Neuroprotective effect of GLP-1 receptor agonist Exendin-4 in rat models of Parkinson’s disease

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DECLARATION

This thesis describes research conducted at The School of Pharmacy, University of London between October 2006 and June 2009 under the supervision of Dr. Peter Whitton. I certify that the research described is original and that any parts of the work that have been conducted in collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by a suitable citation any part of this work that had already appeared in publication.

Signature........................................Date. 2/11/09.
ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder with no current cure and therapies that are directed towards symptomatic relief but have potentially disabling side effect profiles. The need for an alternative therapeutic approach is crucial. Not so long ago it has been demonstrated that the glucagon-like peptide-1 receptor (GLP-1R) agonist exendin-4 (EX-4) originally isolated from saliva of Heloderma suspectum lizard (Eng et al., 1992) is neurotrophic and neuroprotective in vitro leading to the suggestion that stimulation of central GLP-1 receptors could have therapeutic value in neurodegenerative disorders such as PD (Perry et al., 2002b).

Here we have investigated the effects of EX-4 treatment in rats with nigrostriatal lesions following central 6-hydroxydopamine (6-OHDA) or lipopolysaccharide (LPS) injection. Both toxins were delivered stereotactically and animals were then investigated in vivo and ex vivo with a wide range of behavioural, neurochemical and histological tests to assess the integrity of the nigrostriatal dopamine (DA) system. EX-4 was administered twice daily at two different doses seven days after intracerebral toxin injection for the duration of one week. Another week thereafter the lesion severity was quantified based on contralateral circling behaviour following apomorphine injection.

Circling was very pronounced in toxin only treated animals and significantly lower in rats co-treated with EX-4 at both doses (0.1 and 0.5µg/kg) while it was increased again.
in groups co-treated with either GLP-1R antagonist EX-9-39 or the DA D3-receptor antagonist nafadotride. This suggested that the neuroprotective effect of EX-4 involved activation of both GLP-1R and DA D3-receptors. Animals were then implanted with microdialysis probes and extracellular DA was measured using in vivo microdialysis. Both basal and potassium evoked levels of DA were markedly reduced in toxin treated animals but were comparable to control levels in EX-4 treated groups. Groups co-treated with either of the two antagonists resulted in DA levels being comparable to 6-OHDA or LPS only treated animals, which again shows the involvement of both GLP-1Rs and D3-receptors. In addition to these findings, striatal tissue DA, tyrosine hydroxylase (TH) activity and TH immunolabelling were also measured in these groups and all confirmed the behavioural and in vivo data.

In conclusion, EX-4 clearly reverses the loss of extracellular DA and the integrity of the nigrostriatal system in toxin lesioned rats. The mechanism involves both the GLP-1R and D3-receptors. EX-4 readily enters the brain on peripheral administration and is already in use for type 2 diabetes for which it was FDA approved in 2005 and in our view should be clinically tested in human PD patients without further delay.*

* In fact, findings of both Bertilsson et al., 2008 and Harkavyi et al., 2008 have lead to the first EX-4 clinical trial in PD patients. Trial is currently being set up with the support of The Cure Parkinson’s Trust, UK.
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LIST OF ABBREVIATIONS

1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)
3,4-Dihydroxy-l-phenylalanine (L-DOPA)
3’3-diaminobenzidine (DAB)
6-hydroxydopamine (6-OHDA)
acetylcholine (ACh)
adenylate cyclase (AC)
amyloid beta peptide (Aβ)
analysis of variance (ANOVA)
anti-apoptotic B-cell leukemia protein (Bcl2)
aromatic amino acid decarboxylase (AADC)
artificial cerebrospinal fluid (aCSF)
blood brain barrier (BBB)
brain derived neurotrophic factor (BDNF)
bromodeoxyuridine (BrdU)
cAMP response element binding protein (CREB)
catechol-O-methyl transferase (COMT)
central nervous system (CNS)
corticotrophin-releasing factor-1 receptor (CRF-R1)
cyclic adenosine monophosphate (cAMP)
deep brain stimulation (DBS)
dihydroxy-phenyl-alanine (DOPA)
DOPA decarboxylase (DDC)
dopamine (DA)
electrochemical detection (ECD)
exchange proteins directly activated by cAMP (Epac)
exendin-4 (EX-4)
forkhead transcription factor (FoxO1)
glial cell derived neurotrophic factor (GDNF)
glicentin-related pancreatic polypeptide (GRPP)
globus pallidus externus (GPe)
globus pallidus internus (GPi)
glucagon-like peptide 1 (GLP-1)
glucagon-like peptide 1 receptor (GLP-1R)
glucagon-like peptide 2 (GLP-2)
high performance liquid chromatography (HPLC)
insulin receptor substrate 2 (Irs2)
intervening peptide-1 (IP-1)
Intracerebroventricular (ICV)
lateral ventricular wall (LVW)
lipopolysaccharide (LPS)
medial forebrain bundle (MFB)
monoamine oxidase (MAO)
nuclear factor kappa B (NF-κB)
oxyntomodulin (OXM)
pancreatic-duodenal homeobox (PDX-1)
Parkinson's disease (PD)
peak area (PA)
phosphoinositide-3-kinase (PI3K)
phospholipase C (PLC)
protein kinase A (PKA)
protein kinase C (PKC)
striatum (STR)
subgranular zone (SGZ)
substantia nigra pars compacta (SNc)
substantia nigra pars reticulata (SNr)
subthalamic nucleus (STN)
subventricular zone (SVZ)
tyrosine hydroxylase (TH)
urocortin (UCN)
ventral intermediate thalamus (VIT)
γ-aminobutyric acid (GABA)
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Chapter 1

General Introduction
Section 1: Introduction to Parkinson’s disease

1.1 Brief history

Parkinson's disease (PD) has existed in different parts of the world since ancient times. The first clear description of it was found in an old Indian medical system and was referred to as “kampavata” - meaning tremors. Traditional therapies in the form of various herbal preparations containing anticholinergics and monoamine oxidase inhibitors were used in the treatment of PD in India and China (Manyam et al., 1999). However it was not formally recognized and the symptoms were not documented until 1817 when a British physician James Parkinson described the condition as a “shaking palsy” (Parkinson, 2002). PD was then referred to as “paralysis agitans” for a period of time, until French neurologist Jean-Martin Charcot coined the term Parkinson’s disease. The underlying biochemical changes were only identified in 1950s owing largely to the work of a Swedish scientist called Arvid Carlsson who won a Nobel Prize for his work. 3,4-Dihydroxy-L-phenylalanine (L-DOPA) therapy was introduced to clinical practice in 1967 (Hornykiewicz, 2002). The first study showing improvements in PD patients receiving L-DOPA was published in 1968 (Cotzias, 1968).

1.2 Epidemiology

PD is the most common neurodegenerative disorder after Alzheimer’s disease (AD). Cases of PD are reported at all ages; although it is quite rare in people younger than 40
and the average age at which symptoms begin is 58-60. It occurs in all parts of the world, but appears to be more common in people of European ancestry than in those of African ancestry. Those of East Asian ancestry have an intermediate risk. It is more common in rural than urban areas, and men are affected slightly more often than women. Twin studies showed that the occurrence of PD in patients under 40 years old is almost always genetically caused (Tanner, 2003). It is relatively widespread with about 100-150 cases per 100,000 of the population in North America. In the UK the age adjusted prevalence is 254 cases per 100,000 but the prevalence rises with age from 0.143% in the 50 to 59 year old population to 1.75% in the population aged 80 years or older (Schrag et al., 2000). There have been several major epidemiological studies carried out in countries such as Finland, Holland, Spain, United Kingdom and United States (de Lau et al., 2004; Marion, 2001). All of these show similar patterns concluding that occurrence increases with age and that it is more common in males than in females with an estimated ratio of 2:1.

1.3 Symptoms

The cardinal signs of PD are: tremor, rigidity, bradykinesia and postural instability and appear when almost all (around 80%) nigrostriatal dopaminergic innervation is lost. Tremor normally occurs when a limb is at rest and improves with voluntary movement. This is the most apparent and well known symptom of PD although 30% of patients have little perceptible tremor and are referred to as akinetic-rigids. Although resting tremor is more common in PD than in other diseases with parkinsonian features, it is not rare in other disorders. Rigidity is characterized by stiffness and increased muscle
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tone. Bradykinesia/akinesia is slowness and absence, respectively, of coordinated voluntary movements and is present in around 80% of patients (Sabatini et al., 2000). Postural instability, which is characterized by a loss of postural reflexes, leads to balance impairment and falls. Other motor symptoms include dystonias which are abnormal, painful muscle contractions usually in the feet but may include other skeletal muscles. Such dystonic episodes are acutely painful and totally disabling for a patient. Since muscles in the face are also affected, the swallowing reflex is impaired therefore PD patients often drool. Impaired ability to swallow can lead to aspiration, pneumonia and ultimately death. Speech disturbances and cognitive decline are common in patients with PD: these include decreased verbal fluency and cognitive disturbance especially related to comprehension of emotional content of speech and of facial expression (Pell, 1996).

Section 2: Pathophysiology

2.1 Dopamine

DA is the predominant catecholamine neurotransmitter in the mammalian brain where it has a variety of roles including cognition, locomotion, emotion, positive reinforcement of learning, food intake and endocrine function. Roles of DA are not restricted to the central nervous system (CNS). In the periphery it serves as a modulator of cardiac function and vascular tone, as well as gastrointestinal motility and renal function. It is also involved in catecholamine release and hormone secretion. DA was
first discovered in 1952 by the Swedish scientist Arvid Carlsson. The name “dopamine” came from its precursor in the biosynthetic pathway which is L-DOPA and also because it is a monoamine. Arvid Carlsson was awarded the Nobel Prize for demonstrating that DA was a neurotransmitter in its own right and not just a precursor of noradrenaline and adrenaline. Classical neurotransmitters like catecholamines are manufactured in one or several biochemical steps. These synthetic pathways are important firstly because they offer a regulatory mechanism for the amount of e.g. dopamine available for release and secondly because the pathway can be altered by adding various drugs or precursors. The rate limiting step in production of DA is catalyzed by tyrosine hydroxylase (TH) where tyrosine is converted to dihydroxy-phenyl-alanine (DOPA). DOPA is then decarboxylated by aromatic amino acid decarboxylase (AADC) to form DA. Neurons that use DA as their neurotransmitter only posses TH and AADC unlike for example noradrenergic nerves that also posses DA β-hydroxylase which in turn adds a hydroxyl group to DA to form noradrenaline. There are several mechanisms of transmitter inactivation after release. The major mechanism is reuptake back into the nerve terminal via DA transporters present in the membrane. Released DA also undergoes enzymatic breakdown by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT).

