) according to the standard rat brain atlas of Paxinos and Watson (1986).

Under isoflurane general anaesthesia (induction: 4% in 100% O₂; maintenance 1.5-3% in 100% O₂, depending on individual animals) and following loss of pedal-withdrawal and corneal reflexes, rats were placed in a Kopf small animal stereotaxic frame with the mouth piece set at −3.3mm below the ear bars. The head of the animal was secured, and a skin incision made with a sterile scalpel blade along the midline in an anteroposterior direction. The skin and periosteum were separated from the skull by use of a cotton bud and skin clamps and the
surface of the skull exposed. The desired stereotaxic location was then marked for both cannulae tips and two cannula holes drilled through the skull. Further holes (2 anterior and 1 posterior) were drilled surrounding the original two and micro screws (size M2, Clerkenwell Screws, UK) inserted into these. Cannulae were then implanted 2mm above the SNpr, and secured using dental cement (De Trey, UK), thus anchoring the cannulae to the micro screws and skull surface. The cannula holder was then withdrawn and a plastic collar (cut from the end of a 1 ml pipette) was secured in place with a further mould of dental cement which was used to protect the protruding cannulae ends. 30-gauge stainless steel stylets were inserted into each cannula to keep them as patent as possible. A rehydrating solution of 0.9% saline (5 ml kg⁻¹, i.p.) was then administered and any loose skin around the dental cement was sutured together. Following completion of surgery, animals were allowed to recover on a thermostatically-controlled heated blanket until fully conscious.

4.2.2.2 Induction of akinesia with reserpine

After a minimum of 48 hours following surgical recovery, animals were rendered akinetic through injection of the catecholamine depleting agent, reserpine (5mg kg⁻¹, s.c. in a dose volume of 1ml kg⁻¹). Reserpine solution was prepared as 5ml of a 5 mg ml⁻¹ solution in warmed 18 MΩ water, containing 0.85% (v/v) acetic acid. To facilitate injection of this acidic solution, rats were lightly anaesthetised (4% isoflurane in 100% O₂ until loss of righting reflex) and then received both an injection of reserpine and a rehydrating solution of 0.9% saline (5 ml kg⁻¹, i.p.). Following injection, rats were replaced in cages on top of thermostatically controlled heated mats for 18 hours during which time the akinesia was allowed to develop. Following successful akinesia induction, the effects of group III mGlu receptor modulation were assessed.
### Assessment of reversal of akinesia

Behavioural assessment of motor activity was made from video-recorded observations of rats in 40cm diameter, flat-bottomed hemispherical bowls (Amee, Manchester, UK). Following a 15 min acclimatisation period, baseline activity was monitored for 30 min. Suitably akinetic animals (< 4360° rotations 30 min⁻¹) then received a single unilateral injection of test compounds into the SNpr. The following agents were examined:

<table>
<thead>
<tr>
<th>Name</th>
<th>Mechanism of action</th>
<th>Dose-Range (nmol)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-SOP</td>
<td>Broad-spectrum group</td>
<td>250 - 750</td>
<td>MacInnes et al., 2003</td>
</tr>
<tr>
<td></td>
<td>III mGlu receptor agonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-AP4</td>
<td>Broad-spectrum group</td>
<td>3 - 300</td>
<td>Not yet performed in SNpr, i.c.v. performed by Valenti et al., 2003</td>
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<tr>
<td></td>
<td>III mGlu receptor agonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S)-3,4-DCPG</td>
<td>Selective mGlu8</td>
<td>0.3 - 300</td>
<td>Not yet performed in SNpr. Characterised by Thomas et al., 2001</td>
</tr>
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<td>PHCCC</td>
<td>Selective mGlu4</td>
<td>75</td>
<td>Not yet performed in SNpr, i.c.v. performed by Marino et al., 2003</td>
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<td>5 - 75</td>
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<td>Broad-spectrum group</td>
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<td>Not yet performed in SNpr. Characterised by Miller et al., 2003</td>
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</tbody>
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Agonists were dissolved in 2.5 (L-SOP), 2 (L-AP4) or 0.5 (S-3,4-DCPG) µl phosphate buffered saline (PBS; (mM); NaCl 137, KCl 2.7, KH₂PO₄ 1.8, Na₂HPO₄ 10) and pH adjusted to 7.4 with NaOH. PHCCC was dissolved in 0.5 µl of 40% DMSO in 0.85% NaCl. The relatively large volumes needed for L-SOP and L-AP4 reflected solubility restraints previously reported (MacInnes et al., 2004h). Injections were made manually at 1µl per minute via 30 gauge stainless steel needles that extended 2mm below the tip of the guide cannulae and were attached with tubing (Portex) to a 10µl Hamilton micro syringe. Animals were videotaped for a further 30 - 60 min following each injection. Full (360°) contraversive rotations which were counted manually in 10 min time bins from the videotaped recordings were measured as an index of relief of akinesia. Where receptor specificity was assessed, the selective group III mGlu receptor antagonists M-SOP, UBP1112 or CPPG were administered 30 min prior to agonist and behaviour recorded as above for the duration of antagonist pre-injection period and agonist injection period. Antagonists were dissolved in 2.5 µl PBS (M-SOP) or 0.5 µl (UBP112 and CPPG) PBS / NaOH at pH 7.4. Following behavioural testing, animals were killed by isoflurane overdose and cervical dislocation. Brains were rapidly removed and snap frozen in isopentane at −45°C (cooled by solid CO₂) and stored dessicated at −70°C. Cresyl violet post mortem histological verification of cannulae placement was undertaken.

4.2.3 Studies in the unilateral 6-hydroxydopamine (6-OHDA) rodent model of Parkinson’s disease

30 min prior to surgery animals were injected with a combined solution of pargyline (5 mg kg⁻¹; i.p) and desipramine (25 mg kg⁻¹; i.p) dissolved in 18 MΩ water. As discussed in Chapter 2 of this thesis, pargyline and desipramine were included to elevate 6-OHDA availability and specificity for dopaminergic neurons. Under isoflurane general anaesthesia (induction: 4% in 100% O₂; maintenance 1.5-3% in 100% O₂, depending on individual animals) and following loss of pedal-withdrawal and corneal reflexes, rats were placed in a
Kopf small animal stereotaxic frame with the mouth piece set at −3.3mm below
the ear bars. The head of the animal was secured in place using blunt ear bars,
and a skin incision made with a sterile scalpel blade along the midline in an
anteroposterior direction. The skin and periosteum were separated from the
skull by use of a cotton bud and skin clamps and the surface of the skull
exposed. The stereotaxically determined location was then marked for injection
and a hole drilled through the skull. A single injection of 6-OHDA (12.5 µg in 2.5
µl sterile water containing 0.9 % saline and 0.02 % ascorbic acid) was then
made through the burr hole into the left median forebrain bundle (co-ordinates
from bregma, due to the more dorsal location of the median forebrain bundle
compared to the SNpr: AP − 0.28, DV − 0.90 and ML + 0.2 cm). The dose of 6-
OHDA was chosen on the basis of previous studies within our laboratory to
produce a full lesion of the nigrostriatal tract (Johnston et al., 2003). The
injections were made using a 5 µl Hamilton syringe held within an automated
nano pump (KD Scientific from Presearch, UK), attached to the stereotaxic
manipulator. The flow rate for injections was set to 0.5 µl min⁻¹, meaning each
injection took a total of 5 min. Following 6-OHDA infusion, a rehydrating solution
of 0.9% saline (5 ml kg⁻¹, i.p.) was administered and the skin around the wound
was sutured together. Upon completion of surgery, animals were allowed to
recover in a heated incubator until fully conscious.

4.2.3.1 Behavioural assessment of 6-OHDA lesion efficacy

The extent of the nigrostriatal tract lesion was assessed in vivo two weeks post-
surgery. 6-OHDA lesioned animals were habituated in 40 cm flat-bottomed
hemispherical observational bowls (Amee, Manchester, UK) for 15 min. Animals
were then injected with apomorphine hydrochloride (1 mg kg⁻¹; i.p) and
proceeded 24 h later with vehicle. Apomorphine was dissolved for injection in 18
MΩ water. Rotational behaviour was video-recorded and the total 360°
contraversive rotations quantified for 30 min post-injection. Those 6-OHDA
lesioned rats exhibiting ≥ four net contraversive rotations per minute were
considered to have over 90% loss of striatal dopamine terminals (Hefti et al.,
1980a). These suitably lesioned animals were then allowed to a one week recovery and apomorphine wash-out period, whereupon they underwent cannulation surgery. This surgical procedure was identical to that described in section 4.2.2.1 apart from unilateral (left side) cannulation was performed. The co-ordinates from interaural line were therefore AP + 0.32, DV + 0.36 and ML + 0.20 cm) according to the standard rat brain atlas of Paxinos and Watson (1986). A minimum of four days after cannulation, animals were then used for behavioural locomotor tests as described in section 4.2.2.3. In this case however, only the effects of a single dose of L-SOP were examined (the dose of L-SOP was taken from prior studies with reserpine-treated rats). Cannula placement was again ascertained post-mortem as detailed below

### 4.2.3.2 Injection site verification

Frozen brains were sectioned (30 µm) on a Bright OTF cryostat and microscopic assessment of these sections was made to identify those animals in which the injection needle placed through the indwelling cannulae was correctly placed. At room temperature, slides containing 3 sections of SNpr processed in triplicate were fixed in PFA (4%) and dehydrated in a rising series of ethanols (70%, 5 min; 90%, 5 min; 100%, 5 min). They were then defatted in xylene (10 min), rehydrated in a descending series of ethanol (100%, 5 min; 90%, 5 min; 70%, 5 min) before being immersed in dilute cresyl violet stain (0.1%, Sigma) for between 20 – 25 min. Sections were then rinsed in water before being dehydrated once again in a rising series of ethanols (70%, 5 min; 90%, 5 min; 100%, 5 min). Lastly, sections were placed for 10 min in histoclear (BDH) and coverslipped using DPX mounting medium (BDH) before being allowed to dry overnight and viewed under a light microscope (Leica). An example of monochromatic cresyl violet-stained image is shown in figure 4.2.1.
4.2.4 Data analysis

Only behavioural data from sections showing correct needle placement (approximately 80%) were included for subsequent data analysis. In all cases rotational data were quantified per 10 min time bin. For time course data, a two-way ANOVA with Bonferroni’s post-hoc test was used to determine whether differences between vehicle and the effects of group III mGlu receptor modulation were significant at each time point. The effect of increasing doses of group III mGlu receptor agonists (and M-SOP) were investigated using a one way ANOVA and Student-Newman-Keuls post-hoc test. The effect of PHCCC compared to vehicle was analysed using an unpaired 2-tailed t-test. In all cases, $P < 0.05$ was taken to be significant.
4.3 Results

4.3.1 Locomotor effects following intranigral injection of group III mGlu receptor agonists in the reserpine-treated rat.

4.3.1.1 L-SOP

Animals receiving L-SOP injections into regions surrounding the SNpr failed to show any remission from akinesia and data from these animals were not included in the final analysis. Throughout baseline recordings all reserpine-treated rats displayed minimal circling behaviour (0 – 1 rotation 30 min⁻¹) and were therefore deemed suitably akinetic for inclusion in subsequent studies. Vehicle injection produced no change in rotational behaviour compared to baseline. Injection of L-SOP (750 nmol) produced contraversive rotations in the reserpine-treated rats which were significantly increased compared to vehicle. These effects had both commenced and reached their peak within the first 10 min following injection and had returned to baseline levels after 30 min (Figure 4.3.1.1a). Accordingly total rotational behaviour was assessed for the full dose range of L-SOP over 30 min post L-SOP injection. Analysis of the total rotational behaviour revealed that injection of L-SOP (250 – 750 nmol) produced a dose-dependent increase in net contraversive rotations 30 min⁻¹, which peaked at 66 ± 18 rotations (mean ± s.e.m.) at the highest dose tested (Figure 4.3.1.1b). EC₅₀ values for L-SOP could not be generated due to a failure to achieve the full maximal response; animals receiving doses of L-SOP higher than 750 nmol exhibited motor dysfunctions, manifesting as seizures.
Figure 4.3.1.1: Locomotor effects following intranigral L-SOP injection in reserpine-treated rats.

(a) Time course of locomotor activity induced by a maximally effective dose of the group III mGlu receptor agonist L-SOP (750 nmol in 2.5 µl PBS) or vehicle (2.5 µl PBS) *** P < 0.001 (2-way ANOVA and Bonferroni's post-hoc test) and (b) total locomotor effects of L-SOP (250 - 750 nmol in 2.5 µl PBS) or vehicle (2.5 µl PBS) following unilateral injection into the substantia nigra pars reticulata in reserpine-treated rats. All values represent mean ± s.e.m. (n = 6 animals per dose). * P < 0.05, ** P < 0.01 and *** P < 0.001 indicate the significant differences from vehicle-treated rats; ## indicates a significant difference compared to 750 nmol L-SOP (1-way ANOVA with Student-Newman-Keuls post-hoc test, P < 0.05)
4.3.1.2 L-SOP and CPPG

As previously observed, intranigral injection of L-SOP (750 nmol) alone produced a significant increase in net contraversive rotations compared to baseline motor activity. Administration of the broad-spectrum group III mGlu receptor antagonist CPPG (75 nmol) alone produced no change in contraversive rotations compared to CPPG vehicle (PBS). However, in the continuing presence of CPPG (75 nmol) compared to vehicle, the subsequent number of net contraversive rotations produced by L-SOP (750 nmol) was significantly inhibited by 80%. Analysis of the time course revealed significant differences between CPPG and CPPG vehicle-treated groups at 10 and 20 min which were abolished by 30 min post L-SOP injection, Figure 4.3.1.2 (a). Figure 4.3.1.2 (b) shows the total number of contraversive rotations elicited by either treatment group.
Figure 4.3.1.2.: Locomotor effects following intranigral injection of L-SOP following 30 min pre-treatment with the group III mGlu receptor antagonist, CPPG or vehicle.

Figure 4.3.1.2: (a) Time course of locomotor activity and (b) total locomotor activity induced by unilateral injection into the substantia nigra pars reticulata of L-SOP (750 nmol in 2.5 µl PBS), following 30 min pre-treatment with either the group III mGlu receptor antagonist, CPPG (75 nmol in 2.5 µl PBS) or vehicle (2.5 µl PBS) in reserpine-treated rats. Values represent mean ± s.e.m. (n = 6 animals per treatment). *** P < 0.001 and ** P < 0.01 indicates a significant difference from CPPG-treated rats (2 way-ANOVA with Bonferroni's post-hoc test for (a) and a significant difference from vehicle-treated rats (b) (unpaired 2 tailed t-test).
4.3.1.3 L-AP4

Animals receiving L-AP4 injections into regions surrounding the SNpr failed to show any remission from akinesia and data from these animals were not included in the final analysis. Only those rats deemed suitably akinetic were included in final data analysis. Vehicle injection produced no change in rotational behaviour compared to baseline. Injection of L-AP4 (300 nmol) produced contraversive rotations in the reserpine-treated rats which were significantly increased compared to vehicle. These effects had both commenced and reached their peak within the first 10 min following injection and had returned to baseline levels after 30 min (Figure 4.3.1.3a). Accordingly total rotational behaviour was assessed for the full dose range of L-SOP over 30 min post L-AP4 injection. Analysis of the total rotational behaviour revealed that injection of L-AP4 (3 – 300 nmol) produced a dose-dependent increase in net contraversive rotations 30 min⁻¹, which peaked at 37 ± 5 rotations (mean ± s.e.m.) at the highest dose tested (Figure 4.3.1.3b). EC₅₀ values for L-AP4 could not be generated due to a failure to achieve the full maximal response, limits of L-AP4 solubility were reached at 300 nmol thus preventing further doses being investigated.

4.3.1.4 L-AP4 and CPPG

Administration of the group III mGlu receptor antagonist CPPG (75 nmol) alone produced no change in contraversive rotations compared to CPPG vehicle (PBS). However, in the continuing presence of CPPG (75 nmol) compared to CPPG vehicle, the subsequent number of net contraversive rotations produced by L-AP4 (300 nmol) was significantly inhibited by 71 %. Analysis of the time course revealed significant differences between CPPG and CPPG vehicle-treated groups at 10 and 20 min which were abolished by 30 min post L-AP4 injection, Figure 4.3.1.4(a). Figure 4.3.1.4(b) shows the total number of contraversive rotations elicited by either treatment group.
Figure 4.3.1.3: Locomotor effects following intranigral L-AP4 injection in reserpine-treated rats.