2.2 Dopamine receptors

There are five subtypes of DA receptors. D₁ and D₅ are stimulatory and are known as D₁-like. They are similar to each other in that both stimulate the production of cyclic adenosine monophosphate (cAMP), whereas D₂, D₃ and D₄ represent a separate group
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called $D_2$-like and inhibit cAMP production (Kebabian et al., 1979). All DA receptors are metabotropic meaning they are G-protein-linked. $D_1$ and $D_2$ were the first to be discovered and are the most common types found in brain areas such as striatum (STR) and the nucleus accumbens which are the major termination sites of the nigrostriatal and mesolimbic DA pathways. Although DA receptors are widely distributed in the brain, different areas have different receptor type densities, reflecting different functional roles. $D_1$ are excitatory postsynaptic receptors whereas $D_2$ are inhibitory autoreceptors and are coupled negatively to adenylate cyclase (AC). These $D_2$ autoreceptors are normally found presynaptically except in case of pituitary gland, one of the functions of which is to secrete the hormone prolactin. Here, $D_2$-receptors are found postsynaptically and their function is to inhibit prolactin release by the pituitary. DA receptors are not only expressed in the CNS. For example DA receptor subtypes $D_1$, $D_2$, $D_4$ and $D_5$ have been characterized in the human pulmonary artery where they are thought to mediate vascular tone (Ricci et al., 2006). In rats, DA receptors were found in blood vessels of most major organs and more specifically dopamine $D_4$ receptors were shown to be present in the atria of both rat and human hearts. DA has positive inotropic but not chronotropic effects on the heart and increases cardiac output (Hussain et al., 2003; Ricci et al., 1998). All types of DA receptors are also found in the kidneys. DA acting via the receptors found in renal vasculature and the renal parenchyma is able to modulate kidney function (Aperia, 2000).
2.3 Functional anatomy

The basal ganglia form a complex network of parallel loops that integrate cerebral regions, basal ganglia nuclei and the thalamus (Alexander et al., 1986). The motor circuit is that which is mostly related to the pathophysiology of movement disorders. Cortical motor areas project to the STR where they establish excitatory, glutamatergic synaptic connections with medium spiny neurons containing \( \gamma \)-aminobutyric acid (GABA). These neurons give rise to two pathways that connect the STR to the output nuclei of the basal ganglia, namely the globus pallidus internus (GPi) and the substantia nigra pars reticulata (SNr). Neurons of the direct pathway, project directly from the striatum to the GPi/SNr. These neurons have \( D_1 \) receptors, co-express peptides such as substance P and dynorphin and have a direct inhibitory effect on GPi/SNr neurons. Striatal neurons in the indirect pathway connect the STR with the GPi/SNr through synaptic connections in the globus pallidus externus (GPe) and the subthalamic nucleus (STN). These contain \( D_2 \) receptors and the peptide enkephalin. Projections from STR to GPe and from the GPe to STN are GABAergic and inhibitory. Neurons which originate in the STN on the other hand use glutamate as a neurotransmitter and activate neurons in the GPi/SNr. Stimulation of these neurons in the indirect pathway leads to inhibition of the GPe, disinhibition of the STN and excitation of the GPi/SNr. Therefore, the output activity of the basal ganglia is influenced by the opposing effects of inhibitory inputs from the direct and the excitatory effect of the indirect pathways (Figure 1.1). This in turn provides an inhibitory effect on the brainstem and the thalamic output to the cortex which are involved in motor activities. Experiments in primates demonstrate that facilitation of movement is associated with pauses in activity of GPi/SNr neurons.
and that activation of neurons of the direct and indirect pathways facilitates and suppresses motor activity, respectively (Chevalier et al., 1990). Thus the direct and indirect pathways have opposite effects on the output functions of the basal ganglia (Alexander et al., 1990). It is thought that DA modulates glutamate activity on corticostriatal inputs by exerting a dual effect on striatal neurons i.e. exciting D₁ receptor-expressing neurons in the direct pathway and inhibiting D₂ receptor-expressing neurons in the indirect pathway (Cepeda et al., 1993).

2.4 The parkinsonian state

Symptoms of PD result from a progressive loss of dopaminergic neurons in the substantia nigra (SN) in the brain. Pigmentation is lost in the pars compacta (SNC) region of the SN due to the loss of neuromelanin containing DA cells. Neuromelanin is thought to be a by product of monoamine degradation. The neurons from the SNC project to the STR and their loss leads to alterations in the neural circuits in the basal ganglia which are responsible for movement. According to the classic model of PD (Albin et al., 1989), the GPi has an increased inhibitory drive to the thalamus that reduces the excitatory thalamic drive to the cerebral cortex. This has been confirmed by various brain imaging techniques. The model predicts that reduced activation of DA receptors in the striatum caused by DA deficiency results in reduced inhibition of neurons in the indirect pathway and reduced excitation of neurons in the direct pathway. The STR modulates voluntary movement by these two pathways. Reduced inhibition of the indirect pathway leads to overinhibition of the GPe, disinhibition of the STN and increased excitation of the GPi/SNr neurons, whereas decreased activation
of the direct pathway causes a reduction in its inhibitory influence in the GPi/SNr. The net result of these series of excitations and inhibitions is excessive activation of basal ganglia output neurons accompanied by excessive inhibition of motor systems leading to the parkinsonian state (Figure 1.1).
Figure 1.1: Simplified version of the neural circuitry in the basal ganglia. Normal state compared to reduced striatal DA innervation like seen in Parkinson’s disease. Loss of DA leads to alterations in the neural circuits, ultimately leading to excessive inhibition of the thalamus and therefore reduced excitation of the motor cortex. Drawn based on text from (Obeso et al., 2000).
Section 3: Causative factors

3.1 Genetic

PD has been thought of as a prototypic non-genetic disorder for a long time. A major step in understanding the underlying mechanisms of the pathology involved in PD came from identification of inherited forms of the condition that are clinically identical to the idiopathic forms. The identification of the α-synuclein gene as the first gene involved in familial PD revealed a close relationship between inherited and idiopathic forms of the disease and indeed α-synuclein turned out to be the major component of Lewy bodies not only in rare familial forms but also in the brains of idiopathic cases without any family history of the disease (Duda et al., 2002). Lewy bodies are accumulations of α-synuclein proteins which have a central hydrophobic region therefore the protein has a high tendency to aggregate and initially forms an intermediate ring structure called an oligomer and ultimately these come together to form an insoluble polymer or fibril (Giasson et al., 2001). Alpha-synuclein is abundantly expressed as a cytosolic and lipid-binding protein in the vertebrate nervous system and in its normal state is unfolded. In presynaptic nerve terminals monomeric α-synuclein exists in equilibrium between free and plasma membrane bound states. The physiological functions of this protein are still unclear but it is thought to be involved in synaptic vesicle recycling, storage and regulation of neurotransmitters (Vekrellis et al., 2004). However some of the genetically caused forms of PD do not comply with the histopathological criteria for idiopathic cases since in some patients with parkin gene
mutations there appear to be no Lewy bodies (Hayashi et al., 2000; Zimprich et al., 2004). Parkin's protein product is a ligase enzyme responsible for adding ubiquitin chains to misfolded proteins marking them for destruction by the proteosome (Mata et al., 2004). Several other genes and their protein products have been identified which are thought to be involved in the molecular pathology of PD. Mutations have been identified in a gene called DJ-1 (Bonifati et al., 2003). It encodes a ubiquitous, highly conserved protein which is thought to be involved in chaperone function and cell redox potentials. It appears that overexpression of this gene protects cells against the effect of mitochondrial complex I inhibitors and oxidative stress caused by hydrogen peroxide and thus it is likely that mutations in it may lead to a higher risk of PD. Another important gene linked to familial PD is dardarin or leucine rich repeat kinase 2 (LRRK2). This large multidomain protein is a complex kinase and pathogenic mutations can occur in several of the protein domains. Cell biological experiments have shown that one of the most common mutations linked to PD is in the kinase domain and leads to toxicity by a simple gain of kinase function (Greggio et al., 2006).

3.2 Environmental

The earliest hypothesis on the pathogenesis of PD was based upon a fact that mitochondrial complex I inhibitors such as 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) and rotenone were able to induce parkinsonian symptoms leading to a selective loss of dopaminergic neurons in mouse (Seniuk et al., 1990) and primate models (Forno et al., 1986) of the disease. The toxic effect of the former compound was first inadvertently demonstrated in humans when a group of heroin
addicts self administered incorrectly synthesised heroin which in fact was MPTP leading to them developing symptoms of PD overnight (Langston et al., 1983). The early animal models did not fully mimic the condition because there were no Lewy bodies; however some of the recent ones which used continuous infusion of rotenone and MPTP are a better representation of the pathology. This suggests that sporadic PD might be caused by environmental toxins acting on mitochondrial complex I and inhibiting it. Inhibiting complex I has two major consequences, firstly ATP depletion and therefore impairment of all energy dependent processes and secondly generation of free radicals. This has been shown by analyzing post mortem PD brains which show increased levels of lipid peroxidation markers and protein nitration in the SN suggesting increased levels of oxidative stress (Andersen, 2004).

3.3 Inflammatory

In addition to these genetic and environmental factors, the idea that PD might be caused by neuroinflammation had recently received much attention. The evidence was first presented by McGeer and his group in 1988 (McGeer et al., 1988) where they showed that major histocompatibility complex proteins were upregulated in postmortem brains of PD patients. It was later reported that a wide array of proinflammatory cytokines were released by activated microglial cells in the brains of PD sufferers, including, tumor necrosis factor-alpha, interleukin-1, interleukin-6, epidermal growth factor and transforming growth factor-alpha (Mogi et al., 1994; Muller et al., 1998). Upregulation of inducible nitric oxide synthase and cyclooxygenase-2 were also reported (Przedborski et al., 1996; Rockwell et al., 2000). The inflammatory response is further
amplified and sustained by factors released from dying dopaminergic cells leading to a lethal cascade and irreversible destruction of the nigrostriatal pathway (Orr et al., 2002). Although commonly inflammatory reactions in the CNS are considered detrimental, there is evidence to suggest that they can be beneficial and even neuroprotective (Hohlfeld et al., 2006). For example it was demonstrated that T cells and other cells of the immune system are capable of producing neurotrophic factors e.g. BDNF which promotes neuronal growth and survival (Kerschensteiner et al., 2003). It is now becoming increasingly evident that immune cells can release both neurodestructive and neuroprotective molecules during CNS inflammation and it is the balance between the two that ultimately determines the net outcome of a neuroimmune interaction (Kerschensteiner et al., 2009)
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Section 4: Current treatments

4.1 Pharmacological

4.1.1 L-DOPA

As mentioned previously, the symptoms of PD only appear when around 80% of dopaminergic neurons are lost; therefore, by the time the pharmacological treatment is started, it is purely symptomatic and directed at minimizing the motor impairments associated with the condition. L-DOPA is the amine precursor of DA which is administered orally and is absorbed from the small intestine. It remains the most common pharmacological treatment. L-DOPA in the periphery can be metabolized to DA and not reach its desired destination, therefore it must be administered with peripherally active DOPA decarboxylase (DDC) inhibitors e.g. carbidopa or benserazide. L-DOPA is also partly metabolized by COMT and an enzyme antagonist such as entacapone may also be given to maximize L-DOPA delivery to the target site. The peripheral DDC inhibitors are designed not to cross the blood brain barrier (BBB) therefore when L-DOPA reaches the brain it is only then converted to DA. Although L-DOPA treatment seems like the most sensible option there are numerous complications. Potential adverse effects associated with L-DOPA treatment include nausea and vomiting, hypotension, arrhythmias, extreme emotional states and psychotic episodes. Long term problems include dyskinesias and motor fluctuations which appear after 2-5 years of treatment in about 30-50% of patients and in alarming 80-100% of patients.
after 10 years of treatment (Uitti et al., 1996). Other risks associated with L-DOPA treatment that have been reported include an increased incidence of malignant melanoma (Fiala et al., 2003) and higher incidence of stroke and coronary heart disease due to elevated levels of homocysteine (Postuma et al., 2004).

4.1.2 Dopamine agonists

Another strategy in PD treatment is to stimulate DA receptors in the STR directly. Several DA agonists are available clinically and include bromocriptine, pergolide, pramipexole and ropinirole. The advantage of these drugs is that they are not affected by pharmacokinetic shortcomings seen with L-DOPA. There is no competition with dietary neutral amino acids for absorption in the gut and no competition for penetration of the BBB. DA agonists have substantially longer elimination half-lives than L-DOPA, which allows for more prolonged stimulation of receptors. Since these drugs are specific at targeting DA receptors it was hoped they would provide more selective therapeutic benefits and reduce some of the unwanted effects seen in long term L-DOPA therapy. In fact they were shown to produce more behavioural complications including psychosis and autonomic side effects like orthostatic hypotension as well as more frequent gait disorders such as freezing episodes. But they still do provide a better therapeutic window and a reduced profile of diskynesias compared to L-DOPA therapy (Factor, 1999; Watts, 1997). Other safety issues concerning the use of DA agonists include excessive daytime somnolence and sleep attacks (Factor et al., 1990), pathological gambling and related impulse control disorders where there is an inability
to resist an impulse desire despite negative consequences. These include compulsive shopping, compulsive eating and hypersexuality (Galpern et al., 2007).