(a) Time course of locomotor activity induced by a maximally effective dose of the group III mGlur receptor agonist L-AP4 (300 nmol in 2 µl PBS) or vehicle (2 µl PBS) *** P < 0.001 (2-way ANOVA and Bonferroni's post-hoc test) and (b) total locomotor effects of L-AP4 (3 – 300 nmol in 2 µl PBS) or vehicle (2 µl PBS) following unilateral injection into the substantia nigra pars reticulata in reserpine-treated rats. All values represent mean ± s.e.m. (n = 6 animals per dose). * P < 0.05, ** P < 0.01 and *** P < 0.001 indicate the significant differences from vehicle-treated rats; ## indicates a significant difference compared to 750 nmol L-SOP (1-way ANOVA with Student-Newman-Keuls post-hoc test, P < 0.05)
Figure 4.3.1.4: Locomotor effects following intranigral injection of L-AP4 following 30 min pre-treatment with the group III mGlu receptor antagonist, CPPG or vehicle.

(a) Time course of locomotor activity and (b) total locomotor activity induced by unilateral injection into the substantia nigra pars reticulata of L-AP4 (300 nmol in 2 µl PBS), following 30 min pre-treatment with either the group III mGlu receptor antagonist, CPPG (75 nmol in 2 µl PBS) or vehicle (2 µl PBS) in reserpine-treated rats. Values represent mean ± s.e.m. (n = 6 animals per treatment). *** P < 0.001 and ** P < 0.01 indicates a significant difference from CPPG-treated rats (2 way-ANOVA with Bonferroni's post-hoc test for (a) and a significant difference from vehicle-treated rats (b) (unpaired 2 tailed t-test).
4.3.1.5 Locomotor effects following intranigral injection of mGlu4 positive allosteric modulator PHCCC in the reserpine-treated rat.

Animals receiving PHCCC injections into regions surrounding the SNpr failed to show any remission from akinesia and data from these animals were not included in the final analysis. Throughout baseline recordings all reserpine-treated rats displayed minimal circling behaviour (0 - 1 rotations 40 min\(^{-1}\)) and were therefore deemed suitably akinetic for inclusion in subsequent studies. Vehicle injection produced no change in rotational behaviour compared to baseline. Injection of PHCCC (75 nmol) produced contraversive rotations in the reserpine-treated rats which were significantly increased compared to vehicle. These effects had both commenced and reached their peak within the first 10 min following injection and had returned to baseline levels after 40 min (Figure 4.3.1.5a). Accordingly total measurements were made over 40 min post PHCCC injection. Injection of a single dose of PHCCC (75 nmol) produced a significant increase in net contraversive rotations 40 min\(^{-1}\) compared to vehicle treatment (Figure 4.3.1.5b).

Due to time constraints in finishing this Ph.D. thesis, only the effects of a single dose of PHCCC were investigated. However, a full dose-response study is being performed within this laboratory on the anti-akinetic effects of PHCCC.
Figure 4.3.1.5: Locomotor effects following intranigral PHCCC injection in reserpine-treated rats.

(a) Time course of locomotor activity induced by a maximally effective dose of the mGlu4 receptor positive allosteric modulator PHCCC (75 nmol in 0.5 µl 40% DMSO in 0.85% NaCl) or vehicle (0.5 µl 40% DMSO in 0.85% NaCl) and (b) total locomotor effects of (PHCCC (75 nmol in 0.5 µl 40% DMSO in 0.85% NaCl) or vehicle (0.5 µl 40% DMSO in 0.85% NaCl) following unilateral injection into the substantia nigra pars reticulata in reserpine-treated rats. Values represent mean ± s.e.m. (n = 6 animals per treatment). *** P < 0.001 indicates a significant difference from vehicle-treated rats (2-way ANOVA for (a) and an unpaired 2 tailed t-test (b)).
4.3.1.6 PHCCC and CPPG

Administration of the group III mGlu receptor antagonist CPPG (75 nmol) alone produced no change in contraversive rotations compared to CPPG vehicle (PBS). However, in the continuing presence of CPPG (75 nmol) compared to CPPG vehicle, the subsequent number of net contraversive rotations produced by PHCCC (75 nmol) was significantly inhibited by 69%. Analysis of the time course revealed significant differences between CPPG and CPPG vehicle-treated groups at 10, 20 and 30 min which were abolished by 40 min PHCCC injection, Figure 4.3.1.6(a). Figure 4.3.1.6(b) shows the total number of contraversive rotations elicited by either treatment group.
Figure 4.3.1.6: Locomotor effects following intranigral injection of PHCCC following 30 min pre-treatment with the group III mGlu receptor antagonist, CPPG or vehicle.

(a) Time course of locomotor activity and (b) total locomotor activity induced by unilateral injection into the substantia nigra pars reticulata of PHCCC (75 nmol in 0.5 µl PBS), following 30 min pre-treatment with either the group III mGlu receptor antagonist, CPPG (75 nmol in 0.5 µl PBS) or vehicle (0.5 µl PBS) in reserpine-treated rats. Values represent mean ± s.e.m. (n = 6 animals per treatment). *** P < 0.001 and ** P < 0.01 indicates a significant difference from CPPG-treated rats (2 way-ANOVA with Bonferroni's post-hoc test for (a) and a significant difference from vehicle-treated rats (b) (unpaired 2 tailed t-test).
4.3.1.7  (S)-3,4-DCPG

Animals receiving (S)-3,4-DCPG injections into regions surrounding the SNpr failed to show any remission from akinesia and data from these animals were not included in the final analysis. Throughout baseline recordings all reserpine-treated rats displayed minimal circling behaviour (0 – 2 rotations 30 min\(^{-1}\)) and were therefore deemed suitably akinetic for inclusion in subsequent studies. Intranigral injection of 300 nmol (S)-3,4-DCPG produced contraversive rotations in the reserpine-treated rats which were significantly increased compared to vehicle. Analysis of the time course revealed these effects had both commenced and reached their peak within the first 10 min following injection and had returned to baseline levels after 40 min (figure 4.3.1.7a). Accordingly total measurements were made over 30 min post (S)-3,4-DCPG injection. Injection of a single dose of (S)-3,4-DCPG (0.3 – 300 nmol) produced a dose-dependent increase in net contraversive rotations 30 min\(^{-1}\) (figure 4.3.1.7b). Higher doses were not investigated due to both cost and time constraints. Similarly, no antagonist studies were performed against the actions of (S)-3,4-DCPG due to time constraints.
Figure 4.3.1.7: Locomotor effects following intranigral (S)-3,4-DCPG injection in reserpine-treated rats.

(a) Time course of locomotor activity induced by a maximally effective dose of the mGlu8 receptor agonist (S)-3,4-DCPG (300 nmol in 0.5 µl PBS) or vehicle (0.5 µl PBS) and (b) total locomotor effects of (S)-3,4-DCPG (0.3 – 300 nmol in 0.5 µl PBS) or vehicle (0.5 µl PBS) following unilateral injection into the substantia nigra pars reticulata in reserpine-treated rats. Values represent mean ± s.e.m. (n = 4 animals per dose). *** P<0.001, ** P<0.01 and * P<0.05 indicate the significant difference from vehicle-treated rats; ### P<0.001, ## P<0.01 and # P<0.05 indicates a significant difference compared to 0.3 nmol (S)-3,4-DCPG. —— P<0.01 and —— P<0.01 indicates a significant difference to 300 nmol (S)-3,4-DCPG (2 way-ANOVA with Bonferroni’s post-hoc test for (a) and 1-way ANOVA, P < 0.0001) (b).
In initial studies attempting to demonstrate group III mGlu receptor specificity for the L-SOP-mediated reversal of akinesia two further group III mGlu receptor antagonists, M-SOP and UBP 1112 were used. Surprisingly, intranigral injection of M-SOP alone produced a biphasic dose-dependent increase in net contraversive rotations 30 min⁻¹ (Figure 4.3.1.7a). Further, a 75 nmol M-SOP pre-treatment evoked an increase of 265% in the net contraversive rotations of a subsequent L-SOP (750 nmol) injection carried out 30 min post M-SOP injection. In animals pre-treated with M-SOP vehicle, 30 min prior to L-SOP injection a response of similar magnitude to that obtained with L-SOP alone in previous studies (see Figure 4.3.1.1) was produced. (Figures 4.3.1.7 b & c). Animals were unable to be injected with L-SOP following 50 nmol M-SOP injections due to excessive central nervous system activation resulting in seizures and convulsions in all animals tested with M-SOP and L-SOP. Both 5 and 15 nmol M-SOP failed to evoke significant contraversive rotations alone, but similarly failed to significantly affect subsequent L-SOP responses.
Figure 4.3.1.7: Locomotor effects following intranigral injection of M-SOP in reserpine-treated rats.

(a) Net contraversive rotations 30 min$^{-1}$

(b) Net contraversive rotations 10 min$^{-1}$
Figure 4.3.1.7 (a) Total locomotor effects of M-SOP (5 – 75 nmol in 2.5 µl PBS) or vehicle (2.5 µl PBS) following unilateral injection into the substantia nigra pars reticulata in reserpine-treated rats. Values represent mean ± s.e.m. (n = 6 animals per dose). ** P < 0.001 and * P < 0.05 indicate the significant difference from vehicle-treated rats; ### P < 0.001 indicates a significant difference compared to 50 nmol M-SOP. ∞∞ P < 0.01 and ∞ P < 0.05 indicates a significant difference to 75 nmol M-SOP (1-way ANOVA, P < 0.0001). (b) Time course of locomotor activity induced by pre-treatment with a dose of the group III mGlu receptor antagonist M-SOP (75 nmol in 2.5 µl PBS) or vehicle (2.5 µl PBS) on subsequent L-SOP-induced locomotor behaviour. Values represent mean ± s.e.m. (n = 6 animals per treatment). * P < 0.05 indicates a significant difference from vehicle-treated rats (2-way ANOVA with Bonferroni’s post-hoc test). (c) Total locomotor effects induced by unilateral injection into the substantia nigra pars reticulata of L-SOP (750 nmol in 2.5 µl PBS), following 30 min pre-treatment with either the group III mGlu receptor antagonist, M-SOP (75 nmol in 2.5 µl PBS) or vehicle (2.5 µl PBS) in reserpine-treated rats, n = 6 (unpaired 2 tailed t-test)
Interestingly, intranigral injection of another selective broad-spectrum group III mGlu receptor agonist, UBP1112 produced similar results to those observed following M-SOP injection. Both doses of UBP112 (5 and 50 nmol) tested resulted in no significant increases in contraversive rotations when given alone. However, at the both doses tested, responses to L-SOP (750 nmol) injection 30 min post UBP1112 administration were elevated compared to 30 min prior treatment with vehicle, in a similar fashion to that seen with 75 nmol M-SOP, figure 4.3.3.5.

Figure 4.3.3.5: Locomotor effects following UBP1112 intranigral injection in reserpine-treated rats.

![Figure 4.3.3.5](image_url)

**Figure 4.3.3.5:** Total locomotor effects induced by unilateral injection into the substantia nigra pars reticulata of the group III mGlu receptor antagonist UBP1112 (5 or 50 nmol in 0.5 µl PBS). Effects of 30 min pre-injection with either vehicle (2.5 µl PBS) or 5 or 50 nmol UBP1112 (0.5 µl PBS) on subsequent L-SOP (750 nmol in 2.5 µl PBS) induced total motor activity in reserpine-treated rats. ***P < 0.05 indicates a significant difference from Vehicle and L-SOP-treated rats (1 way ANOVA, P < 0.001).
4.3.4 Studies in the 6-OHDA lesioned rat model of Parkinson's disease.

4.3.4.1. Behavioural assessment of lesion efficacy

Injection of apomorphine (1 mg kg$^{-1}$, i.p.) into 6-OHDA lesioned rats produced a significant increase in net $360^\circ$ contraversive rotations in 30 min, compared to vehicle (18 MΩ water). Thus, apomorphine produced $208 \pm 5$ rotations in 6-OHDA lesioned rats, but only $4 \pm 1$ rotations in vehicle treated rats (Figure 4.3.4.1).

**Figure 4.3.4.1:** Locomotor effects following apomorphine injection in 6-OHDA lesioned rats.

![Bar graph showing locomotor effects](image_url)

**Figure 4.3.4.1:** Total locomotor effects of intraperitoneal injection of apomorphine (1 mg kg$^{-1}$) or vehicle (18 MΩ water) in 6-OHDA lesioned rats. Values represent mean ± s.e.m. (n = 8 animals per treatment). ***P < 0.001 indicate the significant differences from vehicle-treated rats; (paired t-test)
4.3.4.2. L-SOP

Injection of L-SOP (750 nmol) produced a significant increase in net contraversive rotations 30 min\(^{-1}\) compared to vehicle (PBS) injection in 6-OHDA lesioned rats (lesion on the left hemisphere, injection of L-SOP into the left hemisphere (ipsilateral)). These effects had both commenced and reached their peak within the first 10 min following injection and had returned to baseline levels after 30 min (Figure 4.3.4.2a). Accordingly total rotational behaviour was assessed for the full dose range of L-SOP over 30 min post L-SOP injection. Analysis of the total rotational behaviour revealed that injection of 750 nmol L-SOP produced a significant increase in net contraversive rotations 30 min\(^{-1}\), producing 237 ± 23 rotations (mean ± s.e.m., Figure 4.3.4.2b). Surprisingly, the duration of this time course was identical to that observed in reserpine-treated rats (Figure 4.3.1.1) although the magnitude of this response in 6-OHDA lesioned animals was significantly increased.

This study investigated the effects of a single dose of L-SOP only, since this was a preliminary study designed to demonstrate efficacy in a second animal model of PD. Similarly, the relatively long duration of these experiments from animals arriving, undergoing 6-OHDA lesion, apomorphine testing, cannulation and then behavioural testing meant associated costs were higher than those experiments performed in reserpine-treated rats.
Figure 4.3.4.2: Locomotor effects following intranigral L-SOP injection in 6-OHDA lesioned rats.

(a) Time course of locomotor activity induced by the group III mGlu receptor agonist L-SOP (750 nmol in 2.5 µl PBS) or vehicle (2.5 µl PBS) and (b) total locomotor effects of L-SOP (750 nmol in 2.5 µl PBS) or vehicle (2.5 µl PBS) following unilateral injection into the substantia nigra pars reticulata in 6-OHDA-lesioned rats. Values represent mean ± s.e.m. (n = 8 animals per treatment). **P < 0.001 indicates a significant difference from vehicle-treated rats (a) 2-way ANOVA and Bonferroni's post-hoc test (b) unpaired 2 tailed t-test.
4.4 Discussion

These studies have revealed that administration of the broad-spectrum group III mGlu receptor agonists L-SOP and L-AP4 directly into the SNpr bring about relief of akinesia in the reserpine-treated rat. That the responses to L-SOP and L-AP4 were blocked by the selective group III mGlu receptor antagonist, CPPG confirms the involvement of group III mGlu receptors in mediating these responses. Furthermore, since a positive allosteric modulator of mGlu4 (PHCCC) and the mGlu8 selective agonist (S)-3,4-DCPG were able to reverse akinesia when given alone this suggests an involvement of both the mGlu4 and mGlu8 receptor subtypes in mediating these effects. The anti-parkinsonian effects following intranigral administration of L-SOP were also observed in a more chronic model of PD, the 6-OHDA lesioned rat. These observations will be discussed in detail below, but taken together they clearly imply that activation of group III mGlu receptors in the SNpr in vivo do possess anti-parkinsonian efficacy.

The narrow effective dose range observed for L-SOP is consistent with previous behavioural studies using intranigral L-SOP (MacInnes et al., 2004g). However the maximally effective dose reported in these studies for L-SOP (750 nmol) is slightly lower than that previously reported (1 µmol) by MacInnes et al. Indeed, this greater dose of L-SOP produced uncontrollable motor activity in all animals tested, exemplified by seizures and convulsions. The reasons behind these adverse effects are unknown at present, but may be reflective of the reserpine-induced lowering of seizure threshold previously documented (Azzaro et al., 1972; Wenger et al., 1973; Ogren and Pakh, 1993; Kitano et al., 1996). These effects may also be due to the nature of the compounds being investigated, since group III mGlu receptor agonists have been reported to have a short (circa 15 min) pro-convulsant initial effect, followed by a longer lasting (upto 3 days) anti-convulsant effect (Ghauri et al., 1996b). This pro-convulsant effect may be due to activation of hetero-receptors by group III mGlu receptor agonists,
serving to reduce GABA release in target nuclei. A further possibility is that the site of injection (SNpr) is heavily involved in regulating seizure susceptibility, and represents a major site of action of the anticonvulsant effects of GABAergic drugs (Gale, 1985). Disruption to this nucleus may therefore result in epileptiform behaviour. However, recent studies have shown anti-convulsant effects following inhibition of the rat STN efferents by bilateral injection of muscimol (Dybdal and Gale, 2000c). Thus, inhibition of glutamatergic afferents (as would be expected following L-SOP injection) within the SNpr would be predicted to be anti-convulsant. The increased incidence of seizures reported in this study may reflect slight differences in the site of L-SOP injection. Whilst post-mortem histological verification demonstrated all animals received unilateral injection into the SNpr, the exact location of the injection needle placement within this anatomically heterologous nucleus was not noted. This seizure effect was not noted in 6-OHDA lesioned-rats, although the pro-convulsant 1 µmol dose of L-SOP was not tested in these animals.