4.1.3 Anticholinergic drugs

Muscarinic receptor antagonists were the first drugs introduced for the treatment of PD (Lang et al., 2002) and are still in the clinic especially in the early stages of the condition. The pharmacological rationale for this approach was the demonstration of functional antagonism between the DA and acetylcholine (ACh) systems in the STR (Duvoisin, 1967). Indeed, D₂ receptor activation reduces the release of ACh. In PD, DA depletion is accompanied by an increased activity of the striatal cholinergic system, subsequent rearrangement of the striatal circuitry and appearance of the motor symptoms. Recent evidence suggests that loss of dopaminergic afferents does not exert its effect on cholinergic signalling directly by diminished D₂ receptor activity. Rather, it has been proposed that DA depletion triggers a reduction in the efficacy of the M₄ autoreceptors and these are responsible for increases in ACh signaling (Ding et al., 2006). Hence anticholinergic agents were intended to correct this imbalance, and although were shown to be beneficial, were also associated with various neuropsychiatric side effects which often led to patient withdrawal which in turn led to worsening of the symptoms (Katzenschlager et al., 2003).
4.1.4 Catechol-O-methyltransferase (COMT) inhibitors

COMT is one of two enzymes that are responsible for DA metabolism. It is an intracellular enzyme located in postsynaptic neurons. Any compound having a catechol structure is a substrate, including L-DOPA. COMT inhibitors act peripherally to inhibit metabolism of L-DOPA to 3-O-methyl-DOPA (Jorga et al., 1997). The strategy here is to increase the half-life and bioavailability of L-DOPA increasing the duration of its effect. There are two drugs in this class which have been approved for PD treatment. Tolcapone was introduced in 1997, followed by entacapone in 1999. Both drugs increase the bioavailability of L-DOPA but also have a number of adverse effects. The major side effects of tolcapone include diarrhoea, which was the most frequent reason given for patient withdrawal from long-term trials. The other event that occurs between 6 to 12 weeks after the start of tolcapone is the elevation of alanine aminotransferase and aspartate aminotransferase, with three times the normal elevation. Although enzyme levels return to normal in two to four weeks, three deaths from acute hepatic failure have been reported in association with the use of tolcapone. After that incident the use of tolcapone became limited (Olanow, 2000). Prescribing requirements include liver-function testing before starting the drug and monitoring every two weeks for one year and every four weeks for six months and every two months for the lifetime of the patient (Lew et al., 2007). Entacapone has been documented to cause hypotension, benign urine discoloration, constipation and diarrhoea but no liver toxicity has been reported (Rinne et al., 1998).
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4.1.5 Monoamine oxidase (MAO) inhibitors

MAO is an intracellular enzyme located in the outer mitochondrial membrane which plays a major role in oxidative deamination of monoamines such as DA. There are two isoforms of the enzyme, MAO-A and MAO-B. The B-isoform is primarily present in the brain and is found in astrocytes but not neuronal cells (Westlund et al., 1985). Inhibition of the enzyme would be expected to increase amounts of both intracellular and extracellular DA, and therefore serve as another pharmacotherapeutic strategy in the treatment of PD. The most well known MAO inhibitor used in PD is selegiline which was introduced in 1989 followed by one new agent more recently, rasagiline, a more B-type selective compound and also a new formulation of the oral form of selegiline branded Zydis (Clarke et al., 2003). One of the main problems with this class of agents is the so called “cheese reaction”. Foods rich in a tyramine such as chocolate, red wine and most notably cheese may cause episodes of hypertensive crises. This is because tyramine is similar in structure to noradrenaline and if ingested in high amounts with the impaired function of MAO, causes displacement of noradrenaline from synaptic vesicles leading to increased heart rate and vasoconstriction. This problem was partly solved with the introduction of selective type-B agents. The other potential dangerous interaction may arise if a non-selective MAO drug is used in conjunction with a tricyclic antidepressant or a selective serotonin re-uptake inhibitor. This will lead to elevated 5-hydroxytryptamine levels and may lead to the so called “serotonin syndrome”. Clinical features include motor symptoms, including movement disorders, myoclonus and tremor, muscle rigidity, hyperreflexia; mental status changes, including agitation, confusion, disorientation and restlessness; and autonomic
instability, including low-grade fever, nausea, diarrhoea, headache, shivering, flushing, diaphoresis, tachycardia, tachypnea, blood pressure changes, and pupil dilation (Bodner et al., 1995).

4.2 Surgical

When pharmacological treatment is no longer enough to control the symptoms, surgical intervention may help certain patients. Nowadays the most common surgical procedure performed is deep brain stimulation (DBS) involving implantation of electrodes and selective stimulation of certain brain areas. DBS has progressively replaced brain lesioning such as thalamotomies and pallidotomies over the past twenty years. The first target in the modern era of DBS was the ventral intermediate thalamus (VIT) (Benabid et al., 1987). VIT stimulation provided a positive effect on tremor but no improvement in rigidity, gait or balance. Limited effectiveness of VIT simulation led to application of this procedure to other brain areas in a search for a better target. Following an observation that MPTP induced lesion of the STN appeared to improve the symptoms of PD (Bergman et al., 1990), the STN has progressively become the most popular target for DBS. Although stimulation of the STN appears to have a positive effect on all symptoms of PD, it does come at a price. As expected for a procedure of this level of complexity the disadvantages are numerous. The first and most obvious one is the cost. Secondly there are many potential complications during surgery. The main risks associated with the procedure itself are intracranial bleeding and infection (Wider et al., 2008). In rare cases seizures are also observed. Hardware problems such as lead fracture and battery failure have also been reported (Blomstedt et
al., 2005). In addition there are also target specific side effects which for the STN may include motor speech disturbances, neuropsychiatric problems such as mood changes, confusion and apathy, eyelid opening apraxia and weight gain (Limousin et al., 2008).

4.3 Experimental non-DA strategies

Many different classes of drugs were tested in the clinic after promising animal model studies. These include serotonin receptor antagonists after observations that serotonergic system was overactive in PD (Fox et al., 2000); N-methyl-D-aspartate receptor antagonists and GABA_A agonists based on the fact that there is an imbalance of the glutamate/GABA system in the basal ganglia; adenosine A_2a receptor antagonists because they were shown to influence both the striatal cholinergic and GABA systems; α_2-adrenergic antagonists based on beneficial effects in animals models; opioid antagonists, after observations that opioid peptide transmission was upregulated in PD sufferers (Piccini et al., 1997) and finally cannabinoid receptor antagonists because these are expressed on GABAergic neurones and inhibit the release of GABA. However, none were proven truly beneficial. Some were associated with severe and unpredictable side effect profiles, while others gave very limited efficacy (Linazasoro, 2004).
4.4 Regenerative therapies

4.4.1 Glial cell derived neurotrophic factor (GDNF)

Neurotrophic factors are essential for neuronal survival and differentiation during development and for maintenance of normal function during adulthood. GDNF is one of the most potent neurotrophic factors for dopaminergic neurons as demonstrated in animal models (Goke et al., 1993). GDNF delivery is however a major problem due to its inability to cross the BBB. Intracerebroventricular (ICV) delivery has been explored as the method for delivering GDNF into the brain, but poor diffusion from the site of injection into the brain parenchyma and the occurrence of serious side-effects such as nausea, loss of appetite, cutaneous sensory disturbances and psychiatric symptoms limit this delivery approach in PD patients (Kordower et al., 1999; Nutt et al., 2003). Both animal and human studies demonstrate that direct intrastriatal administration reduces the occurrence of unwanted effects seen with the ICV method, although the degree of symptomatic relief in clinical trials varies from major improvements to no significant differences. Likely that for a noticeable clinical benefit, GDNF probably has to be delivered continuously for several years (Yasuhara et al., 2007). Direct localised delivery can be performed using several approaches such as a microinfusion pump, cell-based or direct gene delivery. Though, efficient CNS delivery using a pump will require implantation of several catheters on each side of the brain in addition to an infusion pump implanted in the abdominal cavity, making this procedure cumbersome and prone to complications (Zurn et al., 2001). Direct GDNF gene delivery to the target
site leads to efficient local synthesis, but does not offer possibility to completely turn off protein synthesis in case of adverse effects. The same problem limits the use of neural progenitor cells to deliver GDNF to the target site. In addition, the ability of these cells to migrate poses a risk of further complications (Yasuha et al., 2007).

4.4.2 Cell transplantation

The idea of developing transplant cell therapies for PD is based firstly on assumptions that it is the nigrostriatal dopaminergic neuronal pathway that degenerates, and that if new neurones are somehow introduced into the SNC they would re-grow and re-innervate the denervated STR. Extensive animal studies have demonstrated that indeed transplanted neurons are able to survive and re-establish striatal connections and restore baseline DA synthesis and release (Barker et al., 1994). Very promising results were demonstrated in 6-hydroxydopamine (6-OHDA) treated rats where there were improvements with both the motor problems and L-DOPA induced dyskinesias (Lee et al., 2000). As for human studies, initially a series of small open-label ones were conducted to assess the therapeutic potential of this strategy. Foetal dopaminergic neurones were used and showed remarkable long-lasting benefits in many PD patients. However, following these results, two double-blind sham surgery controlled clinical studies failed to reproduce the previously obtained data and there was only limited efficacy. In addition a significant number of patients developed severe off-state dyskinesias after the procedure (Freed et al., 2001; Olanow et al., 2003).
Animal models are important tools in experimental medical science to better understand the pathogenesis of human diseases. Once developed, these models can be exploited to test therapeutic approaches for treating functional disturbances observed in the disease of interest. On the basis of experimental and clinical findings, PD was the first neurological disease to be modelled and subsequently treated with neurotransmitter replacement therapies. Agents that cause selective damage to catecholaminergic neurones have been used to mimic the condition in experimental animals. There are several models of PD that are used to screen potential new therapeutics. For example the reserpine model. It was observed that systemic administration of this compound to rabbits caused akinesia by depleting the levels of catecholamines in the brain; furthermore it was shown that levodopa alleviated these symptoms suggesting that behavioral recovery depended on DA (Carlsson et al., 1957; Hornykiewicz, 1963). The main problem with this model is that changes in catecholamine levels are temporary and reserpine does not cause any morphological changes in the nigrostriatal tract. As mentioned previously, the effect of MPTP was first inadvertently demonstrated in humans when a group of heroin addicts consumed incorrectly synthesized heroin which was in fact MPTP and developed clinical symptoms similar to sporadic PD overnight (Langston et al., 1983). Researchers went on to develop an animal model. After administration MPTP crosses the blood brain barrier and is converted by astrocytes to
its active metabolite MPP\(^+\) which is taken up selectively by dopaminergic neurons via its affinity for the DA transporter. MPP\(^+\) toxicity is believed to be due to inhibition of mitochondrial complex I and thus resulting in oxidative stress (Nicklas et al., 1985).

The problem with MPTP is that it does not produce consistent lesions in all animal species and is more commonly used in mice and primate but not rats. For this project 6-hydroxydopamine (6-OHDA) and lipopolysaccharide (LPS) models were chosen and are described in more detail in the next subsection.