Whilst no studies to date have reported the behavioural effects of intranigral L-AP4 injection in the reserpine-treated rat, the effects of intracerebroventricular (i.c.v.) L-AP4 administration have been published (Valenti et al., 2003e; Maclnnes et al., 2004f). Thus this study investigated the effects of intranigral L-AP4 in the reserpine-treated rat. Both L-SOP and L-AP4 were able to dose-dependently inhibit reserpine-induced akinesia in this model of PD, although the dose of L-AP4 required to reverse akinesia in this study is quantitatively lower than of L-SOP. This is in agreement with results published in Chapter 3 of this thesis, suggesting L-AP4 is the more potent of the two agonists; a suggestion corroborated by electrophysiological evidence in the SNpr (Wittmann et al., 2001a).

The reasons underlying the narrow dose-response range observed for both L-SOP and L-AP4 are not yet known. One previous suggestion is this may be reflective of the pharmacokinetic properties of the drugs themselves, with the
balance of diffusion and washout contributing to a restriction in the peak concentration reaching the desired target sites (MacInnes et al., 2004e).

All experiments described above used the reserpine-treated rat model of acute akinesia. However, given the relative crudeness of this model due to the global depletion of all catecholamines rather than a majority loss of dopamine, caution must be exerted when interpreting the relevance of these results into the overall context of PD. To ensure the anti-akinetic effects following intranigral group III receptor modulating compounds was not solely restricted to the reserpine-treated rat model; a more chronic model of dopamine depletion (the 6-OHDA lesioned rat) was investigated. In this model rats bearing a stable 6-OHDA lesion received a single unilateral injection of L-SOP at a dose previously shown to be effective in the reserpine-treated rat. Injection of L-SOP into the dopamine-depleted SNpr produced significant contraversive rotations when compared to vehicle injection, confirming that group III mGlu receptor modulation may be beneficial in another animal model of PD. Whilst the mechanism of L-SOP-induced circling is not currently understood, intranigral injection of competitive NMDA receptor antagonists have also been reported to induce contraversive rotations in both acute and chronic models of dopamine depletion (St-Pierre and Bedard, 1994a; St-Pierre and Bedard, 1994b; Kaur and Starr, 1997). Since activity levels of the STN have been shown to control the activity levels of the SNpr (Robledo and Feger, 1990), the explanation most favoured by authors is the inhibition of glutamatergic STN afferents either post-synaptically (by NMDA receptor antagonists) or pre-synaptically (by group III mGlu receptor antagonists). In the 6-OHDA-lesioned hemisphere of the CNS, a number of changes in basal ganglia network activity occur including under-activity of the GABAergic striatonigral pathway and over-activity of the glutamatergic subthalamonigral pathway. Injection of group III mGlu receptor agonists, such as L-SOP is hypothesised to normalise or reduce subthalamonigral pathway activity. However, due to the decreased striatonigral pathway, overall activity of SNpr afferents may be decreased compared to the non-lesioned hemisphere.
This may then lead to an imbalance in SNpr activity between the two hemispheres. This imbalance in SNpr activity may then manifest behaviourally as contraversive rotations, although experimental evidence for this is lacking and as such this explanation remains entirely speculative at this stage.

Other evidence supports the therapeutic potential of group III mGlu modulation in chronic models. This includes the finding that i.c.v. injection of L-AP4 in 6-OHDA treated rats reduced the forelimb asymmetry associated with this model (Valenti et al., 2005a). Indeed, in this study i.c.v. L-AP4 was found to be as effective as the archetypal anti-parkinsonian compound L-DOPA, confirming that modulation of group III mGlu receptors could be a viable therapeutic strategy in the management of PD.

The receptor specificity of L-SOP and L-AP4 were further examined using the group III mGlu receptor antagonist, CPPG. CPPG shows an approximate 20 fold higher selectivity at group III mGlu (IC50 = 2.2) versus group II mGlu receptors (IC50 = 46.2 nM) (Toms et al., 1996b). Whilst endogenous activation of group III mGlu receptors is believed to occur, this activation by itself is insufficient to restore normal motor function in behavioural tests. Therefore antagonism of this endogenous tone would be predicted to have no behavioural effect, as was observed following intranigral CPPG injections. Prior administration of CPPG produced a marked (circa 80 % of L-SOP and L-AP4) inhibition of agonist-induced responses suggesting the involvement of group III mGlu receptors in mediating these anti-akinetic effects. The concentration of CPPG used in these studies (75 µM) was similar to that used in another behavioural study (100 µM) confirming receptor specificity for the actions of the group III mGlu receptor agonists or positive allosteric modulators (Valenti et al., 2005b). Taken together, these data suggest the observed inhibitory effects of CPPG are believed to represent inhibition of the group III mGlu receptor-mediated effects.
In Chapter 3 of this thesis, another broad-spectrum group III mGlu receptor antagonist (M-SOP) was found to inhibit [3H]-D-aspartate release from rat nigral sections in a similar manner to that seen with the agonists L-SOP and L-AP4. Furthermore, addition of M-SOP was able to potentiate the inhibition of [3H]-D-aspartate seen upon subsequent L-SOP administration. To further investigate whether in vivo administration of M-SOP would result in a similar agonist-like profile; intranigral injections of M-SOP were performed. Confirming these previous in vitro findings, intranigral injections of M-SOP alone produced a dose-dependent increase in net contraversive rotations. Furthermore, prior injection of M-SOP resulted in a significant increase in subsequent L-SOP-mediated responses.

However, M-SOP (50 nmol) has been reported to be an antagonist at group III mGlu receptors within the SNpr in vivo, reducing the effect seen on subsequent L-SOP injection (MacInnes et al., 2004d). The reasons behind the marked disparity between this study and that of MacInnes et al. are presently unclear. In the present study, this dose of M-SOP produced a greater number of contraversive rotations 30 min\(^{-1}\) than those produced following injection of 750 nmol L-SOP. One possible explanation for the difference observed could be that M-SOP may be acting at (as yet unidentified) inhibitory pre-synaptic receptors present on STN terminals within the SNpr. That prior treatment with 75 nmol M-SOP was able to significantly enhance the subsequent response to 750 nmol L-SOP lends credence to this theory, since pre-treatment with an antagonist acting at group III mGlu receptors should compete for binding with L-SOP and serve to reduce, not enhance this subsequent effect.

In order to determine whether this augmentation of L-SOP-induced motor behavioural response was an artefact of M-SOP use, a further selective group III mGlu receptor antagonist, UBP1112 was investigated (Miller et al., 2003b). Surprisingly, UBP1112 alone did not evoke significant contraversive rotations over the dose range tested (5 and 50 nmol). Nonetheless, a 30 min pre-
treatment with both doses of UBP1112 tested significantly elevated the motor response evoked by L-SOP. These data suggest that whilst M-SOP may be acting as an agonist within the SNpr of reserpine-treated rats, another antagonist, UBP1112 is similarly capable of augmenting the motor response to subsequent intranigral L-SOP. Thus, similar to that suggested for in vitro experiments, one possibility for the anti-akinetic actions of these group III mGlu receptor antagonists is that they may be acting as partial and selective agonists at mGlu4 or mGlu8, possibly with mGlu7 antagonist actions.

Since previous in vitro studies, coupled with behavioural studies in the GP and SNpr suggest the involvement of mGlu4 and mGlu8 in mediating the observed effects, this study then investigated whether indeed mGlu4 or mGlu8 are involved in mediating the anti-akinetic effects seen following L-SOP or L-AP4 injection.

Whilst the presence of mGlu8 on subthalamonigral terminals has yet to be confirmed by immunohistochemical studies, mRNA encoding mGlu8 has been detected in the STN (Messenger et al., 2002a). Furthermore binding studies in mGlu4 receptor knock-out mice revealed a significant (80%) proportion of [3H]-L-AP4 binding remaining in the SNpr (Thomsen and Hampson, 1999d). The concentration of [3H]-L-AP4 used in this study was unlikely to be sufficient to bind to mGlu7, therefore this remaining selective binding is likely to be due to mGlu8. To test whether mGlu8 is involved in mediating a part of the responses seen following L-SOP or L-AP4 injection, the selective mGlu8 agonist, (S)-3,4-DCPG was investigated. (S)-3,4-DCPG has previously been reported to powerfully inhibit glutamate release in the rat neonatal spinal cord, with an EC50 of 31 nM at the mGlu8 receptor (Thomas et al., 2001d). In the present study, intranigral injection of (S)-3,4-DCPG produced a dose-dependent increase in contraversive rotations, indicative of the involvement of mGlu8. This response appeared to be biphasic in nature, suggesting that at the higher dose of 300 nmol (S)-3,4-DCPG may be activating other receptors apart from mGlu8.
However, (S)-3,4-DCPG has previously been shown to have relatively low efficacy at mGlu1-7 receptors, with IC\textsubscript{50} or EC\textsubscript{50} values greater than 3.5 \textmu M on RGT cells expressing the appropriate receptor (Thomas \textit{et al.}, 2001c). Interestingly, these authors report a similar biphasic response to (S)-3,4-DCPG in a rat neonatal spinal cord preparation. Whilst this previous study suggests that this low-affinity effect of (S)-3,4-DCPG is unlikely to be mediated by actions at either ionotropic or metabotropic glutamate receptors (other than mGlu8), the authors were unable to positively identify which receptor type was mediating this effect (Thomas \textit{et al.}, 2001a). Similarly, the apparent biphasic nature of the anti-akinetic response observed in these studies may reflect a missing data point, with no studies carried out at 100 nmol, due to time considerations.

A recent report investigating the effects of (S)-3,4-DCPG on KCl-evoked [\textsuperscript{3}H]-D-aspartate release in the rat cerebral cortex failed to find any significant reduction of [\textsuperscript{3}H]-D-aspartate release (Lee \textit{et al.}, 2003). Furthermore, an electrophysiological study investigating the relative contribution of mGlu8 in the inhibition of EPSPs generated following STN stimulation in the SNpc failed to find any role for the mGlu8 receptor (Valenti \textit{et al.}, 2005c). As both studies were conducted in different anatomical regions to that studied in this thesis, this could be one explanation for the discrepancy between these studies and the results reported here. Furthermore, the study by Valenti \textit{et al.} used slice preparations from 15 to 20 day old rats compared to the 8 – 10 week old rats used in these studies. Thus age-related alterations in mGlu receptor expression may occur, such as have been demonstrated in other brain regions including the trapezoid body (Elezgarai \textit{et al.}, 1999) and the superior colliculus (Lacey \textit{et al.}, 2005).

To further investigate whether mGlu4 is involved in mediating the observed reversal of reserpine-induced akinesia, PHCCC a positive allosteric modulator for mGlu4 was investigated. The concentration of PHCCC chosen for this study is identical to that used in a behavioural study where PHCCC was injected into the 3\textsuperscript{rd} ventricle of reserpine-treated rats (Marino \textit{et al.}, 2003f). Similar to the
results found by Marino et al., intranigral PHCCC alone was able to significantly reverse reserpine-induced akinesia compared to vehicle. Since PHCCC is devoid of intrinsic agonist properties (Marino et al., 2003c), this finding suggests there is a significant amount of glutamate available within the SNpr for activation of the mGlu4 receptors present. This surprising finding further suggests that the reserpine-treated rat model of akinesia accurately reflects some of the pathologically relevant changes in basal ganglia circuitry seen in Parkinson's disease.

Thus intranigral injection of all group III mGlu agents tested (L-SOP, L-AP4, (S)-3,4-DCPG and PHCCC) were able to reverse reserpine-induced akinesia in the rat. Analysis of the time course of locomotor activity revealed remarkable similarity between all agents tested. This similarity may be further evidence that the receptors responsible for mediating the anti-akinetic effects are mGlu4 and mGlu8. It may be that further evidence for this hypothesis may be gained from the use of the newly available mGlu7 AMN 082 dihydrochloride. Unfortunately this compound was unavailable at the time these experiments were performed.

Within the SNpr there are two distinct populations of pre-synaptic group III mGlu receptors upon which L-SOP and L-AP4 may bind and subsequently activate. One population exists as hetero-receptors upon the GABAergic striatonigral terminals; the other as auto-receptors on the glutamatergic subthalamic nigral terminals (Bradley et al., 1999a; Kosinski et al., 1999c). Electrophysiological data has supported the significance of both receptor populations in effecting pre-synaptic inhibition of neurotransmitter release. Thus application of L-AP4 has been observed to inhibit both EPSPs generated following stimulation of the STN and IPSPs generated following stimulation of the striatum in a rat brain slice preparation (recorded from the SNpr) (Wittmann et al., 2002b). To clarify which of these receptor populations is likely to be involved in the observed anti-akinetic actions of both broad-spectrum agonists, it may be useful to reconsider the pathophysiology of the basal ganglia in PD.
The subthalamonigral pathway is considered to be overactive in PD, resulting in an increase in excitation of the SNpr which in turn leads to excessive inhibition of the thalamocortical feedback loop (Blandini et al., 2000a). Therefore activation of pre-synaptic group III mGlu receptors on STN terminals in the SNpr would be expected to reduce excessive glutamate release in this nucleus, thus leading to a normalisation of the basal ganglia motor loop, and subsequent anti-akinetic effects. Conversely, the striatonigral pathway is considered to be underactive in PD due to compensatory increases in GABA-A receptor binding in the SNpr discovered in animal models of PD (Chadha et al., 2000a). Since GABA release at these synapses may already be lower than normal as a consequence of dopamine depletion, pre-synaptic group III mGlu receptor activation may have limited or no effects due to the small amount of GABA being released. Furthermore since this pathway is GABAergic in nature; any reduction of GABA release from terminals in the SNpr would be expected to lead to further excitation of the SNpr through loss of inhibitory neurotransmission. This in turn would lead to enhanced akinesia, rather than the anti-akinetic effects observed. Support for this hypothesis is gleaned from an elegant electrophysiological study which described the loss of pre-synaptic inhibition of GABA release under conditions of dopamine depletion following reserpine-treatment (Wittmann et al., 2002a). However, the pre-synaptic inhibition of glutamate release was unaffected following dopamine depletion in the same study. Since the behavioural studies presented in this chapter were performed in dopamine-depleted rats, it is therefore unlikely that the anti-akinetic effects observed following intranigral injection of broad-spectrum group III mGlu receptor agonists are due to inhibition of GABA release from striatonigral terminals; rather that these effects are due to inhibition of glutamate release from subthalamonigral terminals. This mechanism of action in the SNpr has previously been described for group III mGlu receptor agonists in vitro (Chapter 3 of this thesis).
Valenti et al. hypothesise that the main effects of i.c.v. L-AP4 injection are mediated through the inhibition of overactive striato-pallidal synapses, due specifically to activation of the mGlu4 receptor. Certainly the resultant decrease in excess GABA release in the GP would be expected to enhance the activity of the GABAergic pallidosubthalamic neurons; resulting in inhibition of the STN and a reduction in the excitatory drive to the SNpr, and thus restoring thalamocortical feedback. Support for this hypothesis comes from a series of experiments showing that application of L-AP4 in vitro in mGlu4-receptor knock-out mice does not inhibit striato-pallidal transmission (Valenti et al., 2003c). However the subthalamonigral pathway was not investigated in this study and thus cannot be excluded as an additional target for group III mGlu receptor agonists. Indeed the present findings, together with those of McInnes et al., (2004) suggest both L-SOP and L-AP4 (at least) exert their anti-akinetic effects through activation of group III mGlu receptors residing on subthalamonigral terminals within the SNpr.

In conclusion, the data presented in this chapter establish a role for group III mGlu receptor modulation in rectifying the functional outcome of basal ganglia activity through actions within the SNpr. These finding corroborate the current pharmacological model of the BG circuitry as well as the in vitro findings presented in Chapter 3 of this thesis; that group III mGlu receptor activation can serve to inhibit transmitter release in the SNpr. Furthermore these data describe the involvement of mGlu4 and mGlu8 in mediating the anti-akinetic effects seen following intranigral injection of broad-spectrum group III mGlu receptor agonists. That the effects of L-SOP are seen in more than one animal model of PD suggests group III mGlu receptor agonists are worthy of further consideration investigation in a wider range of rodent and primate models of PD.
Chapter 5: Desensitisation of the effects of stimulation of group III metabotropic glutamate receptor *in vivo* and *in vitro*. 
5.1 Introduction

In Chapter 4, the ability of acute injections of group III mGlu receptor agonists to alleviate reserpine-induced akinesia was explored. These agents were found to be effective at reversing akinesia in the reserpine-treated rat when given directly into the intended site of action. However, only single injections of group III mGlu receptor modulating compounds were examined. In order to further explore the likely therapeutic potential of this class of drug, studies were planned to examine the efficacy of the drugs when given for extended periods. This would have enabled us to explore the likelihood of these drugs eliciting unwanted side-effects (e.g. hyperkinesia or dyskinesia) with repeated administration – a problem that seriously limits the benefit of the current gold standard therapy, levodopa. Thus, here, drugs were administered repeatedly to the same animals and at the same site. During the course of initial experiments, however, it quickly became apparent that, contrary to producing an increase in locomotor activity with repeated injection (as would be witnessed with levodopa), these drugs failed to produce a similar magnitude of akinesia reversal when given a second time. The outcome of these initial studies led to the design of experiments outlined in this chapter, to start to explore this reduction in efficacy, whether it be representative of a desensitisation of receptors or not. Certainly, receptor desensitisation remains the most plausible explanation for the preliminary observations.