5.2 6-hydroxydopamine

6-OHDA was the first chemical agent discovered that had specific neurotoxic effects on catecholaminergic pathways (Sachs et al., 1975). The compound uses the same DA transport system as DA and noradrenaline and therefore produces specific neurodegeneration. Systemically administered 6-OHDA cannot cross the BBB therefore it has to be delivered stereotactically directly into the SN, medial forebrain bundle (MFB) or the STR (Perese et al., 1989). Neurons start degenerating within 24 hours and striatal DA becomes depleted in 2-3 days. Normally 6-OHDA is injected into one hemisphere to produce a unilateral lesion, firstly because bilaterally lesioned animals are difficult to maintain and secondly because the intact side serves as an internal control. Unilateral lesions also lead to asymmetric circling behaviour when tested with a DA agonist such as apomorphine. The number of turns that the animal performs indicates the severity of the lesion and can be easily quantified (Ungerstedt, 1968). Although 6-OHDA produces lesions in many different species of animals, the most commonly used are rats due to well established stereotaxic techniques and also
low maintenance costs. The mechanism of 6-OHDA toxicity is thought to be both an increase in oxidative stress during its metabolism yielding an increase in reactive oxygen species (ROS) production and also its ability to inhibit mitochondrial complex I (Perumal et al., 1992; Sachs et al., 1975). The acute nature of this experimental model differs from the progressive degeneration of neurons in the actual PD and it does not accurately mimic all the clinical and pathological feature characteristic of the condition. One such example would be the absence of cytoplasmic inclusions like those observed in human patients, but despite these limitations it is widely used to test the effects of experimental compounds, evaluate the efficacy of cell transplantation and various neurotrophic factors that promote degenerating nigral neuron survival (Barker et al., 1994).

5.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is a component of gram-negative bacterial cell wall and is recognised as a pathogen by cells of the immune system including microglia (Gehrmann et al., 1995). It is now well established as an effective initiator of dopaminergic neuronal loss and PD symptoms in experimental animal models (Castano et al., 1998). The toxin works by binding to a cell membrane component known as a toll-like receptor 4 on microglial cells triggering an inflammatory response (Liu et al., 2000). Interestingly, this microglia-mediated neuronal cell loss is only observed when LPS is injected into the SN and not for example hippocampus or cortex. This is thought to be due to abundant presence of these cells in the SN and not other regions (Kim et al., 2000). What is also interesting is that in the single injection LPS model of PD
where there is between 50-80% loss of striatal dopaminergic innervation after one week of treatment, serotonin and GABA levels remained unchanged (Herrera et al., 2000).

Section 6: Glucagon family and exenatide

6.1 Introduction to the glucagon family of peptides

Processing of the proglucagon gene gives rise to 29 amino acid glucagon itself and a number of biologically active peptides including glicentin, oxyntomodulin (OXM), glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2). Both OXM and glicentin contain the whole 29 amino acid sequence of glucagon and a C-terminal 8-amino acid extension called intervening peptide-1 (IP-1). Compared to OXM (37 amino acids), glicentin (69 amino acids) also contains the N-terminal extension called glicentin-related pancreatic polypeptide (GRPP). OXM has recently been found to suppress appetite and a recent clinical study found that it could be used as a treatment for obesity (Wynne et al., 2006b). The mechanism of action of OXM is poorly understood. It has been shown to bind to both the glucagon-like peptide 1 receptor (GLP-1R) and the glucagon receptor, but it is likely that its effects are mediated by a novel receptor (Wynne et al., 2006a). Effects of glicentin are even less well understood, but it is thought to be implicated in the growth of intestinal mucosa by mechanisms involving the GLP-1R (Ayachi et al., 2005). On the other hand, a lot more is known about functions of GLP-1 and its mechanisms of action. GLP-1 functions are discussed further in this section and summarized in figure 1.3.
Figure 1.2: Structure of proglucagon gene fragment contains sequences coding for several biologically active peptides: Glicentin related pancreatic polypeptide (GRPP), glucagon, intervening peptide 1 (IP1), glucagon-like peptide 1 (GLP-1), intervening peptide 2 (IP2) and glucagon-like peptide 2 (GLP-2), Figure also summarizes the known functions of GLP-1, adapted from (Drucker, 2001).

6.2 Exenatide

Exendin-4 (EX-4) which is a naturally occurring form of exenatide was originally isolated from salivary secretions of the lizard Heloderma suspectum (Eng et al., 1992) also known as the Gila monster. In the lizard, EX-4 circulates during ingestion of its prey. Since the lizard only feeds about four times a year, it uses EX-4 to “switch on” its pancreas to start producing insulin after a meal. EX-4 has 53% homology to mammalian GLP-1 and exerts its effect by activating GLP-1Rs. As previously mentioned, in mammals GLP-1 is processed from a proglucagon gene found in L-cells
in the small intestine, whereas EX-4 is transcribed from a distinct gene only expressed in the salivary glands of the lizard (Chen et al., 1997). In mammals, GLP-1 is degraded by dipeptidyl-peptidase-IV and its plasma half life is around 1.5 minutes, but EX-4 appears to show resistance to degradation by the same enzyme and has a much longer plasma half life. Some of the physiological effects of EX-4 are not only glucose-dependent enhancement of insulin secretion but also inhibition of glucagon secretion and reduction of food intake by slowing gastric emptying (Kolterman et al., 2003) which is normally accelerated in people with type 2 diabetes. EX-4 was approved by the FDA in 2005 as adjunctive therapy for patients with type 2 diabetes who have not achieved optimal glycaemic control on metformin or sulfonylurea. What appears to be most fascinating is that EX-4 has been shown to increase β-islet mass by promoting its proliferation and neogenesis from precursor cells in both \textit{in vitro} and \textit{in vivo} models (Tourrel et al., 2002). EX-4 appears to also exert its effects in the CNS. There is evidence suggesting that GLP-1 present in the bloodstream can cross the BBB and enter the brain (Orskov et al., 1996). Although it is a large molecule, it exhibits a high degree of hydrogen bonding and is also lipophilic (Kastin et al., 2003).

\textit{6.3 Glucagon-like peptide 1 receptor}

The GLP-1R is a classic seven transmembrane domain G-protein coupled receptor. It was first cloned and characterized from the rat pancreatic islets and later from human pancreatic insulinoma and gut tumor cell lines (Dillon et al., 1993; Thorens, 1992). The rat and the human GLP-1Rs show 95% amino acid homology and are 90% identical,
differing at 42 amino acid positions (Tibaduiza et al., 2001). Human GLP-1R is a 64-kDa protein and its gene is located on the long arm of chromosome 6p21 (Stoffel et al., 1993). All members of the glucagon family of receptors are positively coupled to AC through G\(_\alpha\) subunit with subsequent production of cAMP, although they are also capable of signalling through other transduction pathways (see next section). The receptor is not only found in the pancreas but also in several places in the CNS in both the human and the rat brain (Satoh et al., 2000). Initial findings demonstrated their presence in the hypothalamus suggesting a role in regulation of food intake, although receptor expression was later demonstrated in several other brain regions such as thalamus, brain stem, lateral septum, the subfornical organ and the area postrema. In addition specific neuronal binding sites have been identified in the striatum, hippocampus and cerebral cortex although at lower densities (Calvo et al., 1995; Goke et al., 1995). GLP-1 is also produced by neuronal cells in discrete regions of the CNS (Jin et al., 1988) and circulating GLP-1 was found to readily enter the brain (Orskov et al., 1996).

### 6.4 Signal transduction

As mentioned previously, GLP-1Rs are metabotropic G-protein coupled receptors. Agonist engagement at the extracellular site activates AC through an interaction of the G\(_\alpha\) subunit with the membrane bound enzyme. Activation of AC gives rise to increased production of cAMP (Drucker et al., 1987) which then activates a signalling cascade. The two most characterized pathways are the cAMP-dependent protein kinase A (PKA) pathway and a PKA independent – exchange proteins directly activated by
cAMP (Epac) pathway. PKA is a ubiquitous serine/threonine phosphorylating enzyme (Kolterman et al., 2003). In its inactive form it is composed of one regulatory subunit bound to two catalytic subunits. PKA mediated activation of cAMP response element binding protein (CREB) was shown to be involved in the protective effects associated with GLP-1R activation in β-cells (Sarkar et al., 2007). The PKA independent portion of the signalling cascade forms a part of a large family of related nonkinase effectors which have been shown to activate the Ras superfamily of effector proteins. There are two variants of this pathway known as Epac 1 and Epac 2 and both exhibit high selectivity for activation by cAMP over other cyclic nucleotides. Both have been found in rat islets and also in β-cell lines. Although these can be distinguished as two separate pathways, their precise roles and importance are still elusive (Leech et al., 2000; Ozaki et al., 2000). The neuroprotective mechanism involving GLP-1R is thought to be cAMP dependent, but pancreatic receptors are also known to utilize other signalling cascades (summarized in figure 1.4). The activation of important transcription factor known as pancreatic-duodenal homeobox (PDX-1) that was shown to be involved in β-cell survival, is stimulated via PKA which induces its translocation to the nucleus (Stoffers et al., 2000). A G_q subunit was shown to activate phospholipase C (PLC) pathway, leading to protein kinase C (PKC) activity and an increase in intracellular Ca^{2+} (Wan et al., 2004). PKC is thought to activate nuclear factor kappa B (NF-κB) which appears to be anti-apoptotic (Wang et al., 1996). The phosphoinositide-3-kinase (PI3K) pathway activated by the G_{pγ} subunit activates another important pathway implicated in β-cell growth and survival (Buteau et al., 2003).
Figure 1.3: Diagram to demonstrate possible events downstream of GLP-1R activation. GLP-1Rs are capable of signaling through several signal transduction pathways including PLC/NF-κB, MAPK/ERK, PI3 kinase, cAMP/CREB pathways. *PDX-1 transcription factor is specific to β-cells as well as Ca²⁺ induced insulin vesicular fusion.
6.5 Rationale for using exendin-4 in PD

6.5.1 Control of β-cell proliferation

Initially, positive effects on β-cell proliferation following GLP-1R activation were demonstrated *in vitro* (Buteau et al., 2001) and *in vivo* in partial pancreatectomy model of type 2 diabetes (Stoffers et al., 2000). It was later shown that growth promoting effect of GLP-1R in β-cells was dependent on transactivation of epidermal-growth factor-receptor leading to PI3K expression and ultimately activating its downstream effectors (figure 1.4). The more long term effect of GLP-1 on β-cell proliferation is thought to be associated with upregulated expression of insulin receptor substrate 2 (Irs2) gene. Irs2 prevents diabetes in mice via promoting β-cell replication, survival and function especially during metabolic stress. In these experiments, EX-4 was shown to promote β-cell function, but not in Irs2 knockout mice, although it was able to stimulate the remaining β-cells to secrete insulin and thereby delay the onset of diabetes (Park et al., 2006). GLP-1R stimulation has also been shown to inhibit the expression of a forkhead transcription factor (FoxO1) through phosphorylation-mediated nuclear exclusion and exendin-4 had no effect on β-cell proliferation in transgenic mice constitutively expressing nuclear localization of FoxO1 (Buteau et al., 2006). In fact it was shown that nuclear localization of PDX-1 and FoxO1 are mutually exclusive (Kitamura et al., 2002) thus demonstrating another mechanism by which GLP-1R stimulation may lead to PDX-1 upregulation and ultimately β-cell proliferation. Mice with specific ablation of PDX-1 expression fail to respond to EX-4 treatment, and there
is no positive trophic effect on β-cells (Li et al., 2005). The actions of GLP-1 on cell proliferation are complemented by also promoting cell survival. Treatment with EX-4 has been shown to protect β-cells against apoptosis and consequently preserve cell mass (Wang et al., 2002). β-cell survival was accompanied by a reduction in caspase-3 expression (Farilla et al., 2002) and upregulated expression of an anti-apoptotic B-cell leukemia protein (Bcl2) through NF-κB activation (Li et al., 2003).