Desensitisation of receptors, whereby activation of a receptor leads to a reduced ability to produce a response in the future, provides an important physiological role by acting as a feedback mechanism; limiting both acute and chronic over-stimulation of GPCR signal transduction cascades (Dale et al., 2002). Whilst this forms a useful physiological purpose in limiting signalling events, receptor desensitisation may also significantly limit the therapeutic usefulness of many receptor agonists. Receptor desensitisation is also believed to be the cellular mechanism through which tolerance to agonists such as morphine occurs (Connor et al., 2004a). The mechanisms underlying receptor
desensitisation are complex and involve (amongst others) receptor phosphorylation, uncoupling from G proteins, internalization and ultimately receptor down-regulation. The process of receptor desensitisation is already well-recognised for mGlu receptors of the group I phenotype.

Many studies have investigated the mechanisms underlying group I mGlu receptor desensitisation, since over-activation of these receptors may be involved in the excitotoxic process which may subsequently lead to neuronal cell death (Calabresi et al., 1999). Interestingly, reciprocal interactions between NMDA receptor activation and group I mGlu (mGlu5) receptors have been implied whereby low concentrations of NMDA enhance the response to group I mGlu receptor agonists in cortical and hippocampal neurons (Alagarsamy et al., 1999b). The mechanisms responsible for both group I mGlu receptor desensitisation and NMDA-receptor cross talk appear to involve (amongst other things) alterations in protein kinase C (PKC) activation. Indeed, in Purkinje cell cultures taken from mice which have had a single amino acid residue substituted in the PKC binding site of the mGlu1 receptor, desensitisation following prolonged exposure to the group I specific agonist DHPG was reduced compared to wild-type mice (Sato et al., 2004). Regardless of the mechanism underlying these responses, it is evident from the above experiments that group I mGlu receptors are capable of undergoing both homologous and heterologous desensitisation. Homologous desensitisation indicates when one receptor is activated by the agonist, this mechanism desensitises the subsequent response of the same receptor only. In contrast, heterologous desensitisation indicates that stimulation by one agonist attenuates the response to multiple distinct agonists, operating through different receptor types (Chuang et al., 1996).

Whilst the ability of group I metabotropic glutamate receptors to undergo both heterologous and homologous desensitisation has been relatively well studied, less is known about the group II and III subtypes. Indeed, only a handful of studies have investigated whether group II mGlu receptors undergo
desensitisation at all. In one study from this laboratory, repeated intracerebroventricular injections (24 hours apart) of the group II mGlu receptor agonist, LY379268 in the reserpine-treated rat resulted in a significant decrease of the anti-akinetic actions of this compound (Murray et al., 2002a). This observation suggests that, akin to their group I counterparts, group II mGlu receptors may also undergo homologous desensitisation. Studies using activators of PKC, such as phorbol esters, have shown prior activation of PKC at the corticostriatal synapses can inhibit group II mGlu receptor-mediated inhibition of glutamate release (Swartz et al., 1993). These studies confirm a role for PKC in mediating group II (in addition to group I) desensitisation. Other studies suggest activation of group II receptors can affect other alternative receptor-mediated systems, such as the production of tolerance to morphine. Thus, in rats which had previously been induced to have morphine tolerance, pre-treatment with the group II receptor agonist LY354740, prevented the development of morphine tolerance, without affecting the antinociceptive properties of morphine (Popik et al., 2000). Whilst this experiment did not investigate heterologous desensitisation per se, it seems likely that receptor cross-talk may be involved in mediating these anti-tolerance effects produced by LY354740.

Fewer studies still have investigated the possibility that group III mGlu receptors may undergo homologous or heterologous desensitisation; and of those few studies on group III mGlu receptor desensitisation published to date, none have investigated the behavioural effects seen following repeated administration of agonists. Three previous in vitro experiments have shown responses to group III mGlu receptor agonists to be inhibited by prior activation of the adenosine A₃ receptor (Macek et al., 1998a); a Gₛ-coupled receptor, the β-adrenergic receptor (Cai et al., 2001b) and more recently, a Gₛ-coupled receptor, the α₁-adrenergic receptor (Gordon and Bains, 2003b). Thus it appears that, at least in vitro, group III mGlu receptors may also undergo heterologous desensitisation. However at
the present time, no studies have investigated whether group III mGlu receptors undergo homologous desensitisation either *in vivo* or *in vitro*.
5.1.2 Aims

The aim of the studies outlined in Chapter 5 was to examine whether the anti-akinetic effects of intranigral group III mGlu receptor modulating compounds, presented in Chapter 4, were able to retain efficacy upon repeated intranigral injections, or whether these responses displayed homologous desensitisation in vivo. In the event of homologous desensitisation of group III mGlu receptor agonists or positive allosteric modulators being apparent, a second aim of these studies was to investigate whether heterologous desensitisation also occurred between group III mGlu receptors and a second class of G\(_i/G\_o\)-coupled receptors, the GABA\(_B\) receptor. Previous studies from this laboratory have shown intranigral injections of baclofen, a GABA\(_B\) receptor agonist, is able to reverse reserpine-induced akinesia in the rat (Johnston and Duty, 2003e) and electron microscopy studies have shown GABA\(_B\) receptors to be present on presynaptic terminals of the subthalamonigral pathway (Charara et al., 2000). Therefore studies here aimed to deduce whether cross-talk expected between the group III mGlu receptors and the GABA\(_B\) receptors occurred, after first confirming that GABA\(_B\) receptor activation displayed homologous desensitisation like the group III mGlu receptors. In vitro studies were also performed, to investigate whether the in vivo findings translated to an in vitro situation regarding inhibition of glutamate release in the SNpr (the previously proposed mechanism of action).
5.2 Methods

5.2.1 Animals
Male Sprague-Dawley rats (280-320g) were housed in a temperature- and humidity-controlled environment with a 12h light/dark cycle with access to food and water ad libitum. All procedures were performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986, and all efforts were made to minimise animal suffering and the number of animals used.

5.2.2 Intranigral bilateral cannulation
Intranigral cannulations were performed as per methods detailed in Chapter 4, section 4.2.2.1.

5.2.3 Induction of akinesia with reserpine
Akinesia was induced as per methods detailed in Chapter 4, section 4.2.2.2.

5.2.4 Assessment of homologous desensitisation in reserpine-treated rats.
Agents listed in table 5.1 were investigated for their ability to induce homologous desensitisation (all compounds were adjusted to pH 7.4 with NaOH). Doses of agents were chosen on the basis of previous experiments performed in this laboratory. Behavioural assessment of motor activity was made from video-recorded observations of rats in 40cm diameter, flat-bottomed hemispherical bowls (Amee, Manchester, UK). Following a 15 min acclimatisation period, baseline activity was monitored for 30 min. Suitably akinetic animals (< 4 360° rotations 30 min⁻¹) then received a single unilateral injection of test agent into the SNpr. Injections were made manually at 1µl per minute via a 30-gauge
stainless steel needle that extended 2mm below the tip of the guide cannulae and was attached with tubing (Portex) to a 10µl Hamilton micro syringe.

Table 5.1: List of agents investigated for homologous desensitisation.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Compound type</th>
<th>Agent dissolved in</th>
<th>Dose Injected (nmol)</th>
<th>Volume Injected (µl)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-SOP</td>
<td>Broad-spectrum group III mGlu receptor agonist</td>
<td>PBS</td>
<td>750</td>
<td>2.5</td>
<td>(MacInnes et al., 2004c)</td>
</tr>
<tr>
<td>L-AP4</td>
<td>Broad-spectrum group III mGlu receptor agonist</td>
<td>PBS</td>
<td>300</td>
<td>2</td>
<td>Chapter 4, this Thesis</td>
</tr>
<tr>
<td>PHCCC</td>
<td>mGlu4 positive allosteric modulator</td>
<td>40% DMSO in 0.85% NaCl</td>
<td>75</td>
<td>0.5</td>
<td>Chapter 4, this Thesis</td>
</tr>
<tr>
<td>(S)-3,4-DCPG</td>
<td>mGlu8 agonist</td>
<td>PBS</td>
<td>300</td>
<td>0.5</td>
<td>Chapter 4, this Thesis</td>
</tr>
<tr>
<td>Baclofen</td>
<td>GABA&lt;sub&gt;B&lt;/sub&gt; receptor agonist</td>
<td>PBS</td>
<td>800 ng</td>
<td>0.5</td>
<td>(Johnston and Duty, 2003d)</td>
</tr>
</tbody>
</table>

Phosphate buffered saline (PBS; (mM); NaCl 137, KCl 2.7, KH<sub>2</sub>PO<sub>4</sub> 1.8, Na<sub>2</sub>HPO<sub>4</sub> 10) and pH adjusted to 7.4 with NaOH.

Animals were videotaped for a further 30 - 60 min following injection of each compound. Full (360°) contraversive rotations, measured as an index of relief of akinesia, were counted manually in 10 min time bins from the videotaped
recordings over 30 - 60min following injection. Following the initial injection of agent, animals were replaced in their cages and allowed to rest. 4 hours after initial testing, animals then received a further injection of the same compound, used 4 h earlier, in the same injection site. Animals were then videotaped for a second 30 – 60 min period and full contraversive rotations counted. For studies using L-SOP, the locomotor effects of further repeat injections were made at 8 and 12 hours post initial injection. For these 12 hour studies, owing to variability of responses between animals over time, data were transformed to percentages, whereby each animal’s initial response was set at 100% and subsequent responses given as a percentage of this initial response. In a similar 12 hour study using L-SOP the dose of the final injection was double (1500 nmol) that received on the three injections previously (750 nmol).

Due to Home Office project license considerations limiting the number of injections to four per animal, time-points over 12 hours were not able to be investigated. Similarly since reserpine is a reversible VMAT-2 inhibitor, concerns were raised over the validity of performing multiple injections over periods longer than 12 hours, since this may have entered the time at which the akinetic effects of reserpine start to wear off. However, the loss of normal motor movements induced by reserpine are stable for up to 2 days post reserpine-injection (Naudon et al., 1995). Therefore, the maximal behavioural testing performed in these studies (30 hours post-reserpine injection) were well within this testing period. Furthermore, striatal dopamine levels are reduced to 1-2% of pre-reserpine levels for 4 – 48 hours following reserpine treatment, as measured by HPLC and electrochemical detection (Trugman and James, 1992a). In addition, at all time points tested (4, 8 & 12 hours post initial L-SOP injection), all animals displayed a stable level of akinesia. To investigate periods longer than 12 hours post initial L-SOP injection, a more chronic model of parkinsonism, the 6-OHDA lesioned rat model bearing nigrostriatal tract degeneration was employed (section 5.2.6 below).
Upon completion of behavioural testing, animals were killed by isoflurane overdose and cervical dislocation. Brains were rapidly removed and snap frozen at \(-45^\circ\text{C}\) (cooled by solid \(\text{CO}_2\)) in isopentane and stored dessicated at \(-70^\circ\text{C}\). Cresyl violet post mortem histological verification of cannulae placement was undertaken.

5.2.5 Assessment of heterologous desensitisation in reserpine-treated rats.

Behavioural assessments were made as per section 5.2.4 with the following modifications. Suitably akinetic animals received a single intranigral injection of either L-SOP (750 nmol in 2.5\(\mu\)l PBS), baclofen (800 ng (equating to 3.7 nmol) in 0.5\(\mu\)l PBS) or equivalent volume of vehicle (PBS) and the number of 360\(^\circ\) net contraversive rotations were quantified for 30 – 60 min. Following initial injections, animals were then replaced in their cages and allowed to rest. Four hours later, those animals which received an initial intranigral injection of L-SOP then received subsequent injection of baclofen (800 ng), whilst those animals which received an initial injection of baclofen then received a subsequent injection of L-SOP (750 nmol). In order to ensure any effects of pre-treatment with agonists were due to receptor cross-talk, and not merely due to damage at the site of injection, those animals receiving an initial injection of vehicle then received a subsequent injection of either L-SOP or baclofen. Animals were then videotaped following the second injection for 30 – 60 min and full contraversive rotations counted. Upon completion of behavioural testing, animals were killed by isoflurane overdose and cervical dislocation. Brains were rapidly removed and snap frozen at \(-45^\circ\text{C}\) (cooled by solid \(\text{CO}_2\)) in isopentane and stored dessicated at \(-70^\circ\text{C}\). Cresyl violet post mortem histological verification of cannulae placement was undertaken.
5.2.6 Assessment of homologous desensitisation to L-SOP, in rats bearing a stable 6-OHDA lesion of the median forebrain bundle.

Rats underwent stereotaxic surgery to induce nigrostriatal tract degeneration as detailed in Chapter 4, section 4.2.3. Pre-mortem lesion verification was carried out as per section 4.2.3.1. using 1mg kg\(^{-1}\) intraperitoneal apomorphine. Those 6-OHDA lesioned rats exhibiting ≥ four net contraversive rotations per minute were estimated to have over 90% loss of striatal dopamine terminals (Hefti et al., 1980d). Suitably lesioned animals were then allowed a one week recovery and apomorphine wash-out period, whereupon they underwent cannulation surgery. This surgical procedure was identical to that described in section 4.1.2 apart from unilateral (left side) cannulation only was performed. The co-ordinates from interaural line were AP + 0.32, DV + 0.36 and ML + 0.20 cm) according to the standard rat brain atlas of Paxinos and Watson (1986). Following cannulation animals were allowed to recover in a heated incubator until fully conscious. After a minimum of four days, animals were then used for behavioural locomotor tests as described in Chapter 4, section 4.2.2.3. These animals received injections of 750 nmol L-SOP on day 0, day 3 and day 7. Following each injection of L-SOP, animals were videotaped for a further 40 min. Full (360\(^{\circ}\)) contraversive rotations, measured as an index of anti-parkinsonian efficacy, were counted manually in 10 min time bins from the videotaped recordings. Upon completion of behavioural testing, animals were killed by isoflurane overdose and cervical dislocation. Brains were rapidly removed and snap frozen at −45°C (cooled by solid CO\(_2\)) in isopentane and stored dessicated at −70°C. Cresyl violet post mortem histological verification of cannulae placement was undertaken.
5.2.7 Assessment of in vitro homologous desensitisation in slices from the rodent substantia nigra pars reticulata.

[3H]-D-aspartate release studies were performed as detailed in Chapter 3, section 3.2.2 of this thesis with the following alterations.

Following a 1h equilibration period where gassed Krebs’ buffer was superfused at 1.0 ml/min and the eluent discarded, collection of 2 min release fractions commenced. Superfused eluent was collected into 5 ml scintillation vials set underneath the apparatus, and basal [3H]-D-aspartic acid release was determined over the first 8 min. This was followed by an initial 2 min stimulation (S1) with Krebs’ buffer containing 25mM KCl. The 25mM KCl solution was equimolarly substituted by reducing the concentration of Na+ present, to maintain osmolarity (composition in mM : NaCl, 109; CaCl2 1.3; MgSO4, 1; NaHCO3, 25; KCl, 25; KH2PO4, 1.25; glucose, 10, pH 7.4). An 18 min washout period in Krebs' buffer was then followed by a second 25mM KCl stimulation for 2 min (S2) and a further 10 min in normal Krebs' buffer to the end of the experiment.

Where group III mGlu receptor-mediated homologous desensitisation was investigated, L-SOP (100 µM) was present in Krebs’ buffer both from the start of the experiment until fraction 4 inclusive (0 – 8 min) and at the start of fraction 10 until fraction 14 inclusive (20 - 28 min after the start of the experiment). L-SOP was similarly present in both initial (S1 at fraction 4) and subsequent (S2 at fraction 14) periods of stimulation by 25mM KCl. Thus, L-SOP was present both prior to, and during, both periods of 25mM KCl stimulation.

To ensure that this concentration of L-SOP was able to inhibit [3H]-D-aspartate release, as suggested in Chapter 3, a single incubation of L-SOP (100µM) was performed, where L-SOP was present from fraction 10 until fraction 14 inclusive. Thus, in these experiments L-SOP was only present prior to and during the
second (S2) 25mM KCl stimulation. In all experiments vehicle controls were performed consisting of 25mM KCl at fractions 4 and 14 only.