6.5.2 Cardio-protective effects

Beneficial effects of GLP-1 on the myocardial function are becoming increasingly evident. Both in vitro and in vivo models of myocardial ischaemia demonstrate that infusion of GLP-1 reduces infarct size (Bose et al., 2005). GLP-1 improves myocardial contractility and the uptake of glucose in normal and postischaemic rat hearts (Zhao et al., 2006). In dogs with pacing-induced dilated cardiomyopathy, GLP-1 infusion was able to improve left ventricular performance, stroke volume and cardiac output (Nikolaidis et al., 2004a). It was demonstrated that a 72 hour infusion of GLP-1 improved left ventricular ejection volume in patients after myocardial infarction (Nikolaidis et al., 2004b) therefore in humans with type 2 diabetes and congestive heart failure, GLP-1 may be of dual benefit. More recently, protective effects of EX-4 were demonstrated against ischaemia reperfusion injury in the rat heart where EX-4 reduced infarct size by more than 50% (Sonne et al., 2008).
6.5.3 Neuroprotective effects

Intestinally derived peptides such as GLP-1 are classified as both hormones and growth factors since they can modulate various cellular processes such as cell division, growth and differentiation. The first evidence that EX-4 might have neuroprotective properties appeared in 2002. Both EX-4 and its endogenous peptide analogue GLP-1 were shown to be neuroprotective in a similar manner to nerve growth factor. It was demonstrated that activating GLP-1Rs in PC12 cell lines caused neurite outgrowths and the mechanism appeared to be cAMP dependent where signals which stimulated its production were shown to protect neurons against glutamate induced excitotoxicity (Perry et al., 2002b; Perry et al., 2002c). It was also later demonstrated that EX-4 completely protected cultured hippocampal neurones against glutamate induced excitotoxicity and preserved basal forebrain choline acetyl transferase immunoreactivity in an animal model of Alzheimer’s disease (Perry et al., 2002b). The effects were GLP-1R mediated as co-incubating cells with a specific antagonist blocked the protective effects. The same group later showed that EX-4 decreased the amount of endogenous amyloid beta peptide (Aβ) and protected cultured neurones against apoptosis induced by Aβ and iron (Perry et al., 2003). In the same year it was also demonstrated that EX-4 enhanced spatial learning in mice and protected hippocampal neurones against kainate induced apoptosis (During et al., 2003). Later that year another group showed that GLP-1R activation caused rapid TH transcription in brainstem catecholaminergic neurones and was involved in regulating sympathetic outflow leading to downstream activation of cardiovascular responses in vivo (Yamamoto et al., 2003; Yamamoto et al., 2002). More recently, while investigating
the possibility that GLP-1R stimulation may influence axonal structure and function, beneficial effects of EX-4 were demonstrated in an animal model of peripheral sensory neuropathy induced by pyridoxine (Perry et al., 2007). Very recent experiments by the same group show effectiveness of EX-4 in MPTP mice model of PD and offer further support to the idea that EX-4 works by activating GLP-1R by testing the drug in GLP-1R knock outs (Li et al., 2009). Finally, our own data demonstrate potent dose-dependent neuroprotective and neurorestorative action of EX-4 given peripherally a week after toxin administration in two distinct animal models of PD (Harkavyi et al., 2008).

6.5.4 Modulation of neurogenesis

The majority of neurones are created before birth. The first indication of neurogenesis was presented more than four decades ago but now there is accumulating evidence of ongoing processes that generate new functional neurones from neural stem cells in certain regions of both rodent and human brain. Neurogenesis in the mammalian brain occurs throughout life and has been clearly demonstrated at two locations under normal conditions: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus. Neurones born in the SVZ migrate great distances to the olfactory bulb where they become granule neurones and periglomerular neurones, whereas neurones born in the SGZ migrate into the granule cell layer to become dentate granule cells (Zhao et al., 2008). Not so long ago, several papers presented evidence of a slow rate of neuronal turnover in SN and the STR and it was demonstrated that D3 receptors were involved in modulation of neurogenesis and restoration of the
nigrostriatal pathway in the rat 6-OHDA model of PD (Borta et al., 2007; Van Kampen et al., 2006; Van Kampen et al., 2004). It was also demonstrated that the rate of this turnover appears to be upregulated during partial injury and that dopaminergic fibres originating in the SN project to the SVZ and when damaged, the rate of neurogenesis was reduced (Freundlieb et al., 2006; Zhao et al., 2003). Also, recently it was proposed that EX-4 may promote neurogenesis in the SVZ as measured by cellular bromodeoxyuridine (BrdU) uptake in rats. Moreover, the same group suggested that GLP-1Rs found in neurogenic regions of the brain such as the SVZ may be responsible for neurorestorative effects of EX-4 in vivo (Bertilsson et al., 2008). Although modulation of neurogenesis in the adult brain appears an attractive option, there are debates and controversies over the modulation of adult neurogenesis in neurological disorders especially with studies using BrdU labelling for studying it (Taupin, 2007). For example in brains of human AD patients, it was shown that DNA replication without cell replication precedes neuronal death (Yang et al., 2001) therefore some of the data observed using immunohistochemistry for cell cycle proteins and BrdU labelling in patients and animal models of AD may not demonstrate neurogenesis but rather cells which duplicated their DNA without completing the cell cycle (Yang et al., 2001). Evidence for neurogenesis in the SN is also controversial with some studies reporting increased levels in MPTP models (Zhao et al., 2003) while others when using 6-OHDA treated rats report no evidence of neurogenesis in SN (Frielingsdorf et al., 2004). Results obtained from human PD patients demonstrate reduced cell proliferation in SVZ and SGZ and changes in adult neurogenesis resulting from neurodegenerative disorders likely depend on selected neuronal populations affected and the role of neurogenesis needs further clarification (Zhao et al., 2008)
EX-4 was shown to be neuroprotective against glutamate induced excitotoxicity in vitro. Since then it was suggested that EX-4 may hold potential for treatment of neurodegenerative disorders such as Alzheimer’s disease and PD but its effectiveness in vivo was never assessed. The first and main objective of this project was to verify whether EX-4 was effective in two distinct in vivo models of PD.

The proposed mechanism of action of EX-4 in the pancreas is activation of GLP-1Rs on pancreatic beta islets which causes glucose dependent insulin secretion and also β-cell proliferation and neogenesis. The second objective of this project was to test if neuroprotective effects of EX-4 involve activation of the same receptor.

It was recently demonstrated that GLP-1Rs are expressed in SVZ and proposed that EX-4 might activate these receptors and control proliferation of neural stem cells. My third objective was to test if GLP-1Rs are in fact present in neurogenic regions of the adult rat brain and if so, test if EX-4 had an effect on SVZ neurogenesis.

Accumulating literature evidence suggests the involvement of D3-receptors in adult neurogenesis. D3-receptors were shown to be expressed in STR, SNr and the SVZ. In addition, D3-receptor agonists were shown to be neuroprotective in the 6-OHDA model of PD. Therefore my fourth and final objective was to test if D3-receptor activation is involved in the mechanism of action of EX-4.
Chapter 2

Materials and methods
2.1 Drugs and reagents

All drugs and reagents were purchased from Sigma-Aldrich Ltd., U.K. unless otherwise specified.

2.2 Animals

Male Wistar rats (250-300g) were purchased from Harlan, U.K. and housed under standard conditions in an animal care facility with controlled temperature, humidity and fixed light/dark cycles. Animals were group housed (n=4) and had free access to food and water. All experimental procedures were carried out in accordance with institutional and Home Office regulations.

2.2.1 Stereotaxic surgery

Rats were anaesthetised using 4% isoflurane (Abbott, UK) v/v in O₂ for induction and then secured on a stereotaxic frame (David Kopf, US). A maintenance dose of 1.5% isoflurane v/v in O₂ was used throughout the duration of the surgery. The scalp was exposed using a sterile surgical blade. This was performed by carefully applying pressure laterally, making an incision and removing excess membranous material and connective tissue so that the surface of the skull was fully accessible and clean. Bregma was then located and marked. This was the point of reference from which all the coordinates were calculated according to rat stereotaxic atlas (Paxinos et al., 1982)
Before any injections were made the skull was penetrated using a dental drill. Injection was made using a 10 µl Hamilton syringe. All injections were performed at an approximate rate of 1 µl per minute, and a 5 minute period was allowed for diffusion of the toxin. The syringe was then slowly retracted to prevent any backflow. The scalp was closed using Michele clips, and rats were returned to their cages and observed until conscious.

2.2.2 Intracerebral drug delivery

In the case of the 6-OHDA model, before the surgery was performed, the animals were injected with pargyline (50 mg/kg) and desipramine (25 mg/kg) intraperitoneally (i.p.). This was done to minimize the metabolism of 6-OHDA and to preserve the noradrenergic system. The animals were then anaesthetised and secured on the stereotaxic frame and injected with 6-OHDA hydrochloride (8µg/2µl) dissolved in saline containing 0.2% ascorbic acid or vehicle into the left medial forebrain bundle (MFB) (from bregma in mm; A -4.3, L 1.4 and V 8.2) according to the rat stereotaxic atlas (Paxinos et al., 1982). In the case of the LPS model, animals were injected with LPS (E.coli, stereotype 0111:B4) (2µg/2µl) or saline into the left SNc (from Bregma in mm; A -5.2, L 2.2 and V 8.3).
2.2.3 Drugs injected intraperitoneally

Animals co-treated with EX-4 received i.p. injections of the compound at 0.1 or 0.5μg/kg or saline twice a day, (10am and 5pm), for a period of one week, seven days after the administration of either of the toxins. EX-9-39 is a selective GLP-1R antagonist which crosses the BBB. Rats were co-treated with EX-9-39, received i.p. injections of the compound at double the dose of EX-4 at 1μg/kg or saline twice a day just before each EX-4 injection. Nafadotride is a selective dopamine D₃ antagonist. Animals co-treated with nafadotride received i.p. injections of the drug at 1mg/kg at which it was shown to selectively block D₃ or saline twice a day also just before each EX-4 injection. 5-bromo-2-deoxyuridine (BrdU) is a brominated thymidine analog which gets incorporated into DNA of actively dividing cells and is a gold standard for measuring neurogenesis. It was injected to investigate if treatment with EX-4 promoted neurogenesis and was measured in the subventricular zone which is one of the neurogenic regions of the brain and also the SN. Different treatments groups were compared to each other to check if EX-4 treatment affects neurogenesis. Animals co-treated with BrdU received i.p. injections of the compound at 50mg/kg or vehicle twice a day with each injection of EX-4 and/or other drugs.
2.3 Assessments

2.3.1 Apomorphine test

Fourteen days after surgery, animals were injected with 0.5mg/kg apomorphine s.c. in saline and after 15 minutes each rat was placed in a circular test arena. Two minutes later, after they became accustomed to the new surroundings, the numbers of turns were counted per 120 second period. The number of turns is indicative of the severity of the neurotoxic lesion.

2.3.2 In vivo microdialysis

Animals were anaesthetised with isoflurane (4% induction v/v in O₂ and 1.5% maintenance) and then secured on a stereotaxic frame. Two microdialysis probes, constructed as described before (Whitton et al., 1991) were inserted stereotaxically into the striatum on both sides of the brain (in mm from bregma A +0.2, L 3, V 8) and fixed with dental acrylic (DuraLay, Reliance, Dental MFG. CO) and anchor screws. After surgery, animals were placed in individual microdialysis cages and allowed to recover for 24 hours before dialysis. The rats were then perfused with artificial cerebrospinal fluid (aCSF) (2.5mM KCl, 125mM NaCl, 1.18mM MgCl₂·6H₂O, 1.26mM CaCl₂) pH 7.4 at a rate of 1μl/min using Harvard Apparatus model 22 syringe infusion pump. Samples were collected every 30 minutes. After the fourth sample the aCSF was switched to one containing 100mM KCl where the Na⁺ concentration was reduced to account for the change in osmolarity. This was done for 30 minutes before returning to normal aCSF
for the rest of the experiment (3 more samples, 1 every 30min). Collected samples were frozen at once at -80°C and analysed for DA within one week using high performance liquid chromatography (HPLC) with electrochemical detection (ECD). HPLC injection volume for microdialysis samples was always 10μl.

2.3.3 HPLC with ECD

The HPLC system consists of a reservoir containing mobile phase which was circulated around by a piston pump (P580, Dionex, Germany). Flow was then directed towards a Triathlon refrigerated (4°C) autosampler (Spark-Holland, Netherlands), then a C18 reverse phase column maintained at 40°C (ODS 3 WM, 4.6 mm I.D. x 100 mm; Rainin Dynamax Instruments Co. INC., U.S.A.) and protected by a Microsorb guard column (C18 5 WM, 4.6 mm I.D. x 15 mm, Rainin Dynamax Instruments Co. INC., U.S.A.). This column with guard column was used when analyzing microdialysis samples and DA in tissue homogenates. Another C18 reverse phase column also maintained at 40°C (ODS 3 WM, 4.6 mm I.D. x 150 mm; Rainin Dynamax Instruments Co. INC., U.S.A.) was used for L-DOPA detection from the TH enzyme assays. The flow then passed to an Antec-Intro electrochemical detector (Antec Leyden BV, Holland) fitted with a VTO3 flow cell (Vcell + 625 mV filtered to 5 abu with range set on 0.5 nA/volt). Data capture was achieved and analysed by a Dell Corporation PC system 310 (Dell Corporation, U.S.A.) equipped with Chromperfect for Windows software (Justice Innovations chromatography data systems, CA, U.S.). Chromatograms were printed using an Epson LX-300 printer. All separations were isocratic and the mobile phase
was composed of: sodium acetate (90mM), citric acid (35mM), EDTA (0.34mM), 1-octane-sulfonic acid (ion paring reagent; 0.06mM), 5.5% methanol in deionised water and pH was adjusted to 4.2 using citric acid. The mobile phase was de-gassed using an in-line de-gassing unit (Jour Research) and pumped at a flow rate of 0.65ml/min. The HPLC system was recalibrated and standards run each time the cell required cleaning or there was a need to change the mobile phase. This was done at least weekly.
2.3.3.1 Internal standard

An internal standard was added at a known quantity to each striatal homogenate sample used in the TH assays to account for the loss of analyte during the procedure. DA was used as internal standard for measuring the amounts of L-DOPA. A calibration curve was created using peak area (PA) ratios of DA and L-DOPA plotted against known L-DOPA concentrations (Figure 2.1)

Figure 2.1: Calibration curve for estimating amounts of L-DOPA in striatal samples used to measure synthetic capacity of TH enzyme. The curve was generated with DA as an internal standard through fixing the amount of DA used and altering the amounts of L-DOPA. The displayed line equation was used to calculate the amount of L-DOPA produced using a known PA ratio after L-DOPA detection in a striatal homogenate
The external standard method was used for quantifying DA measured in microdialysis samples as well as in striatal homogenates since an appropriate internal standard compound could not be selected. The external standard used was DA itself and different concentrations were injected and run in the HPLC to construct a calibration curve which was then used to convert the actual sample peak areas to DA concentrations. Calibration curve used is presented in figure 2.2.

![Dopamine standard curve](Dopamine_standard_curve.png)

**Figure 2.2**: Calibration curve for estimating amounts of DA in striatal homogenates and microdialysis samples. The curve was generated with DA as external standard. The displayed line equation was used to calculate the amount of DA produced using a known peak area detected.
2.3.3.3 Chromatogram examples

Microdialysis control

![Microdialysis control graph]

Microdialysis lesioned

![Microdialysis lesioned graph]
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Microdialysis lesioned with exendin

TH assay control
Figure 2.3: Example (1) typical chromatogram from sham surgery microdialysis group. Peaks were identified by comparing them to authentic monoamine standards. DA – dopamine; DOPAC – dihydroxy phenyl acetic acid; 5-HT – 5-hydroxytryptamine; 5-HIAA – 5-hydroxy indole acetic acid; HVA – homovanillic acid. (2) demonstrates a typical trace from a 6-OHDA treated group. (3) demonstrates a group co-treated with EX-4. (4) typical chromatogram obtained with TH enzyme assay control group. (5) no L-DOPA can be detected, indicating loss of TH activity. (6) demonstrates recovered TH synthetic capacity in groups co-treated with EX-4.
2.3.4 Termination and tissue handling

Animals were always sacrificed on completion of microdialysis. This was normally after a period of two weeks since the initial toxic insult. Animals were first lightly anaesthetised with 5% isoflurane v/v in O₂ then placed under a guillotine and decapitated. Brains were very quickly removed, flash frozen on dry ice and stored at -80°C until use. One day before experiment, frozen brains were taken from -80°C storage and stored at -20°C overnight. On the day of the experiment brains were placed on a ceramic tile over ice for a few minutes to defrost. Then the striata were removed using a stainless steel punch. The dissected striata were then placed into microcentrifuge tubes containing 1ml ice cold 0.01M PBS, immediately homogenised and frozen at -80°C until further use. Brain blocks containing SN were refrozen at -80°C and retained for immunohistochemistry.

2.3.5 Tissue dopamine assay

Striatal homogenates were defrosted and divided into two equal parts for tissue DA and TH enzyme assays. On the day of the experiment the 500μl portions were centrifuged at 9000xg for 15 minutes and duplicates of 50μl aliquots of the supernatant were treated with 0.2M perchloric acid containing ascorbic acid (0.2μM) and EDTA (0.2μM) to precipitate the cell debris. Then 50μl homogenate + 10μl perchloric acid combined aliquots were centrifuged again at 9000xg for 15 minutes at 4°C, and 25μl of this supernatant was injected in the HPLC system to determine tissue DA amount. (See
Figure 2.4. DA peak areas were converted to DA amounts using the external standard method and expressed as amounts of DA in pg per gram of striatal tissue.

![Diagram](image)

**Figure 2.4**: Tissue assays experimental details. Striatal homogenates were split into two equal portions each containing 500µl. Half was used to directly measure tissue DA content after centrifuging and treatment with perchloric acid. The remaining half was used to incubate with L-tyrosine to investigate TH synthetic capacity.

### 2.3.6 TH enzyme assay

TH enzyme activity was measured as described in the method of (Naoi et al., 1988). Homogenate 500µl aliquots were centrifuged at 9000xg for 15 minutes and then duplicates of 40µl were incubated with 200µM L-tyrosine in a total reaction mixture volume of 100µl. This consisted of the following components: 100mM sodium acetate-acetic acid buffer (pH 6.0), 2mM ferrous ammonium sulphate, 1mM 6MPH₄ which is a co-factor, 10µg of catalase and 1mM benserazide which is an AADC inhibitor. 6MPH₄ solution was made as 10mM in 1M mercaptoethanol. The incubation mixture, except for L-tyrosine and the cofactor, was preincubated with homogenates at 37°C for 5
minutes, and the reaction was initiated by addition of the L-tyrosine and 6MHP4. After incubation at 37°C for 10 minutes which was found to be the optimal temperature, the reaction was terminated by adding 100μl perchloric acid (0.1M, containing 0.4mM sodium metabisulphite and 0.1mM disodium EDTA). The samples were then vortexed and left to stand on ice for 10 minutes, then centrifuged again at 9000xg for a further 10 minutes. The supernatant was diluted 100μl in 1ml of mobile phase and 25μl were injected in to the HPLC to measure the amount of L-DOPA produced. A similar reaction mixture containing D-isomer of tyrosine and 100μM 3-iodo-L-tyrosine was used as a blank. Total protein content was estimated using BCA reagent (Pierce Biotech. Inc. USA) according to the manufacturer’s instructions.

2.3.7 TH Immunohistochemistry

Slide mounted 12μm thick cryostat sections from frozen brain blocks were removed from the freezer and allowed to equilibrate to room temperature for 30 minutes before fixation in 4% w/v paraformaldehyde, containing 1% w/v gluteraldehyde in deionised water for 5 minutes at 0°C. Following rinsing in 0.01M PBS for 5 minutes, sections were dehydrated through graded alcohols and endogenous peroxidase activity was blocked by incubation in 0.3% H2O2 in methanol for 10 minutes. The sections were then rehydrated and non-specific immunoreactivity was blocked using 10% swine serum in 0.01M PBS for 10 minutes. Sections were then incubated in primary antibody (anti TH IgG raised in rabbit at 1:700 in 0.01M PBS) for 15 hours at 4°C. After rinsing, the sections were incubated sequentially in biotinylated swine anti-rabbit antibody 1:250 in 0.01M PBS and ABC (Vector Labs ltd.) complex was applied for 30 minutes
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at room temperature following the manufacturer's instructions. Immunoreactivity was visualized through incubation in 0.5mg/ml 3’3-diaminobenzidine (DAB), containing 0.009% H₂O₂ in 20ml of DAB solution, for 2 minutes at room temperature. The sections were counterstained in Harris haematoxylin, dehydrated, cleared and mounted for examination. Sections were viewed under a light microscope using 5X magnification. Digital images were captured using a Leica DC500 system and the manufacturer's software.

2.3.8 Quantification of TH slides

Digital images from TH immunohistochemistry slides were analysed for colour intensity at using Lucia G image analysis software, Prague, Czech Republic. The software transforms the digital images into histograms and assigns values for color intensity based on pixel analysis. Six sections, one from each brain, from each n=6 treatment group were selected for analysis. Whole images as presented in results chapters were used for intensity measurements. While sectioning the brains in the cryostat care was taken to ensure that each section stained and analysed for TH represented the same anatomical region of the SN.

2.3.9 BrdU and GLP-1R immunohistochemistry

Slide mounted 12µm thick cryostat sections from frozen brain blocks were removed from the freezer and allowed to dry for 24 hours before fixation in 75% acetone and
25% absolute alcohol for 5 minutes. Slides intended for BrdU immunodetection were also pre-incubated in 2M HCl for 30min at 37°C to denature DNA for antigen retrieval. The same procedure was then followed as for TH immunodetection. The GLP-1R antibody used was a mouse monoclonal antibody raised against human GLP-1R with rat cross reactivity (1/500 dilution), followed by a secondary goat polyclonal antibody with a biotin tag (1/250); BrdU antibody used was a goat monoclonal raised against BrdU (1/500) followed by a secondary rabbit-anti goat polyclonal antibody also with a biotin tag (1/250).

Figure 2.5: A general timeline summarizing experimental protocols. Each animal went through each stage of the procedure except for chapter 5 where animals were used exclusively for immunohistochemistry. Apomorphine tests were performed early in the morning. Microdialysis probes were implanted an hour later. Animals were sacrificed on completion of microdialysis. Brains used for tissue assays maximum within one month.
2.4.0 Animal usage

Most animals went through all stages of experimental procedures from behavioural to neurochemical to histological. However because *in vivo* microdialysis is a complicated technique, the experiment does not always go according to plan e.g. microdialysis probes become blocked after implanting or occasionally rats do not recover from surgery, resulting in loss of n-numbers. But complications during *in vivo* dialysis do not lead to loss of data from other assessment methods e.g. behavioural, because it is performed prior to dialysis, but also the neurochemistry and histology because *in vitro* assays obviously no longer rely on animals being alive. Therefore in most cases more animals had to be treated and dialysed to accumulate n-numbers for dialysis but not necessarily used for all other tests if there were enough n-numbers for those to carry out the statistics. As a rule the experiments would stop for a particular treatment group as soon as n=6 were accumulated for each technique. Since dialysis was the only method really prone to complications, in theory there were more n-numbers for behavioural, tissue assays and histological assessments, but these were not carried out if the animals were only dialysed to accumulate the n-numbers. This explains the numbers of animals used in each chapter.