In order to examine whether the GABA<sub>B</sub> receptor agonist, baclofen, could similarly lead to a reduction in 25mM KCl-evoked [³H]-D-aspartate release from rat nigral sections, pilot experiments were performed investigating a single concentration of baclofen (100µM). These experiments were performed in a similar manner to single L-SOP incubations detailed above. Thus, in these experiments, baclofen was only present prior to (fraction 10) and during (until fraction 14) the second (S2) 25mM KCl stimulation.

Whilst receptor specificity has previously been demonstrated for L-SOP in this experimental set up (Chapter 3, section 3.3.6), receptor specificity had not yet been demonstrated for baclofen. Therefore the GABA<sub>B</sub> receptor antagonist, CGP46381 was employed (Johnston and Duty, 2003c) on a single concentration (100µM) of baclofen. For studies using CGP46381, this was present at fraction 8 until fraction 14 (inclusive; 16 min after the start of the experiment) together with baclofen (present from fractions 10 – 14 inclusive).

Once these studies involving single incubations of baclofen were concluded, the experimental procedures detailed above for repeated mGlu receptor-mediated homologous desensitisation were repeated for GABA<sub>B</sub> receptors. Thus, baclofen (100 µM) was present in Krebs' buffer both from the start of the experiment until fraction 4 inclusive (0 – 8 min) and at the start of fraction 10 until fraction 14 inclusive (20 - 28 min after the start of the experiment). Baclofen was similarly present in both initial (S1 at fraction 4) and subsequent (S2 at fraction 14) periods of stimulation by 25mM KCl. Thus, baclofen was present both prior to, and during, both periods of 25mM KCl stimulation.

At the end of the experiment 3 ml scintillation fluid was added to the approximate 2 ml of collected eluent. Finally the tissue suspension and filter paper discs from the superfusion chamber were added to 3 ml scintillation fluid
with 950 µl Krebs’ buffer. This resulted in 252 scintillation vials, comprising 20 fractions per reaction chamber and 12 tissue chambers. These scintillation vials were then analysed for [³H]-D-aspartic acid content by liquid scintillation spectroscopy. Data handling for in vitro experiments was performed as described in Chapter 3, section 3.2.2.1 and section 3.2.2.2 of this thesis. Note that heterologous desensitisation was not examined in vitro owing to limitation of time.

5.2.8 Data analysis

For homologous desensitisation studies performed in vivo, total net 360° contraversive rotations were compared for each test compound between the initial injection and the second injection four hours later by a paired two-tailed t-test. For longer time-course experiments involving repeated L-SOP dosing for up to 12 hours, the effects of repeated administration were compared using a repeated measures ANOVA and a Dunnett’s post-hoc test. To assess the effects of increasing the final dose of L-SOP (1500 nmol) following 3 previous injections of 750 nmol L-SOP a 1-way ANOVA and Dunnett’s post-hoc test was used. For studies investigating the effects of L-SOP over 7 days, a 1-way ANOVA and Student-Newman-Keuls post-hoc test was used to evaluate differences between time points.

To assess the effects of a single concentration of baclofen versus vehicle treatment, a 2-tailed unpaired t-test was used. In a similar manner, a 2-way ANOVA and a Bonferroni post-hoc test was used to assess the relationship between time-course data produced following intranigral baclofen injection compared to vehicle.

To assess for heterologous desensitisation in vivo, responses to L-SOP or baclofen obtained 4 hours after either vehicle or reciprocal agonist injection were compared using an unpaired 2-tailed t-test.
To investigate the effects of a single concentration of baclofen compared to vehicle in vitro, a two-tailed unpaired t-test was used. Similarly, to confirm receptor specificity seen following pre-treatment with CGP46381 on baclofen-induced inhibition of [$^3$H]-D-aspartate release, a one way ANOVA and Student-Newman-Keuls post-hoc test was used.

To investigate homologous desensitisation of group III mGlu and GABA$_B$ receptor-mediated responses in vitro, repeated treatment with either agonist was compared to single agonist treatment and vehicle treatment through the use of a 1-way ANOVA and Student-Newman-Keuls post-hoc test.
5.3 Results

5.3.1 Effects of repeated injection of group III mGlu receptor modulating compounds in the reserpine-treated rat.

5.3.3.1 Repeated intranigral injection of 750 nmol L-SOP

Similar to the data presented in Chapter 3, section 4.3.1.1 initial intranigral injection of L-SOP (750 nmol) produced a number of contraversive rotations over the time period measured. The magnitude of this initial response to L-SOP was 58 ± 16 rotations in 30 minutes, compared to the 66 ± 18 rotations reported in Chapter 3. However, the response to a subsequent intranigral injection of the same dose of L-SOP into the same animals four hours after the initial injection was significantly reduced (1.5 ± 1 rotation 30 min⁻¹). This almost total abolition of behavioural responses to repeated injection of agonist can be seen in Figure 5.3.3.1 (a) overleaf.

5.3.3.2 Repeated intranigral injection of 300 nmol L-AP4

Similar to the data presented in Chapter 3, section 4.3.1.2 initial intranigral injection of L-AP4 (300 nmol) produced 82 ± 18 contraversive rotations in 30 minutes. However, the response to a subsequent intranigral injection of the same dose of L-AP4 into the same animals four hours after the initial injection was significantly decreased (down to 23 ± 7 rotations 30 min⁻¹). The striking reduction in behavioural responses to repeated injection of agonist can be seen in Figure 5.3.3.1 (b) overleaf.
Figure 5.3.3.1: Locomotor effects observed following repeated intranigral injection of L-SOP (a) and L-AP4 (b) in the reserpine-treated rat.

(a)

Net contraversive rotations 30 min⁻¹

Initial L-SOP

L-SOP 4h later

(b)

Net contraversive rotations 30 min⁻¹

Initial L-AP4

L-AP4 4h later

Figure 5.3.3.1: Total locomotor effects of (a) L-SOP (750 nmol in 2.5 µl PBS; n = 7 animals) and (b) L-AP4 (300 nmol in 2 µl PBS; n = 6 animals) given via unilateral injection into the SNpr of reserpine-treated rats at time 0 and 4 h later. Values represent mean ± s.e.m. (n = animals). * P < 0.05 indicates a significant difference compared to initial injection (paired 2-tailed t-test).
5.3.3.3 Repeated intranigral injection of 300 nmol (S)-3,4-DCPG

Similar to the data presented in Chapter 3, section 4.3.1.3, initial intranigral injection of (S)-3,4-DCPG (300 nmol) produced a number of contraversive rotations over the time period measured. The magnitude of this initial response to (S)-3,4-DCPG was 64 ± 14 contraversive rotations in 40 minutes, compared to the 65 ± 16 rotations reported in Chapter 3. However, the response to a subsequent intranigral injection of the same dose of (S)-3,4-DCPG into the same animals four hours after the initial injection was significantly reduced (9.5 ± 4 rotations 30 min⁻¹). This almost total abolition of behavioural responses to repeated injection of agonist can be seen in Figure 5.3.3.2 (a) overleaf.

5.3.3.4 Repeated intranigral injection of 75 nmol PHCCC

Similar to the data presented in Chapter 3, section 4.3.2, initial intranigral injection of PHCCC (75 nmol) produced 67 ± 5 contraversive rotations in 40 minutes, compared to the 70 ± 15 rotations reported in Chapter 3. However, the response to a subsequent intranigral injection of the same dose of PHCCC into the same animals four hours after the initial injection was again significantly reduced (30 ± 11 rotations 40 min⁻¹). This almost total abolition of behavioural responses to repeated injection of agonist can be seen in Figure 5.3.3.2 (b) overleaf.
Figure 5.3.3.2: Locomotor effects observed following repeated intranigral injection of (a) (S)-3,4-DCPG and (b) PHCCC in the reserpine-treated rat.

(a) Total locomotor effects of (a) (S)-3,4-DCPG (30 nmol in 0.5 µl PBS; n = 4 animals) and (b) PHCCC (75 nmol in 0.5 µl PBS; n = 6 animals) given via unilateral injection into the SNpr of reserpine-treated rats at time 0 and 4 h later. Values represent mean ± s.e.m. * P < 0.05 indicates a significant difference compared to initial injection (paired 2-tailed t-test).
5.3.3.5 Repeated intranigral injection of 750 nmol L-SOP over 12 hours

Following initial injection of L-SOP (750 nmol), rotational behaviour was observed as expected in all animals, in a very similar manner to that seen in Chapter 4, section 4.3.1.1 (66 ± 18 contraversive rotations 30 min\(^{-1}\)) which was then set to 100% for each individual animal, for clarity. Repeat injections of L-SOP produced a marked reduction in magnitude of this behavioural response as shown in Figure 5.3.3.3a. Thus, following a second injection of L-SOP (750nmol) 4h after the first, the behavioural response was almost abolished with circa 4% of the initial response remaining. Following the third injection 4h later, the response to L-SOP had recovered slightly, but not significantly (P>0.05) to around 25% of the initial response, where it remained for the final injection at 12 hours following initial exposure. These reductions in response to L-SOP at 4, 8 and 12h were highly significant compared to the initial response at time 0 (P<0.001).

5.3.3.6 Repeated intranigral injection of 750 / 1500 nmol L-SOP over 12 hours

Following initial L-SOP injection (750 nmol), rotational behaviour was observed in all animals (65 ± 15 rotations 30 min\(^{-1}\)), similar to that previously observed. Once again, repeated administration of L-SOP produced a marked reduction in the magnitude of this response. Thus, following a second injection of 750 nmol L-SOP 4h after the first, the behavioural response elicited by L-SOP was significantly reduced to 21 ± 9 rotations 30 min\(^{-1}\). A further injection of 750 nmol L-SOP 4h later (8h from the start of the experiment) similarly produced a significant reduction in contraversive rotations (17 ± 7 rotations 30 min\(^{-1}\)) compared to the initial injection. However, upon final injection of a higher dose (1500 nmol, a dose previously untested, as doses over 1000 nmol resulted in seizures and uncontrollable motor activity), the number of contraversive rotations produced (61 ± 11 rotations 30 min\(^{-1}\)) returned to those previously observed following the initial 750 nmol L-SOP injection (Figure 5.3.3.3b).
Figure 5.3.3.3: Locomotor effects observed following repeated intranigral injections over 12 hours of (a) 750 nmol L-SOP and (b) 750 / 1500 nmol L-SOP in the reserpine-treated rat.

**Figure 5.3.3.3:** Total locomotor effects after repeated unilateral injections of L-SOP into the substantia nigra pars reticulata in reserpine-treated rats. (a) 750 nmol L-SOP in 2.5 µl PBS. (b) 750 nmol L-SOP (time 0, 4 & 8) and 1500 nmol L-SOP (12 hours post initial injection). Values represent mean ± s.e.m. (n = 6 animals). *** $P < 0.001$ and ** $P < 0.01$ indicates a significant difference compared to initial L-SOP injection (1 way ANOVA and Dunnett's post-hoc test).
5.3.2 Effects of repeated intranigral injection of a group III mGlu receptor agonist in the 6-OHDA lesioned rat

Following initial L-SOP injection (750 nmol), rotational behaviour was observed in all animals (342 ± 30 contraversive rotations 30 min⁻¹), similar to that previously observed (236 ± 23 rotations 30 min⁻¹, Chapter 4, section 4.3.4). Repeated administration of L-SOP after 3 days produced a marked, significant reduction in the magnitude of this response to 160 ± 10 rotations 30 min⁻¹. However, upon final injection of 750 nmol L-SOP 4 days later (7 days from the start of the experiment) a return towards the initial contraversive rotations (291 ± 45 rotations 30 min⁻¹) was observed. This response was not significantly different compared to that produced after the initial injection (Figure 5.3.2).

**Figure 5.3.2:** Locomotor effects observed following repeated intranigral injections over 7 days of 750 nmol L-SOP in the 6-OHDA lesioned rat.

**Figure 5.3.3.3:** Total locomotor effects after repeated unilateral injections of 750 nmol L-SOP in 2.5 µl PBS into the substantia nigra pars reticulata of 6-OHDA lesioned rats. Values represent mean ± s.e.m. (n = 6 animals). **P < 0.01 and * P < 0.05 indicates a significant difference compared to day 3 L-SOP injection (1-way ANOVA and Student-Newman-Keuls post-hoc test).
5.3.3 Locomotor effects following intranigral injection of the GABAB receptor agonist baclofen in the reserpine-treated rat.

Unilateral intranigral injection of vehicle (0.5 μl PBS) produced no change in rotational behaviour compared to baseline over the entire period monitored. Conversely, unilateral intranigral injection of the GABAB receptor agonist baclofen (800 ng in 0.5 μl PBS) produced a significant increase in net contraversive rotations. Figure 5.3.3(a) shows the time-course of this behaviour. The effects had both commenced and reached their peak within the first 10 min following injection and had returned to baseline levels after 60 min. Accordingly, quantification of total circling behaviour was conducted over a 60 min period. Injection of baclofen (800 ng) produced a significant increase in net contraversive rotations 60 min⁻¹ (124 ± 12 contraversive rotations 60 min⁻¹) compared to vehicle treatment, shown in Figure 5.3.3(b).

A single dose of baclofen was investigated on the basis of previous studies within this laboratory indicating this dose of intranigral baclofen was able to reverse reserpine-induced akinesia in the rat (Johnston and Duty, 2003b). The full dose-response relationship was not investigated further for two reasons. Firstly, these studies (both homologous and heterologous behavioural desensitisation) only required a single dose of a second Gi / Go-coupled receptor agonist that was able to reverse akinesia and secondly time constraints restricted the number of experiments that could be performed within a given time period.
Figure 5.3.3: Locomotor effects following intranigral baclofen injection in reserpine-treated rats.

(a) Time course of locomotor activity induced by the GABA\textsubscript{B} receptor agonist baclofen (800 ng in 0.5 µl PBS) or vehicle (0.5 µl PBS) and (b) total locomotor effects of baclofen (800 ng in 0.5 µl PBS) or vehicle (0.5 µl PBS) following unilateral injection into the substantia nigra pars reticulata of reserpine-treated rats. Values represent mean ± s.e.m. (n = 6 animals). For (a) *** P < 0.001 indicates a significant difference to vehicle treatment (2-way ANOVA with Bonferroni's post-hoc test) (b) *** P < 0.001 indicates a significant difference compared to 800 ng baclofen (two-tailed unpaired t-test).
5.3.4 Effects of repeated injection of GABA\textsubscript{B} receptor agonist into the SNpr of the reserpine-treated rat

Similar to the data presented in section 5.3.3 above, initial intranigral injection of the GABA\textsubscript{B} receptor agonist baclofen (800 ng in 0.5 µl) produced a number of contraversive rotations over 60 min post-injection. The magnitude of this initial response to baclofen was $150 \pm 35$ (mean ± s.e.m.) rotations in 60 minutes, compared to the $124 \pm 12$ rotations reported in section 5.3.3. However, the response to a subsequent intranigral injection of the same dose of baclofen into the same animals four hours after the initial injection was decreased by approximately 75\% compared to the initial injection ($35 \pm 9$ rotations 60 min\textsuperscript{-1}). This significant attenuation of the behavioural response can be seen in Figure 5.3.4 below.

**Figure 5.3.4:** Locomotor effects observed following repeated intranigral injections of the GABA\textsubscript{B} receptor agonist, baclofen in reserpine-treated rats.

![Figure 5.3.4](image)

**Figure 5.3.4:** Total locomotor effects of initial injection of baclofen and subsequent baclofen injection 4 hours later, after unilateral injection into the substantia nigra pars reticulata in reserpine-treated rats. (800 ng baclofen in 0.5 µl PBS). Values represent mean ± s.e.m. (n = 6 animals). *P < 0.05 indicates a significant difference compared to initial baclofen injection (paired 2-tailed t-test).
5.3.5 Heterologous desensitisation following pre-treatment with the GABA\(_B\) receptor agonist, baclofen in the reserpine-treated rat

Figure 5.3.5 shows the effect of pre-treatment with either baclofen (800 ng in 0.5 µl PBS) or vehicle (0.5 µl PBS) on the locomotor response to subsequent intranigral injection of L-SOP (750 nmol in 2.5 µl PBS). As previously reported, initial injection of baclofen resulted in an increase in net contraversive rotations to 105 ± 10 rotations 60 min\(^{-1}\) (comparing favourably with 124 ± 12 reported in section 5.3.3 and 150 ± 35 reported in section 5.3.4). Initial injection of vehicle produced negligible locomotor activity of 1.5 ± 1 rotation 60 min\(^{-1}\). A subsequent injection of L-SOP, into those animals pre-treated 4 h earlier with baclofen, lead to a significant reduction in locomotor responses (7.5 ± 3 rotations 60 min\(^{-1}\)) when compared to the response of L-SOP in those animals pre-treated with vehicle (31 ± 6 rotations 60 min\(^{-1}\)).

Figure 5.3.5: Locomotor effects observed following pre-treatment with intranigral injections of either the GABA\(_B\) receptor agonist baclofen or vehicle on subsequent L-SOP-induced motor activity in reserpine-treated rats.