2.4.1 Statistical analysis

The data obtained from the behavioural apomorphine test, tissue DA assay, TH enzyme assay and TH immunohistochemistry were all subjected to one way analysis of variance
(ANOVA) and a post hoc Bonferroni’s multiple comparison tests to compare difference between selected treatments. Microdialysis data were subjected to a two way ANOVA with a post hoc Bonferroni’s multiple comparison test. In both cases data were expressed as mean ± standard error of the mean (n=6). GraphPad Prism 4 software was used for all of the statistical manipulations.
Chapter 3

Effect of exendin-4 in two rodent models of PD
Introduction and experimental details

EX-4 was shown to be neurotrophic and neuroprotective in both in vitro and in vivo models of neurodegenerative disorders. In this chapter EX-4’s neuroprotective potential was assessed using two popular distinct models of PD. The two toxins 6-OHDA and LPS work by different mechanisms but ultimately lead to neuronal degeneration mimicking some aspects of PD pathology. Animals were initially treated with either of the two toxins intracerebrally and allowed a week for the lesion to develop. Control animals received injection of vehicle which was saline in the case of LPS and 0.2% ascorbic acid in the case of 6-OHDA. EX-4 was then injected i.p. twice daily for another week, morning and evening at two different doses. Two weeks after the initial neurotoxic insult, all groups of animals were subjected to tests to assess the integrity of the nigrostriatal DA system. The first test was the behavioural apomorphine challenge. Here the numbers of apomorphine induced turns were recorded in all treatment groups. Reduction of apomorphine induced turning behaviour indicates reversal of the toxin induced changes. Extracellular levels of DA were then measured using in vivo microdialysis. Here a reversal of toxin induced decrease in extracellular DA would indicate a potential antiparkinsonian effect. The idea was to measure as many parameters as possible mainly to get as much evidence as possible but also to make best use of experimental animals. We therefore also measured striatal DA and TH enzyme activity in brain homogenates of these treatment groups as well as immunodetection of TH protein in 12μm sections through in the SN region of the brain. The total number of animals used in this chapter was 106 although every effort was made to minimize the use.
3.1 Neuroprotective effect of exendin-4 in two models of PD

3.1.1 Apomorphine induced circling

This test is based on the observation that DA receptors in the striatum become upregulated in order to compensate for the lack of DA release caused by toxin induced nerve damage. If the toxin is only injected into one cerebral hemisphere as in this case, the animals develop an imbalance of DA release on the two sides of the brain. Apomorphine is a non selective DA agonist which activates all DA receptor subtypes and when injected in such animals causes more receptor activation on the lesioned side compared to the intact side. This causes contralateral turning behaviour. Therefore apomorphine induced circling is a behavioural way of quantifying nigrostriatal lesion severity following toxin injection and attenuation of such circling behaviour indicates normalisation of DA imbalance which can be seen in hemiparkinsonian rats. Figure 3.1.1(a) demonstrates apomorphine induced circling displayed by different treatment groups in the 6-OHDA model of PD. Results demonstrate very pronounced contralateral turning behaviour in toxin only treated animals both after one week 17 ± 2 turns in 120 seconds and after two weeks 26.5 ± 4 turns in 120 seconds in 6-OHDA only treated groups compared to shams 0 ± 0 turns. This was significantly reduced when co-treated with exendin-4 at both doses 8 ± 2 turns for the 0.1µg kg⁻¹ dose which is approximately a 70% reduction and 1 ± 1 turns which is a 93% reduction for the 0.5µg/kg⁻¹ EX-4 dose. Apomorphine induced circling was also tested in the EPS model of PD to ensure the effects of EX-4 that were observed previously were not model
specific. Results are again presented as the number of full contralateral turns counted over a 120 second period. LPS only treated rats demonstrated very pronounced contralateral turning behaviour both after one week (14 ± 2 turns in 120 seconds) and after two weeks (20 ± 3 turns in 120 seconds) compared to sham surgery controlled animals. This was significantly reduced when animals were co-treated with EX-4 at both doses. The numbers of turns recorded were 3 ± 2.2 turns which was almost a 10 fold reduction for the 0.1μg kg\(^{-1}\) dose and 1 ± 1 turns for the 0.5μg/kg\(^{-1}\) dose which was nearly a 100% reduction. Treatment with exendin-4 dose reduced the number of contralateral turns following apomorphine challenge in the LPS model of PD. The data are shown in Figure 3.1.1(b). After apomorphine testing animals were advanced to the next stage of assessment which measured extracellular DA levels using \textit{in vivo} microdialysis.
Figure 3.1.1(a): Effect of EX-4 (0.1 and 0.5 μg/kg) on apomorphine-induced rotational behaviour in 6-OHDA lesioned rats. EX-4 was administered twice daily for seven days, one week after toxin injection. Circling was measured for 120s 15 min after apomorphine injection. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare differences between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA (14) only group (ANOVA F[4,25] = 25.25, *p[7] < 0.001, *p[14] < 0.001, +p[0.1] < 0.001, +p[0.5] < 0.001, n=6 per group, confidence interval set at 95%)
Figure 3.1.1(b): Effect of EX-4 (0.1 and 0.5 µg/kg) on apomorphine-induced rotational behaviour in LPS lesioned rats. EX-4 was administered twice daily for seven days, one week after toxin injection. Circling was measured for 120s 15 min after apomorphine injection. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare differences between groups. *indicates significant differences compared to control. +indicates significant difference compared to LPS (14) only group (ANOVA F[4,25] = 20.93, *p[7] < 0.001, *p[14] < 0.001, +p[0.1] < 0.001, +p[0.5] < 0.001, n=6 per group, confidence interval set at 95%)
3.1.2 In vivo microdialysis

This technique involves the positioning of a semi permeable membrane that allows free movement of water and small solutes between a solution of interest which in this case is the brain interstitial fluid and the solution lacking the solute of interest in this case artificial CSF. In vivo microdialysis allows the monitoring of neurotransmitter release in any part of the brain. Here the effect of exendin-4 in 6-OHDA lesioned animals was assessed by measuring the amount of both basal and K⁺ evoked extracellular DA. Figure 3.1.2(a) shows DA release measured in the STR in 30 minute intervals over four hours. Data were plotted as the amount of DA in fmoles per 10μl HPLC injection volume over time. Basal DA levels in the sham surgery control groups were approximately 40 ± 8 fmoles/10μl, increasing roughly 20 fold to 791 ± 45 fmol when stimulated with 100mM KCl. Note that the levels return to baseline on removal of the high K⁺ stimulus. In 6-OHDA lesioned animals basal levels of DA were close to the limit of detection at approximately 5 ± 2 fmoles/10μl which was an 8 fold reduction compared to control groups whereas K⁺ stimulated DA only reached 133 ± 25 fmoles/10μl which was a 6 fold reduction compared to controls. Animals lesioned with the toxin and then co-treated with EX-4 for one week show a dose dependent recovery in their extracellular DA levels. In the lower dose 0.1μg kg⁻¹ group the average basal amount of DA showed approximately 55% recovery compared to toxin only treated groups and was 24 ± 4 fmol whereas K⁺ stimulated levels reached 566 ± 46 fmoles which was a 4 fold increase. In the higher dose 0.5μg kg⁻¹ EX-4 group, striatal DA was 43 ± 9 fmol in 10μl basal level and reaching 860 ± 76 fmol when K⁺ stimulated, which is essentially the same as control groups. Effect of EX-4 on extracellular DA levels was
also tested in the LPS lesioned animals. Figure 3.1.2(b) demonstrates DA release measured in the STR in 30 minute intervals over four hours. Data were again plotted as the amount of DA in fmoles per 10μl HPLC injection volume over time. Basal DA levels in the sham surgery control groups were approximately 42 ± 7.5 fmoles in 10μl injection samples, increasing to just over 20 fold to 1008 ± 178 fmol when stimulated with 100mM KCl. DA levels returned to baseline on removal of the high K⁺ stimulus. In LPS lesioned rats, basal DA fell to 6 ± 2 which was a 7 fold reduction and K⁺ induced DA also decreased to 188 ± 34 fmol which was an over 5 fold reduction compared to controls. Animals lesioned with LPS and then co-treated with EX-4 for one week showed a dose dependent recovery in their both basal and K⁺ evoked extracellular DA levels. In the lower dose 0.1μg kg⁻¹ group, the average basal amount of DA released was 25 ± 4 fmol which was a 60% recovery compared to LPS only treated rats. When stimulated with 100mM KCl DA levels reached 478 ± 45 which was a 2.5 fold increase compared to LPS lesioned rats. In the higher dose 0.5μg kg⁻¹ EX-4 group, striatal DA was comparable to sham surgery groups at 38 ± 8 fmol in 10μl basal level and reaching 987 ± 123 fmol when K⁺ stimulated which is over 90% recovery for both. Data demonstrate a dose dependent restoration of extracellular DA in the STR in the LPS model of PD. What is interesting in both models is that 20 fold difference between basal and K⁺ is preserved in all groups, suggesting that when the toxin presumably kills a certain number of neurons, the rest maintain the same functional capacity. The same appears to apply for groups which recover with EX-4, not only their basal DA recovers, but the 20 fold increase between basal and evoked is also preserved.
Figure 3.1.2(a): Effect of EX-4 (0.1 and 0.5 µg/kg) on amount of extracellular DA in 6-OHDA lesioned rats as measured by in vivo microdialysis. EX-4 was administered twice daily for seven days, one week after toxin injection. Perfusion rate was 1µl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K⁺. Results were analyzed using two way ANOVA and a post hoc Bonferroni multiple comparisons test. *indicates significant difference from control. +indicates significant difference from both, control and 6-OHDA only groups (ANOVA between treatments $F[21,160] = 13.21$ and time $F[7,160] = 161.63$, *$p < 0.001$, +$p < 0.01$, n=6 per group, confidence interval set at 95%).
Figure 3.1.2(b): Effect of EX-4 (0.1 and 0.5 μg/kg) on amount of extracellular DA in LPS lesioned rats as measured by in vivo microdialysis. EX-4 was administered twice daily for seven days, one week after toxin injection. Perfusion rate was 1μl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K⁺. Results were analyzed using two way ANOVA and a post hoc Bonferroni multiple comparisons test. *indicates significant difference from control. +indicates significant difference from both, control and LPS only group (ANOVA between treatments $F_{[21,160]} = 8.84$ and time $F_{[7,160]} = 93.80$, *$p < 0.001$, +$p < 0.01$, n=6 per group, confidence interval set at 95%).
3.1.3 *Striatal tissue DA levels*

After dialysis animals were killed by decapitation. Brains were homogenised in ice cold PBS buffer and total amounts of DA were estimated using HPLC. Results are presented as amounts of DA in pg/g per gram of striatal tissue. In the 6-OHDA model sham surgery controlled groups the average amount DA was $8.2 \pm 1$ pg/g. In 6-OHDA only treated groups tested after one week the amount of DA decreased by over 60% to $3.1 \pm 0.3$ pg/g and after two weeks it was further decreased by around 90% to $0.8 \pm 0.2$ pg/g compared to control. In groups co-treated with $0.1 \mu g$ kg$^{-1}$ of EX-4, the total amount of DA was increased to $4.2 \pm 1$ pg/g which was a 50% recovery and was significantly different from 6-OHDA only treated groups after two but not after one week. With the $0.5 \mu g$ kg$^{-1}$ EX-4 dose the amount of DA was increased by 85% to $7 \pm 1$ pg/g which was significantly different from 6-OHDA only groups both after one and two weeks. These results demonstrate a recovery of striatal tissue DA in groups co-treated with EX-4. In LPS only treated groups tested after one week the amount of DA decreased by over 65% to $2.6 \pm 1$ and after two weeks DA amount was further decreased by almost 90% to $1 \pm 0.4$ pg/g compared to control. In groups co-treated with $0.1 \mu g$ kg$^{-1}$ of EX-4, the total amount of DA was increased to $3.8 \pm 2$ pg/g which was a near 50% recovery but was not significantly different from LPS only treated groups both after one and after two weeks. With the $0.5 \mu g$ kg$^{-1}$ EX-4 dose the amount of DA was increased by nearly 90% to $6.9 \pm 1$ pg/g which was significantly different from LPS only groups both after one and two weeks. Results demonstrate a recovery of striatal tissue DA in groups co-treated with EX-4. Results for both models are shown in Figure 3.1.3(a) and (b).
Figure 3.1.3(a): Effect of EX-4 (0.1 and 0.5 μg/kg) on amount of striatal DA in tissue homogenates of 6-OHDA lesioned rats. EX-4 was administered twice daily for seven days, one week after toxin injection. Striatal tissue DA was measured using HPLC with ED. Results analyzed using one way and a post hoc Bonferroni test to compare differences between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA (14) only group (ANOVA F[4,25] = 14.20, *p[7] < 0.01, *p[14] < 0.001, +p[0.5] < 0.001, n=6 per group, confidence interval set at 95%)
Figure 3.1.3(b): Effect of EX-4 (0.1 and 0.5 µg/kg) on amount of striatal DA in tissue homogenates of LPS lesioned rats. EX-4 was administered twice daily for seven days, one week after toxin injection. Striatal tissue DA was measured using HPLC with ED. Results analyzed using one way ANOVA and a post hoc Bonferroni test to compare differences between groups.