Figure 5.3.5: Total locomotor effects induced by unilateral intranigral injection of L-SOP (750 nmol / 2.5 µl PBS) in animals pre-treated with either unilateral intranigral injection of baclofen (800 ng / 0.5 µl PBS) or vehicle (0.5 µl PBS) in reserpine-treated rats. Values represent mean ± s.e.m. (n = 6 animals / group). ** P < 0.01 indicates a significant difference between L-SOP responses obtained after pre-treatment with baclofen versus pre-treatment with vehicle (unpaired 2-tailed t-test).
5.3.6 Heterologous potentiation following pre-treatment with the group III mGlu receptor agonist, L-SOP in the reserpine-treated rat

Figure 5.3.6 shows the effect of pre-treatment with either L-SOP (750 nmol in 2.5 µl PBS) or vehicle (2.5 µl PBS) on the locomotor response to subsequent intranigral injection of baclofen (800 ng in 0.5 µl PBS). As previously reported, initial injection of L-SOP resulted in an increase in net contraversive rotations to 63 ± 8 rotations 60 min⁻¹ (comparing favourably with previous responses noted earlier). Pre-treatment with vehicle produced negligible rotations (2 ± 1 rotation 60 min⁻¹) A subsequent injection of baclofen, into those animals pre-treated 4 h earlier with L-SOP, lead to a significant increase in locomotor response (329 ± 29 rotations 60 min⁻¹) when compared to the response of baclofen in animals pre-treated with vehicle (106 ± 4 rotations 60 min⁻¹). Interestingly, this response to baclofen treatment is almost identical to the locomotor responses induced by initial injection of baclofen (105 ± 10 rotations 60 min⁻¹) reported in section 5.3.5).

**Figure 5.3.6**: Locomotor effects observed following pre-treatment with intranigral injections of either the group III mGlu receptor agonist L-SOP or vehicle on subsequent baclofen-induced motor activity in reserpine-treated rats.

![Figure 5.3.6: Total locomotor effects induced by unilateral intranigral injection of baclofen (800 ng / 0.5 µl PBS) in animals pre-treated with either unilateral intranigral injection of L-SOP (750 nmol / 2.5 µl PBS) or vehicle (0.5 µl PBS) in reserpine-treated rats. Values represent mean ± s.e.m. (n = 6 animals / group). *** P < 0.0001 indicates a significant difference between baclofen responses obtained after pre-treatment with L-SOP versus pre-treatment with vehicle (unpaired 2-tailed t-test).](image-url)
5.3.7  Group III mGlu-receptor mediated homologous desensitisation of [³H]-D-aspartate release from rat nigral slices.

Repeated stimulation of rat nigral sections with 25 mM KCl produced similar results in vehicle treated slices, with no difference in [³H]-D-aspartate release observed between the first and the second KCl stimulation (S2 / S1 = 0.98 ± 0.015, mean ± s.e.m.). A single incubation of L-SOP from fractions 10 - 14 inclusive produced an approximate 60% reduction in KCl-evoked [³H]-D-aspartate release at S2, when compared to vehicle (0.40 ± 0.03). Neither single nor double incubation with L-SOP (100 µM) or vehicle (Krebs') had any effect on basal [³H]-D-aspartate release (P<0.01; n=6).

However, when L-SOP was given for four fractions prior to both S1 and S2 (with a washout period in between), this reduction in KCl-evoked [³H]-D-aspartate release was lost, shown in figure 5.3.7(a). In this case, a second further stimulation with L-SOP produced a significant increase in [³H]-D-aspartate release to 178 % of S1 shown in figure 5.3.7(b).

5.3.8  GABA₉-receptor mediated inhibition of [³H]-D-aspartate release from rat nigral slices.

Baclofen (100µM) had no effect on basal [³H]-D-aspartate release (P<0.01; n=6), but produced an approximate 40% reduction in KCl-evoked [³H]-D-aspartate release at S2, when compared to vehicle shown in figure 5.3.8. This inhibition of [³H]-D-aspartate release was 64 ± 4 % of vehicle (Krebs') (mean ± s.e.m., n = 6).

Addition of the GABA₉ selective antagonist CGP46381 (50 µM) abolished the inhibition of [³H]-D-aspartate seen following perfusion with 100 µM baclofen, returning [³H]-D-aspartate release levels to vehicle values. Figure 5.3.8 (a) shows the representative release rate curves for vehicle and CGP46381 together with 100 µM baclofen alone. CGP46381 was perfused for 4 min (2
fractions) prior to the addition of baclofen. Addition of CGP46381 (50 µM) abolished the effect of 100 µM baclofen on [³H]-D-aspartate release, shown in Figure 5.3.8 (b).

5.3.9 GABA₉-receptor mediated homologous desensitisation of [³H]-D-aspartate release from rat nigral slices.

Repeated stimulation of rat nigral sections with 25 mM KCl produced similar results in vehicle treated slices, with no difference in [³H]-D-aspartate release observed between the first and the second KCl stimulation (S2 / S1 = 1.03 ± 0.03, mean ± s.e.m.). A single incubation of baclofen from fractions 10 – 14 inclusive produced an approximate 40% reduction in KCl-evoked [³H]-D-aspartate release at S2, when compared to vehicle (S2 / S1 = 0.63 ± 0.05). Neither single nor double incubation with L-SOP (100 µM) or vehicle (Krebs') had any effect on basal [³H]-D-aspartate release (P<0.01; n=6).

However, similar to that data obtained with repeated stimulation of L-SOP, when baclofen was given for four fractions prior to both S1 and S2 (with a washout period in between), this reduction in KCl-evoked [³H]-D-aspartate release was lost, shown in figure 5.3.9(a). In this case, a second further stimulation with baclofen produced a significant increase in [³H]-D-aspartate release to 207 % of S1 shown in figure 5.3.9(b).
Figure 5.3.7: Effects of the repeated administration of the broad-spectrum group III mGlu receptor agonist, L-SOP on 25mM KCl-evoked [3H]-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation showing the effects of single L-SOP (100 µM, black) and dual L-SOP (100 µM, red) on S2 compared to vehicle (Krebs', blue) (n = 6). (b) Integrated mean S2 / S1 ratio data showing effect of repeated administration of L-SOP on [3H]-D-aspartate release. Values represent mean ± s.e.m. (n = 6). * P < 0.05 ** P < 0.01 indicates significant difference compared to vehicle, ### P < 0.001 indicates significant difference compared to single L-SOP administration (1-way ANOVA with Student-Newman-Keuls post-hoc test, P < 0.0005).
Figure 5.3.8: Effects of the GABA<sub>B</sub> receptor agonist, baclofen on 25mM KCl-evoked [³H]-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation showing the reversal of baclofen-mediated inhibition (black) of [³H]-D-aspartate release following pre-treatment with CGP46381 (blue) compared to vehicle (red).

(b) Integrated mean S2 / S1 ratio data showing effects of baclofen alone (100 µM) and baclofen (100 µM) and CPPG (50 µM) together in combination on [³H]-D-aspartate release. Values represent mean ± s.e.m. (n = 4). * (P < 0.05) and indicates significant difference compared to vehicle, there was no significant difference between vehicle treatment and baclofen + CGP46381 (1-way ANOVA, P < 0.0033).
Figure 5.3.9: Effects of the repeated administration of the GABA_B receptor agonist, baclofen on 25mM KCl-evoked [³H]-D-aspartate release from slices of rodent substantia nigra.

(a) Representative percentage release rate curves for dual 25mM KCl stimulation showing the effects of baclofen (100 µM, black) and repeated baclofen (100 µM, red) on S2 compared to vehicle (Krebs', blue) (n = 6). (b) Integrated mean S2 / S1 ratio data showing effect of repeated administration of baclofen on [³H]-D-aspartate release. Values represent mean ± s.e.m. (n = 6). * P < 0.05 ** P < 0.01 indicates significant difference compared to vehicle, ### P < 0.001 indicates significant difference compared to single baclofen administration (1-way ANOVA with Student-Newman-Keuls post-hoc test, P < 0.0005).
Discussion

Studies performed in this laboratory have previously reported single injections of broad spectrum group III metabotropic glutamate receptor agonists within the SNpr are able to reverse reserpine-induced akinesia (Maclnnes et al., 2004b). This finding was backed up and extended to show the involvement of at least mGlu4 and mGlu8 in this response by data presented in Chapter 4 of this thesis. However, no studies to date have investigated whether this therapeutic efficacy is maintained following repeated injections of group III mGlu receptor modulating compounds, as would be desired for pharmacotherapy in the treatment of Parkinson's disease. Here it was quickly realised that this was not the case. Thus, whilst initial injections of L-SOP in the SNpr were able to reverse reserpine-induced akinesia with a magnitude of response very similar to that previously reported (Maclnnes et al., 2004a), subsequent injection of L-SOP 4 hours later produced a much reduced reversal of akinesia. The 4 hour time-point used in the acute studies was chosen on the basis of previous studies investigating in vitro desensitisation of group I mGlu receptors, which have shown circa 50% of cell surface receptors to be internalized after 60 min of agonist treatment (Mundell et al., 2001; Mundell et al., 2003). However, since cellular mechanisms were not investigated in this current study, a period of washout was introduced to avoid possible confounding effects such as receptors still being occupied with subsequent behavioural alterations. This reduction in magnitude of response to repeated injection of the same agonist (in this case L-SOP) may be indicative of homologous desensitisation.

A similar loss of efficacy was also observed for repeated injections of a second broad-spectrum group III mGlu receptor agonist, L-AP4. Thus, whilst initial intranigral injections of L-AP4 produced a significant reduction in reserpine-induced akinesia, this reduction of akinesia was much less upon subsequent L-AP4 application 4 hours later. Interestingly, quantification of the initial L-AP4 injection showed an almost doubling of net contraversive rotations compared to
that previously reported in Chapter 4, section 4.3.1.3. Whilst the reasons behind this are unclear, this enhanced response may reflect more accurate injection site within the anatomically heterologous SNpr in this separate group of animals. However, since injection site verification studies were only performed at a gross level (i.e. injection site within the SNpr or not), this explanation remains purely conjectural at this time. Furthermore, the magnitude of loss of the anti-akinetic response to repeated L-AP4 injections was less than that observed following repeated L-SOP injections. Again the reasons for this difference in apparent degree of desensitisation between the two agonists remain unclear since both agents are broad-spectrum group III mGlu receptor agonists with similar EC50 values at mGlu4, 7 & 8 (Conn and Pin, 1997). One possible explanation is that L-SOP and L-AP4 have different dissociation rates from group III mGlu receptors, a factor that is thought to be important in the difference in desensitisation rates of other G-protein coupled receptors such as the opioid receptors by different agonists (Alvarez et al., 2002a; Woolf and Linderman, 2003).

Since studies performed in Chapter 4 of this thesis have suggested the involvement of both the mGlu8 and mGlu4 receptors in mediating a proportion of the anti-akinetic effects seen following L-SOP or L-AP4 injection, repeated injections of the mGlu8 receptor agonist, (S)-3,4-DCPG and the mGlu4 positive allosteric modulator, PHCCC were investigated. In keeping with the findings for both L-SOP and L-AP4, repeated intranigral injection of (S)-3,4-DCPG brought about a significant decrease in anti-akinetic efficacy. These findings were replicated for the mGlu4 receptor, with a reduction in anti-akinetic efficacy being observed following repeated intranigral PHCCC injection. Given the loss of efficacy observed following repeated intranigral injections of both L-SOP and L-AP4 the observation that a further direct agonist (S)-3,4-DCPG brings about reductions in efficacy is not surprising. However, the finding that repeated PHCCC also leads to a reduction in efficacy is more interesting, since it has been previously suggested that allosteric modulation of G protein-coupled
receptors may offer enhanced selectivity and a reduction in receptor downregulation and receptor desensitisation compared to direct agonists (Marino et al., 2003a). However, results presented in this chapter suggest that, unfortunately, this is not the case and that whilst the magnitude of efficacy loss is indeed lower than that observed for any other compound tested here, there remains a significant loss of anti-akinetic responses following repeated intranigral injections of PHCCC.

This reduced ability of L-SOP, L-AP4, PHCCC and (S)-3,4-DCPG to relieve reserpine-induced akinesia following a second injection is indicative of desensitisation (Alvarez et al., 2002b). However, since the behavioural effects of the second injection were only compared to the initial injection (and not a vehicle control) it remains unknown whether repeated injection of these drugs would still produce a significant reversal of akinesia compared to vehicle (albeit of a lower magnitude). This may indeed be the case with L-AP4 and PHCCC, since the second injection did appear to still produce some relief of akinesia. In order to address this question, further studies are needed which investigate multiple injections of vehicle together with multiple injections of agonist. However, due to the large numbers of animals involved in carrying out such a study (and associated costs), together with the length of time involved, these studies could not be performed within the time-frame permitted for this Ph.D.

The above studies were performed over a relatively short time scale (repeated injections 4 hours after the initial). In order to ascertain whether any further reductions in efficacy were observed after this time-point, studies were carried out with L-SOP alone, giving repeated injections over 12 hours in the reserpine-treated rat and over 7 days in rats bearing a stable 6-OHDA lesion of the nigrostriatal tract. The time-points of 3 days and 7 days were chosen for the 6-OHDA study due to previous experiments carried out within this laboratory on repeated group III mGlu receptor agonist exposure in the globus pallidus of 6-OHDA-lesioned rats (Maclnnes & Duty, unpublished observations).
Furthermore, a previous study investigating the effects of a single intracollicular injection of L-SOP into genetically epilepsy-prone rats described increases in mGlu4 and mGlu7 encoding mRNA together with increases in mGlu7 protein 2 days following drug treatment (Yip et al., 2001b). These results suggest behavioural alterations in repeated L-SOP administration may be present a few days post-initial treatment. The experiment was ended at day 7 due to high costs associated with maintenance of animals throughout the (lengthy) experimental process involved with lesioned and cannulated animals. That the anti-parkinsonian efficacy of L-SOP is similarly lost following a second injection 3 days later in a second model of Parkinson’s disease, the 6-OHDA lesioned rat, confirms the above observations are not merely an epiphenomenon pertaining solely to the reserpine-induced akinesia model. Moreover that a similar decrease in efficacy was noted following repeated injections at day 0 and day 3 into the globus pallidus (Maclnnes and Duty, unpublished observations) indicates this response is not a phenomenon specific to the SNpr. That the L-SOP-mediated anti-akinetic responses in both the globus pallidus (Maclnnes and Duty) and the SNpr, had both returned to the original values after 7 days suggests a period of resensitisation of group III mGlu-receptor mediated responses requires (at least) four days following L-SOP injection.

Since there are reciprocal connections from the STN to the GPe (Parent and Hazrati, 1995o), those group III mGlu receptors present on STN terminals within the SNpr may also be present on STN terminals within the pallidum. In this scenario, activation of pre-synaptic group III mGlu receptors on STN terminals in the GPe may reduce glutamate release (in an auto-receptor manner) and thus lead to a reduction in pallidal activity. These effects may therefore inhibit the group III mGlu-receptor mediated anti-parkinsonian effects produced by inhibiting GABA release from striatopallidal terminals. However, several studies lend support to the hypothesis that it is the increased GABAergic inputs which underlie the alterations in GP firing patterns in PD. For example, an increase in GABA concentrations at the GPe leads to akinesia (Pycock et al., 1976) and
intrapallidal injections of the GABA_A receptor antagonist, bicuculline produces an anti-parkinsonian effect when injected into the GPe of reserpine-treated rats (Maneuf et al., 1994). Furthermore, the predominant anatomical inputs to the GPe are inhibitory, with only relatively sparse (circa 10% of the total) excitatory glutamatergic inputs from the STN (Shink and Smith, 1995). Thus, it remains unlikely that the increased glutamatergic drive to the GPe is solely responsible for the alterations in firing seen in PD. In addition, since activation of group III mGlu receptors within the pallidum leads to a reversal of akinesia, it is most likely that the effects of group III mGlu receptor agonists are to inhibit the release of GABA from over-active striatopallidal terminals (MacInnes et al., 2004n).

In studies investigating repeated intranigral L-SOP administration for up to 12 hours, the loss of behavioural efficacy observed 4 hours post initial injection was subsequently maintained for further injections up to 12 hours post-initial injection. This maintained loss of efficacy over 12 hours suggested possible behavioural desensitisation or induction of cellular tolerance to the effects of L-SOP. Thus, the possibility of tolerance (the need for ever-increasing concentrations over a period of treatment to produce the original effect) was investigated in a separate group of animals who received a double dose of L-SOP at the end of the experiment (12 hours post initial injection). This double dose of L-SOP (1500 nmol) is sufficient to lead to convulsions and seizures in naive reserpine-treated animals (chapter 4 this thesis). In animals pre-treated with 3 injections of L-SOP however this pro-convulsant dose of L-SOP returned the magnitude of anti-akinetic response to original values. This normalised response to an increased dose of agonist is thus indicative of the development of tolerance. Whilst the mechanisms underlying receptor tolerance have yet to be firmly established (see (Finn and Whistler, 2001; Borgland, 2001; Von Zastrow M. et al., 2003; Von Zastrow M., 2004), it would appear from the widely studied field of other G/G_o-coupled recepors, such as the opioid receptors, that desensitisation and internalisation of receptors may be important (Connor et al., 2004n).
A diagram showing pathways involved in global GPCR desensitisation is shown in Figure 5.4.

Figure 5.4: Pathways involved in GPCR desensitisation and resensitisation

Typically activation of a GPCR leads to (1) activation and inhibition of specific signalling pathways in the cell. (2) Short-term desensitisation mediated by phosphorylation of the receptor by G-protein receptor kinases (GRKs), followed by β-arrestin binding to GPCRs that uncouple the receptor at the plasma membrane. (3) Endocytosis of the receptor followed by sorting of the receptor either (4) back to the plasma membrane or (5) to lysosomes for degradation.

Picture adapted from (Kristiansen, 2004)
Such a reduction in response and subsequent reappearance of response may similarly be due to receptor down-regulation or internalisation, followed by receptor resensitisation and recycling to the cell surface as depicted in figure 5.4. In summary, these initial studies have revealed for the first time, that in vivo responses to broad spectrum group III mGlu receptor agonists, mGlu8 agonists and to mGlu4 allosteric modulators, in the reserpine-treated rat and / or the 6-OHDA lesioned rat, appear to desensitise. Worryingly, these data suggest that the use of group III mGlu agonists or allosteric modulators as monotherapy in the treatment of Parkinson's disease may be of limited long-term effectiveness.

Previous work from this laboratory has investigated the activation of a second G; / Go-coupled receptor in the SNpr, the GABA_B receptor as a potential strategy in the treatment of PD. However it was not known whether the effects of GABA_B receptor agonists would be subject to a similar desensitisation, as seen here with group III mGlu receptor agonists. This possibility was therefore examined here. Initial pilot studies were carried out using intranigral baclofen at a dose previously shown to be effective at relieving reserpine-induced akinesia in the rat (Johnston and Duty, 2003a). In a similar manner suggested for group III mGlu receptor agonists in Chapter 4 of this thesis, these authors suggest the population of GABA_B receptors mediating this anti-akinetic response are the GABA_B receptors shown by immunohistochemistry to be present on pre-synaptic asymmetric (glutamatergic) terminals within the SNpr (Boyes and Bolam, 2003). In light of the anatomical connections between glutamatergic inputs to the SNpr, these terminals are most likely to be those arising from the subthalamic nucleus (Parent and Hazrati, 1995p). Indeed, electrophysiological studies have shown that activation of pre-synaptic GABA_B receptors by the agonist baclofen reduces glutamate-mediated EPSPs (Shen and Johnson, 1997a). The results from this pilot experiment were in line with those obtained previously within this laboratory, with regard to both time-course and total locomotor activity (Johnston and Duty, 2003f). Moreover, in a similar manner to that seen with the group III mGlu receptor activation, repeated injection of
baclofen after 4 hours produced a significant reduction in anti-akinetic response, suggestive of behavioural desensitisation.

In common with group I mGlu receptors, repeated pharmacological activation of GABA_B receptors is often associated with a reduced effect, so this finding is not so unexpected. For example the analgesic response to baclofen in rats is lost following 5 days consecutive treatment with baclofen (Enna et al., 1998). Furthermore, repeated dosing of baclofen similarly produces complete tolerance to the hypothermic effects within one week of daily dosing in rats (Lehmann et al., 2003b). Despite the development of tolerance reported in this previous study, no changes in either receptor protein levels or mRNA encoding any GABA_B receptor subunit were found post-mortem (Lehmann et al., 2003c). With regard to attenuation of the response to baclofen, the phenomenon is not related to altered pharmacokinetics which remain unaffected by repeated dosing (Faigle and Keberle, 1972).

Whilst the above studies have shown in vivo tolerance to the effect of repeated baclofen administration over relatively chronic treatment regimes, cellular studies have shown that repeated administration of baclofen also induces short-term changes in receptor expression levels. For example, pre-incubation with GABA in transfected CHO cells resulted in a significant loss of forskolin-induced increases of cAMP which attained significance after 2 hours. In the same study, 2 hours of agonist stimulation led to a significant increase in the number of GABA_B receptors internalized to endosomes (Gonzalez-Maeso et al., 2003). However, the situation for GABA_B receptors internalization is complex, since there similarly exists an internalization-independent mechanism for desensitisation. Experiments in cortical neurons indicate that endogenous GABA_B receptors from cortical cultures fail to internalise when exposed to agonists including baclofen for up to 6 hours (Fairfax et al., 2004). These differences between internalization studies may reflect some lacking component
of receptor stabilising complex or antibody-induced endocytosis in over-expression systems (Couve et al., 2004).

Given the above studies indicating repeated or prolonged GABA<sub>B</sub> receptor agonist exposure can reduce both cellular receptor levels and induce tolerance to behavioural responses, it is not surprising that in this study repeated intranigral injection of baclofen resulted in a significant reduction in anti-akinetic responses. However, most previous behavioural studies have only noticed an attenuation of effect following multiple injections spread over a number of days. Why the anti-akinetic response following repeated intranigral baclofen is lost following a short (4 hour) period is unknown but may reflect alterations in method of administration. Previous experiments investigating the tolerance-inducing actions of baclofen have administered baclofen intra-peritoneally (Enna et al., 1998) or sub cutaneously (Lehmann et al., 2003a), rather than direct administration into the CNS. It may be that such a direct route of administration results in a more pulsatile activation of receptors in a manner similar to that seen in vitro. However, such an explanation remains purely speculative at this stage. Certainly this decreased effect is not believed to merely reflect mechanical damage caused by multiple injections, since previous vehicle injections did not affect subsequent responses to either baclofen or group III mGlu receptor agonists.

On the basis of the results described here, the use of GABA<sub>B</sub> receptor agonists as a monotherapy in the treatment of Parkinson's disease is likely to be restricted, at best. However, only one GABA<sub>B</sub> receptor agonist was investigated in these studies and therefore these results may only describe a baclofen-specific loss of efficacy. Therefore, other GABA<sub>B</sub> receptor agonists, and allosteric modulators need to be investigated in a repeated injection paradigm before such an approach can be completely ruled out. This loss of behavioural response to repeated injections of baclofen (and group III mGlu receptor modulating compounds) is indicative of homologous desensitisation occurring for
both receptor types. However, on the basis of other findings, heterologous desensitisation was also worthy of investigation in vivo.

Previous in vitro studies have suggested activation of other receptor types may affect subsequent responses to both GABA_B and group III mGlu receptor agonists. In the case of pre-synaptic GABA_B receptors, pharmacological inactivation of PKC leads to a reduction in baclofen-mediated inhibitions of IPSPs in cultured nucleus basalis of Meynert neurons (Kubota et al., 2003a). Interestingly post-synaptic interactions between GABA_B and other receptors have also been documented in the literature. Thus prolonged activation of GABA_B receptors leads to a desensitisation of adenosine A_1 receptor-mediated responses in cultured rat hippocampal neurons (Wetherington and Lambert, 2002). Furthermore there are many examples of post-synaptic interactions between group I mGlu receptors and GABA_B receptors, revealed following electrophysiological studies. The nature of these interactions appears to be one of enhancement rather than desensitisation, but it is worthy of mention regarding the complexities of cross-talk between these receptor types. For example, at cerebellar parallel fiber synapses, activation of GABA_B receptors by baclofen resulted in an enhancement of the group I mGlu receptor agonist 1S,3R-ACPD-induced current response and rise in intracellular calcium. In the same study these authors discovered this augmentation of group I mGlu receptor-mediated responses could similarly be enhanced following endogenous GABA release from cerebellar interneurons (Hirono et al., 2001). The authors suggest a mechanism mediating this GABA_B-mediated enhancement is the involvement of G_\gamma/G_\alpha-coupled PLC activation and calcium mobilisation from internal stores, since both PLC and G_\gamma/G_\alpha inhibitors were able to attenuate the enhancement of group I mGlu receptor-mediated responses. More recently it was discovered that extracellular calcium ions bind to GABA_B receptors; this Ca^{2+}-GABA_B receptor complex is then able to enhance the effects of post-synaptic group I mGlu receptors at cerebellar synapses in vitro. Furthermore, in the same study immunoprecipitation experiments suggest the
formation of a GABA\textsubscript{B}-mGlu1 receptor complex in mouse cerebellum (Tabata \textit{et al.}, 2004). Thus, calcium binding to GABA\textsubscript{B} receptors may induce a direct change in the mGlu1 receptor structure, enhancing its response to agonists (Kubo and Tateyama, 2005).

Given that both group I mGlu and GABA\textsubscript{B} receptors have been shown to interact post-synaptically, coupled with results from this thesis reporting repeated intranigral injection of both pre-synaptic group III mGlu and GABA\textsubscript{B} receptor agonists results in decreased anti-parkinsonian efficacy, experiments were designed to investigate the possibility of cross-talk between pre-synaptic group III mGlu and GABA\textsubscript{B} receptors in our experimental paradigm. In support of this approach, pre-injection with the GABA\textsubscript{B} receptor agonist, baclofen, resulted in a significant decrease in anti-akinetic actions of the group III mGlu receptor agonist, L-SOP, when compared against pre-treatment with vehicle and subsequent L-SOP injection. These results suggest that prior activation of pre-synaptic GABA\textsubscript{B} receptors can indeed attenuate the subsequent response to group III mGlu receptor activation.

The mechanisms underlying this receptor cross-talk remain purely speculative at this stage, since such investigations were beyond the scope of this thesis. However, one possible explanation is that prior activation of GABA\textsubscript{B} receptors by baclofen results in a subsequent internalisation of group III mGlu receptors. This in turn would result in a loss of functional receptors at pre-synaptic STN terminals within the SNpr and lead to a reduction in responses elicited by L-SOP. Experiments investigating opioid receptor desensitisation have revealed this explanation would fit the time-course of experiments reported here, where internalisation of receptors is seen from 10 min to beyond 24 hours post-opioid receptor activation (Connor \textit{et al.}, 2004b). However, very recent studies have revealed that repeated administration of L-AP4 in over-expression systems produces no receptor internalisation even after 60 min of agonist treatment. By contrast a modest increase in surface receptors was observed after 15 min
where it remained for up to 6 hours. This increase in cell surface receptors was inhibited by pre-treatment with the group III mGlu receptor antagonist, CPPG (Mathiesen and Ramirez, 2006c). Many studies have reported a PKC-mediated reduction of group III mGlu receptor responses \textit{in vitro} (Macek \textit{et al.}, 1998b; Macek \textit{et al.}, 1999; Cai \textit{et al.}, 2001a; Gordon and Bains, 2003a; Mathiesen and Ramirez, 2006b). However, it is difficult to reconcile how activation of the pre-synaptic GABA\textsubscript{B} receptors by baclofen, with subsequent activation of G\textsubscript{i}/G\textsubscript{o} leads to activation of PKC. One possible further explanation is that pre-treatment with baclofen leads to an inhibition of voltage-dependent calcium channels. This inhibition of calcium channels may result in a temporary increased resistance to further inhibition by L-SOP. Since activation of group III mGlu receptors results in an inhibition of neurotransmitter release that is primarily mediated by a reduction in calcium-channel conductance (Millan \textit{et al.}, 2002c), this may be one further hypothetical explanation for the reduction of efficacy seen with L-SOP.

In stark contrast to the above scenario, pre-injection with the III mGlu receptor agonist, L-SOP resulted in a significant increase in anti-akinetic actions of the GABA\textsubscript{B} receptor agonist, baclofen when compared against pre-treatment with vehicle and subsequent baclofen injection. These results were completely unexpected and suggest that prior activation of pre-synaptic group III mGlu receptors can actually potentiate the subsequent response to GABA\textsubscript{B} receptor activation.

One possible explanation for augmentation of the baclofen response is that pre-treatment with L-SOP is predicted to lead to a decrease in cAMP levels (Conn and Pin, 1997). This decrease in cAMP levels may in turn lead to a decrease in protein kinase A (PKA) activity. Since PKA activation enhances currents through L-type Ca\textsuperscript{2+} channels (Catterall, 2000) a sub-threshold decrease in PKA activation (such as may occur following prior activation of group III mGlu receptors by L-SOP) may lead to a condition whereby subsequent application of
a second G/G\(_o\)-coupled receptor agonist, such as baclofen, further decreases PKA and calcium channel activity, thus inhibiting transmitter release. The ability of GABA\(_B\) receptor agonists to inhibit transmitter release is largely independent of their direct effects on cAMP levels, rather reflecting the decrease in voltage-dependent calcium channel activity and direct calcium-mediated vesicular release (Giustizieri et al., 2005). Therefore, a further decrease in calcium channel activity following a prior inhibition of PKA by L-SOP may lead to an additional inhibition of glutamate release from subthalamic terminals within the SNpr, thus increasing relief from reserpine-induced akinisia.

One additional effect of prior inhibition of PKA may also be to prevent PKA-mediated phosphorylation of Snapin, a protein recently identified as an important regulator of neurotransmitter release (Chheda et al., 2001b). PKA phosphorylation of Snapin increases the binding of this protein to synaptosomal-associated protein-25 (SNAP-25). In an over-expression cell line study, phosphorylated Snapin led to an enhancement of release vesicles (Chheda et al., 2001a). Unfortunately, the role of PKA activity and GABA\(_B\) receptors is currently under debate, with a study showing PKA activity reduces GABA\(_B\) receptor desensitisation and leads to an increased cell-surface stability of GABA\(_B\) receptors (Couve et al., 2002). However, a separate study suggests decreased activity of PKA leads to a reduction in GABA\(_B\) receptor agonist activity (Kubota et al., 2003b). Until the role of PKA in the modulation of responses to GABA\(_B\) receptor agonists is more completely understood, the putative explanation described above remains purely hypothetical.

Furthermore there exists the possibility that, similar to post-synaptic GABA\(_B\) receptors and group I mGlu receptors, a direct steric interaction may occur between pre-synaptic GABA\(_B\) receptors and group III mGlu receptors, altering receptor-mediated responses following prior activation of one component of the complex. However, this explanation too remains purely hypothetical at this stage.
Given the desensitisation of behavioural responses noted in vivo following repeated intranigral injections of either group III mGlu or GABA$_B$ receptor agonists, a final series of experiments were conducted to establish at what level this change in behaviour was likely to be effected. The studies outlined earlier in chapter 3 of this thesis showed how activation of group III mGlu receptors can lead to a decrease in glutamate release from nigral slices. This would provide an explanation for the behavioural effect observed following intranigral group III mGlu receptor injection. Was this effect also desensitised following repeated application of a group III mGlu receptor agonist?

Indeed, repeated administration of the group III mGlu receptor agonist, L-SOP resulted in a loss of inhibition of KCl-evoked [$^{3}$H]-D-aspartate release in vitro. These data suggest that indeed the loss of the efficacious behavioural response seen in vivo following repeated intranigral group III mGlu receptor agonist injections may be due to a loss of glutamatergic release inhibition.

Whilst previous studies from this laboratory have suggested the anti-akinetic actions of baclofen result from pre-synaptic inhibition of glutamatergic terminals within the SNpr, such an explanation had not been demonstrated experimentally (Johnston and Duty, 2003g). Therefore an initial pilot study was performed with an identical concentration of baclofen (100 µM) previously shown to be effective in vitro, in rat brain slices (Knight and Bowery, 1996). Previous studies have suggested baclofen can inhibit elevated KCl-induced [$^{3}$H]-D-aspartate release from cultured granule cells, by approximately 60% (Zhu and Chuang, 1987). These data are similar to the level of inhibition of 40% seen in this current study. The small differences in magnitude of release may be accounted for by the use of fresh intact tissue slices, the decreased concentration of KCl and an elevated concentration of baclofen used in this study.
The receptor specificity of this baclofen-induced inhibition of [³H]-D-aspartate was examined using the group GABA<sub>B</sub> antagonist, CGP 46381. Pre-incubation with CGP 46381 served to reverse the inhibition of evoked [³H]-D-aspartate release seen following baclofen application. Since superfusion of CGP 46381 alone produced no effects on [³H]-D-aspartate release, this suggests GABA<sub>B</sub> receptors are not activated by endogenous glutamate under basal or release conditions within this slice preparation. CGP 46381 is a highly selective GABA<sub>B</sub> antagonist, with an IC<sub>50</sub> of 4.9 µM (Alexander et al., 2006). The concentration of CGP 46381 employed here (50 µM) should therefore be able to inhibit any GABA<sub>B</sub> receptor-mediated effects, due to the excess concentration used. Taken together, these data suggest the observed inhibitory effects of CGP 46381 is believed to represent an inhibition of the GABA<sub>B</sub> receptor-mediated effects of baclofen.

Due to time limitations and the desire to further investigate whether desensitisation could also be observed in vitro, only one concentration of baclofen was investigated in this study. Further studies should therefore be carried out investigating a broader concentration range and using more replications to provide greater reliability. However, clearly these data support the explanation that reversal of akinesia could result from decreased glutamate release from subthalamonigral terminals (Johnston and Duty, 2003h).

Similar to those results obtained by repeated application of L-SOP in vitro, repeated application of baclofen resulted in a loss of inhibition of [³H]-D-aspartate release in rat nigral slices. That the responses to both baclofen and L-SOP appear to desensitise in vitro supports the view that these effects may underlie the loss of behavioural efficacy seen in vivo.

The mechanisms behind this attenuated response to subsequent agonist administration in vitro may be different to those hypothesised for the in vivo results, due to the much shorter time-period able to be studied in vitro. Whilst in
In vivo agonists were administered every four hours due to prolonged behavioural effects, coupled with the necessity for a relatively long wash-out period, in vitro repeated exposure was able to be carried out within 14 minutes. Previous in vitro over-expression studies have suggested mGlu4 does not desensitise upon repeated administration, measured by reductions in forskolin-stimulated cAMP levels (Mathiesen and Ramirez, 2006a). Furthermore, over-expression of the G-protein receptor kinase 2 (GRK2) does not induce any desensitisation of the ability of mGlu4 to inhibit adenylate cyclase. Rather, over-expression of GRK2 induces inhibition of the ability of L-AP4 to stimulate the mitogen-activated protein kinases (MAPK) pathway (Iacovelli et al., 2004). Whilst these results may appear to be in contradiction to those obtained in this thesis, important differences exist between these studies and those presented here. As previously suggested, the ability of group III mGlu receptors to inhibit neurotransmitter release is largely independent of their ability to reduce cAMP levels, rather this result is brought about via a direct inhibition of voltage-dependent calcium channels (Millan et al., 2002b). Therefore, the reduction in response observed in these studies suggest the ability of the βγ subunit to inhibit pre-synaptic calcium channels may be a further component of the group III mGlu receptor signalling cascade that is altered upon repeated administration of agonist. Further experiments are necessary to investigate this novel finding further.

Previous electrophysiological studies have investigated the effects of repeated administration of baclofen in vitro. Prolonged (15 min) application of baclofen (100 µM) led to a progressive return of IPSPs recorded from postnatal 2-5 day old rat hippocampal cultures. These results indicate a rapid decrease in efficacy of pre-synaptic GABA\textsubscript{B} “auto”-receptors. However, in the same study the pre-synaptic “hetero”-receptor effects of baclofen on EPSPs within the hippocampus did not show any decrease in efficacy (Tosetti et al., 2004). These data are in contrast to those presented in this thesis, where repeated baclofen administration results in a significant reduction in GABA\textsubscript{B} receptor-mediated
efficacy at glutamatergic synapses. The reasons for this discrepancy may be accounted for by the age of the animals used in this study (approximately 9 – 12 weeks), and by the region of the CNS under study (SNpr compared to hippocampus). In the neonatal rat hippocampus, GABAAergic transmission is excitatory in nature due to the inversion of the chloride gradient, a fact which is reversed in the adult animal (Ben-Ari et al., 1997). Like their group III mGlu receptor counterparts, little is known about the mechanisms of GABAB receptor desensitisation. Recent studies investigating this phenomenon have found GRK4 to mediate GABAB receptor desensitisation in a phosphorylation-independent manner (Perroy et al., 2003). Whilst the mechanisms of this are as yet unclear they may reflect a direct binding of GRK4 to the Gai/o, leading to an intracellular sequestration of the α-subunit and hence loss of response. However, there currently exists no experimental evidence to support this hypothesis.

Unfortunately, due to time limitations, it was not possible to investigate in vitro the heterologous potentiation seen in vivo. Clearly this set of experiments may provide additional support for the hypothesis of group III mGlu / GABAB receptor cross-talk.

Whilst the observation of desensitisation appears to limit the use of group III mGlu or GABAB receptors as therapies for PD, it is important to note that, due to experimental limitations this may not be the case. The observed desensitisation of both group III and GABAB receptors may be a dose-dependent phenomenon, similar to that observed with mu opioid receptors (Deng et al., 2001). Thus, lower doses of either group III or GABAB receptor agonists may yet display behavioural recovery from reserpine-induced akinesia without the loss of efficacy seen following a second injection. For at least one of the group III mGlu receptor agonists (L-AP4) this appears to be the case, since sub-chronic (7 day) repeated intranigral injections of 10nmol L-AP4 produced no loss of protection against 6-OHDA-induced neurotoxicity (Vernon et al., 2005). However, only the
highest dose of L-AP4 tested (300nmol) produced any significant behavioural effects compared to vehicle in singly injected reserpine-treated rats (Chapter 4, this thesis). Therefore only this effective dose could be used to measure behavioural effects following repeated injection studies. However, L-SOP and (S)-3,4-DCPG were both effective at reducing reserpine-induced akinesia across a range of doses and therefore lower doses of these agonists could be tested in the future. Since a full dose-response curve for PHCCC was not produced, it is currently unknown whether a lower dose of PHCCC may be able to be used. One further experimental limitation is that the behavioural effects of all agents tested were only compared against the initial response, rather than for vehicle controls. Thus, it currently cannot be ascertained whether the anti-parkinsonian effects of these agents remains significant (compared to vehicle) following repeated intranigral injections. In support of this view, data from the study using repeated intranigral injections of L-SOP over 1 week may suggest that a 3 day dosing regime may indeed be effective. However, in order for this hypothesis to be fully tested future experiments would need to use extra groups of animals. In the case of reserpine-treated rats, three extra groups (comprising vehicle & vehicle; agent & vehicle; vehicle & agent) would be needed to fully explore this hypothesis and to refute the hypothesis that repeated injections per se reduced behavioural responses. Each additional group would need to comprise of at least 8 animals (to allow for incorrect cannula placement or postsurgical complications). Therefore these experiments would quickly become extremely time-consuming. For the 6-OHDA study, at least three extra groups of animals would similarly be required (L-SOP, vehicle, L-SOP; vehicle, L-SOP, vehicle and vehicle, vehicle, vehicle). An alteration in the dosing regime may also help to determine the time-course of any reduced effect, with the effects of daily repeated injections being the most obvious suggested protocol.
In conclusion, studies outlined in this chapter serve to demonstrate that repeated activation of both group III mGlu and GABA_B receptors may lead to a reduction in anti-parkinsonian efficacy. Furthermore, these data suggest a novel type of functional interaction between pre-synaptic group III mGlu and GABA_B receptors in the rodent basal ganglia, whereby activation of one receptor type can profoundly influence the ability of the other to induce a response. This implies that other GPCRs could indirectly serve to fine-tune glutamatergic transmission at the subthalamonoigral synapse in vivo. Unfortunately, these findings may shed doubt on the ability of group III mGlu (or GABA_B) receptor agonists to be useful pharmacological therapies for the treatment of Parkinson's disease. Whilst group III mGlu receptors have been suggested as possible therapies for epilepsy (Moldrich et al., 2003b), schizophrenia (Wigmore and Lacey, 1998) and neurotoxicity (Bruno et al., 1996) repeated administration of group III mGlu receptor agonists has not yet been performed in animal models of these conditions. Thus it remains to be seen whether the findings presented in this chapter represent a global reduction in efficacy following repeated administration in vivo, or whether this phenomenon is related specifically to the subthalamonoigral synapse. However, it is unlikely given other studies performed in this group which show desensitisation of group III mGlu-mediated responses at the striatopallidal synapse (MacInnes & Duty, unpublished observations). Additional cellular based studies are needed to further elucidate the exact mechanisms by which both GABA_B and group III mGlu receptors undergo desensitisation in vivo. With this knowledge it may be possible to negate this deleterious effect and restore therapeutic efficacy.
General conclusions
At the current time Parkinson’s disease (PD) remains both without a cure and without adequate symptomatic treatment. Current dopaminergic treatments such as L-DOPA, aimed at reversing striatal dopamine loss only retain efficacy for up to 5 – 10 years, and are plagued with a number of disabling side-effects such as dyskinesias and on-off fluctuations in motor responses. The need to retain anti-parkinsonian efficacy without the side-effects form the principal rationale behind the search for alternative therapies. This search has been greatly enhanced by the recent advances in our understanding of the pathophysiology and alterations in basal ganglia circuitry.

Loss of the dopaminergic nigrostriatal tract initiates a number of changes in downstream basal ganglia motor nuclei. These changes culminate in inhibition of thalamo-cortical feedback which is believed to underlie the motor complications of PD such as tremor, rigidity and bradykinesia. One important change in the intermediary nuclei within the basal ganglia is increased activity of the glutamatergic STN which projects to the inhibitory SNpr and EP (GPI), resulting in increased activity levels of these nuclei. One recently successful method of treating PD involves deep brain stimulation or lesioning of the STN efferents (subthalamotomy). However, such a surgical approach is costly and currently only available in limited centres world-wide. Thus a pharmacological mimic of subthalamotomy may be predicted to show therapeutic efficacy in the treatment of PD, offering both reduced treatment costs and increased availability to these surgical alternatives. Group III mGlu receptors have recently been demonstrated to be present pre-synaptically on these subthalamonigral terminals where they are believed to act as autoreceptors, regulating the release of glutamate. Activation of these receptors may therefore provide a suitable pharmacological means in the treatment of PD. The overall aim of this thesis was to further investigate this hypothesis that group III mGlu receptors may provide an alternative non-dopaminergic therapy in the treatment of PD.
Initial experiments set out to investigate whether group III mGlu receptor agonists and allosteric modulators could indeed bring about a reduction in presynaptic release of glutamate. A previous electrophysiological study investigating the effects of group III mGlu receptor modulation in the SNpr had shown activation of these receptors by a broad-spectrum group III agonist (L-AP4) can reduce the excitability of SNpr neurons (Wittmann et al., 2001). Furthermore these authors suggested that the mechanism underlying this response is pre-synaptic inhibition of glutamate release from STN terminals within the SNpr. However, direct experimental evidence in support of this hypothesis has not yet been published. Therefore initial studies investigated the ability of group III mGlu receptor modulating compounds to inhibit elevated KCl-evoked [3H]-D-aspartate release (a marker of glutamate release) from rat nigral sections.

Both broad-spectrum group III mGlu receptor agonists, L-SOP and L-AP4 reduced 25mM KCl-evoked [3H]-D-aspartate release in a concentration-dependent manner. Furthermore this inhibition of [3H]-D-aspartate release was confirmed to be group III mGlu receptor-mediated, since pre-treatment with the group III mGlu receptor antagonist, CPPG, abolished the effects of both L-SOP and LAP4. Interestingly, another group III mGlu receptor antagonist, M-SOP, did not reduce the effects of L-SOP. Rather, M-SOP led to an unexpected concentration-dependent inhibition of [3H]-D-aspartate release when given alone. When M-SOP was given in combination with L-SOP, a potentiation of the L-SOP-evoked reduction in [3H]-D-aspartate release was observed. These surprising observations are nonetheless consistent with a previous report which indicated another group III mGlu receptor antagonist (M-AP4) inhibited glutamate release from cortical efferents in the striatum (East et al., 1995) and suggests that these agents may possess some agonist-like abilities. Since broad-spectrum agonists were found to be effective at reducing [3H]-D-aspartate release, subsequent studies aimed to start to elucidate which sub-type of group III mGlu receptors (mGlu 4, 7 or 8) might be mediating these effects. Here, the
mGlu4 positive allosteric modulator, PHCCC was used. PHCCC alone was without any effect in modulating [3H]-D-aspartate release confirming its role as an allosteric modulator rather than a full receptor agonist. However, when given in conjunction with a sub-threshold concentration of L-SOP the combination of PHCCC and L-SOP served to significantly reduce [3H]-D-aspartate release. These data support the hypothesis that activation of group III mGlu receptors on these terminals can decrease neurotransmitter release in an autoreceptor manner and indicate that mGlu4 is involved in mediating at least some component of this decrease in neurotransmitter release.

The second series of experiments, described in Chapter 4 of this thesis, explored the anti-parkinsonian potential of group III mGlu receptor modulating compounds in rodent models of the disease. In rats displaying reserpine-induced akinesia, intranigral injection of both L-SOP and L-AP4 brought about a dose-dependent restoration of locomotor activity indicative of anti-parkinsonian efficacy. In a similar fashion, the mGlu4 positive allosteric modulator, PHCCC reduced reserpine-induced akinesia when given alone. That PHCCC did not require a sub-threshold dose of L-SOP here in order to reveal its efficacy most probably reflects the fact that sufficient receptor stimulation is already provided here by the presumed over-activity of glutamatergic STN efferents within the SNpr in this model of PD. The possible involvement of mGlu8 receptors was also explored in these studies. Intranigral injection of the mGlu8 selective agonist, (S)-3,4DCPG similarly evoked a dose-dependent restoration of locomotor activity in reserpine-treated rats. Unfortunately, at the time of performing these experiments no selective mGlu7 agonist was available, thus the possible involvement of mGlu7 could not be examined. Following the success of intranigral L-SOP in reserpine-treated animals, it was reassuring that the anti-parkinsonian efficacy was mimicked in a second behavioural model, the 6-OHDA lesioned rat. In conclusion, these data are strongly supportive of the potential for group III mGlu receptor agonists as anti-parkinsonian compounds and indicate that both mGlu4 and mGlu8 receptors are certainly involved in
mediating these group III mGlu receptor-mediated antiparkinsonian effects, although mGlu7 remains as a potential candidate.

Following the success of single doses of group III mGlu receptor modulating compounds on locomotor behaviour, Chapter 5 set out to determine whether this efficacy was maintained following repeated dosing, as would be desired therapeutically. Firstly, in reserpine-treated rats the behavioural effects of repeated activation of group III mGlu receptors by L-SOP, L-AP4, PHCCC and (S)-3,4-DCPG were measured over four hour time-points. All agents thus tested displayed a significant reduction in anti-akinetic behavioural response upon subsequent injection of the same agonist. To ensure this behavioural tolerance to repeated injections was not an epiphenomenon pertaining to the reserpine-treated rat model of PD, studies were performed in rats bearing a stable 6-OHDA lesion of the nigrostriatal pathway. Here too subsequent injections of L-SOP produced significantly reduced antiparkinsonian responses over a 7 day period. Since previous studies have suggested activation of group III mGlu receptors may lead to alterations in responses to other G-protein-coupled receptors (Mecek et al., 1998; Cai et al., 2001) a further series of experiments were performed investigating the effects of activating (both singly and repeatedly) a further G\textsubscript{i} / G\textsubscript{o}-coupled receptor, the GABA\textsubscript{B} receptor. Baclofen-mediated activation of GABA\textsubscript{B} receptors, in the SNpr of reserpine-treated rats led to a significant reversal of akinesia which was similarly reduced in size following subsequent agonist injection. Further studies were performed on the effects of repeated in vitro administration of baclofen and L-SOP on [\textsuperscript{3}H]-D-aspartate release. Similar to those findings observed in vivo, repeated in vitro application of both agonists served to reduce the inhibition of [\textsuperscript{3}H]-D-aspartate release from rat nigral sections. These data lend support to the behavioural findings of this thesis and together suggest both group III mGlu and GABA\textsubscript{B} receptors undergo homologous desensitisation both in vivo and in vitro.
Finally, a complex cross talk was revealed between activation of GABA_B and group III mGlu receptors in the SNpr *in vivo*. Thus, whilst prior activation of GABA_B receptors by baclofen served to reduce the behavioural response to L-SOP, pre-treatment with L-SOP rather potentiated the behavioural responses to subsequent baclofen injection. Whilst the mechanisms mediating this observed behavioural crosstalk between GABA_B and group III mGlu receptors remain unclear, these data are the first *in vivo* evidence of heterologous desensitisation for group III mGlu receptors.

Broadly defined, the aim of this thesis was to investigate the role and anti-parkinsonian potential of group III mGlu receptor activation in the rodent basal ganglia. Certainly, the results obtained here increase our knowledge of the consequences of group III mGlu receptor activation in the SNpr. Release studies carried out here provide data in support of previous electrophysiological experiments, and lend credence to the suggested mechanism of action following activation of group III mGlu receptors being inhibition of glutamate release from pre-synaptic terminals. The success of behavioural studies, where a range of group III mGlu receptor modulating compounds demonstrated anti-parkinsonian efficacy in both the reserpine-treated rat and the 6-OHDA lesioned rat was encouraging. However, studies to date of all the examined group III mGlu receptor modulating compounds suggested this therapeutic efficacy is lost following repeated injection. In summary, these data widen our knowledge of group III mGlu receptor function in the rodent SNpr under normal and parkinsonian conditions, and suggest that further research is needed into this potentially troubling loss of efficacy seen after repeated injections.
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