* indicates significant differences compared to control.
+ indicates significant difference compared to LPS (14) only group (ANOVA $F_{[4,21]} = 7.97$, *$p[7] < 0.05$, *$p[14] < 0.01$, +$p[0.5] < 0.01$, $n=6$ per group, confidence interval set at 95%)
3.1.4 TH enzyme assay

TH is the rate limiting step in DA synthesis. TH activity reflects neuronal functionality and was measured in the brain homogenates of all treatment groups of rats from both 6-OHDA and LPS models of PD. Data were plotted as amounts of striatal L-DOPA detected after incubation of homogenates with L-tyrosine in pmoles min\(^{-1}\) mg\(^{-1}\) protein. High L-DOPA yield reflects high TH capacity. In sham operated controls the amount of L-DOPA was 60 ± 5 pmoles/min/mg compared to only 4.1 ± 2 in 6-OHDA only treated groups after two weeks. This was a 15 fold reduction in 6-OHDA treated compared to control groups. In 6-OHDA groups after one week there was a 5 fold reduction and L-DOPA amount equal to 12 ± 2.3 pmoles/min/mg. EX-4 treatment increased the amount striatal L-DOPA dose dependently. In the 0.1μg kg\(^{-1}\) EX-4 treated group there was an increase to 21 ± 3 pmoles/min/mg which was higher 5 fold compared to 6-OHDA only groups. In the 0.5μg kg\(^{-1}\) EX-4 group there was an increase in the amount of striatal L-DOPA to 53 ± 6 pmoles which was a recovery of nearly 90%. In LPS model sham surgery controls had 55 ± 7 pmoles/min/mg compared to just 7 ± 2 in LPS only treated groups after two weeks. This was an 8 fold reduction compared to controls. There was a 4 fold reduction down to 14 ± 3 pmoles/min/mg in amount of L-DOPA in LPS groups after one week compared to controls. EX-4 treatment again increased the amount striatal L-DOPA dose dependently. In the 0.1μg kg\(^{-1}\) EX-4 treated group the amount of L-DOPA was 28 ± 2 pmoles/min/mg which was a 4 fold increase compared to LPS only groups. In 0.5μg kg\(^{-1}\) EX-4 groups there is an increase in the amount of striatal L-DOPA to 63 ± 9 pmoles/min/mg which is a 100% recovery compared to LPS only treated animals. Data shown in 3.1.4(a) and (b).
Figure 3.1.4(a): Effect of EX-4 (0.1 and 0.5 µg/kg) on amount of striatal L-DOPA formation of 6-OHDA lesioned rats. EX-4 was administered twice daily for seven days, one week after toxin injection. Striatal L-DOPA was measured using HPLC with ED. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare difference between groups. *indicates significant differences compared to control. +indicates significant difference compared to both 6-OHDA (14) only and 6-OHDA + EX-4 0.1µg/kg groups (ANOVA F[4,25] = 39.49, *p[7] < 0.001, *p[14] < 0.001, +p[0.5] < 0.001, n=6 per group, confidence interval set at 95%)
Figure 3.1.4(b): Effect of EX-4 (0.1 and 0.5 µg/kg) on amount of striatal L-DOPA formation of LPS lesioned rats. EX-4 was administered twice daily for seven days, one week after toxin injection. Striatal L-DOPA was measured using HPLC with ED. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare difference between groups. *indicates significant differences compared to control. +indicates significant difference compared to LPS (14) only and LPS + EX-4 0.1µg/kg groups (ANOVA F[4,25] = 20.79, *p[7] < 0.001, *p[14] < 0.001, +p[0.5] < 0.001, n=6 per group, confidence interval set at 95%)
3.1.5 TH immunohistochemistry

Here we looked at TH expression in the SN using immunohistochemistry. TH enzyme is a neuronal marker for both noradrenergic and dopaminergic neurones. TH expression in the SN suggests possible presence of functional DA neurons and was compared in all treatment groups in both models of PD. One section from each brain in each treatment group was used for analysis. The digital images were analysed using Lucia G image analysis software (LIM laboratory imaging, Prague, Czech Republic). Data plotted was the colour intensity displayed by each entire individual image when it was analysed using the above software package. Data are expressed as mean and error bars represent standard error of the mean for each treatment group (n=6). Images which represented control groups generally had high colour intensity meaning presence of TH protein and reflected by software assigned arithmetic values of 15388 ± 2975 on average. Groups treated with 6-OHDA and tested after two weeks showed a 7 fold decrease in their colour intensity values 2017 ± 1362 reflected by a decrease in DAB labelling indicating loss of TH immunostaining. When tested after one week, there was a more than 2 fold decrease in intensity 5311 ± 2650 compared to control groups meaning there is around 50% loss of TH staining after 1 week of 6-OHDA administration. Groups co-treated with EX-4 showed an increase in DAB staining meaning an increase in TH expression and reflected by intensity values of their images. In the lower 0.1μg kg⁻¹ dose groups, the intensity was increased almost 4 fold 8322 ± 3125 units compared to 6-OHDA only treated groups after two weeks. In 0.5μg kg⁻¹ EX-4 treated groups, the intensity increased to 14890 ± 2935 units which was a near 100% recovery. When analyzing images from the LPS model of PD, it was found that in control groups the average
intensity was 18662 ± 2433 units representing a certain level of TH expression represented by DAB staining. Groups treated with 6-OHDA and tested after two weeks showed almost a 6 fold decrease in TH expression and DAB staining seen on the slides which was reflected by intensity values assigned by the software 3142 ± 1255 to these images. When tested after one week, the level of TH expression showed an almost 3 fold decrease 6512 ± 1872 compared to control groups. Groups co-treated with EX-4 displayed an increase in DAB labelling reflected by the colour intensities of relevant images. In the lower 0.1µg kg⁻¹ dose groups, the intensity was increased nearly 3 fold 8322 ± 3125 compared to LPS only treated groups after 2 weeks. In 0.5µg kg⁻¹ EX-4 treated groups, the intensities were increased to 15384 ± 3119 units which indicated an over 80% recovery and was significantly different from LPS only treated animals. Photomicrograph examples are presented in Figures 3.1.5(a) and (c). Analysed data are shown in 3.1.5(b) and (d).
Figure 3.1.5(a): Photomicrographs of selected 12μm cryostat sections through the rat SN immunoassayed for TH. Nigral cell bodies appear dark brown in colour visualised using DAB staining. The right side is ipsilateral i.e. treated and left is contralateral i.e. untreated. (B) demonstrates the effect of 6-OHDA after one week compared to control (A). (D) represents 6-OHDA lesion assessed after two weeks compared to control (C). (F) and (H) show the effects of 0.1μg/kg and 0.5μg/kg exendin-4 respectively in 6-OHDA treated animals one week post lesion compared to their controls (E) and (G). Scale bars – 100μm.
Figure 3.1.5(b): Effect of EX-4 (0.1 and 0.5 μg/kg) on TH expression in the SN visualised using DAB in 6-OHDA lesioned rats. EX-4 was administered twice daily for seven days, one week after toxin injection. TH immunostaining was quantified using Lucia G image analysis software based on colour intensity. Results analyzed using one way ANOVA and a post hoc Bonferroni's test to compare difference between groups. *indicates significant differences compared to control. + indicates significant difference compared to 6-OHDA (14) only group (ANOVA F[4,25] = 4.78, *p[14] < 0.05, +p[0.5] < 0.05, n=6 per group, confidence interval set at 95%)
Figure 3.2.5(a): Photomicrographs of selected 12μm cryostat sections through the rat SN immunoassayed for TH. Nigral cell bodies appear dark brown in colour visualised using DAB staining. The right side is ipsilateral i.e. treated and left is contralateral i.e. untreated. (B) demonstrates the effect of LPS after one week compared to control (A). (D) represents LPS lesion assessed after two weeks compared to control (C). (F) and (H) show the effects of 0.1μg/kg and 0.5μg/kg exendin-4 respectively in LPS treated animals one week post lesion compared to their controls (E) and (G). Scale bars – 100μm.
Figure 3.2.5(b): Effect of EX-4 (0.1 and 0.5 μg/kg) on TH expression in the SN visualised using DAB in LPS lesioned rats. EX-4 was administered twice daily for seven days, one week after toxin injection. TH immunostaining was quantified using Lucia G image analysis software based on colour intensity. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare difference between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA (14) only group (ANOVA $F_{[4,25]} = 7.76$, $^{*} p[7] < 0.01$, $^{*} p[14] < 0.001$, $+p[0.5] < 0.01$, n=6 per group, confidence interval set at 95%)
Chapter 3: Effect of exendin-4 in two rodent models of PD

3.3 Summary of findings

1. EX-4 reduced the number of contralateral apomorphine induced turns in both 6-OHDA and LPS hemiparkinsonian models of PD.

2. Extracellular DA levels measured by in vivo microdialysis were dose dependently increased in groups co-treated with EX-4 in both models of PD.

3. In groups of animals co-treated with EX-4 the amount of striatal tissue DA was higher compared to toxin only treated animals in both the 6-OHDA and the LPS models.

4. Striatal tissue homogenates from animals which received EX-4 had increased levels of TH enzyme activity. There was a dose dependent increase in the amounts of L-DOPA produced in both models of disease compared to toxin only treated groups.

5. TH protein immunodetection revealed increased levels of TH expression in groups receiving EX-4 compared to vehicle treated animals in both the 6-OHDA and LPS models.
Chapter 4

The effect of Exendin-4 is via GLP-1 receptor activation
Introduction and experimental details

The mechanism of action of EX-4 in animal models of diabetes where the peptide was extensively studied requires the activation of GLP-1 receptors on the β islets of the pancreas which causes glucose dependent insulin secretion from these cells. The main objective of this chapter was to test if the same receptor was involved in the neuroprotective effects that are seen with EX-4 but also to accumulate more evidence for the neuroprotective effects of EX-4 itself. It was decided to use the MFB 6-OHDA model of PD for mechanism of action studies since it is a harsher model and mimics end stage clinical condition. Similar experimental protocol was followed. Briefly, the animals were first injected stereotaxically with 8µg of 6-OHDA in 2µl of saline containing 0.2% ascorbic acid into the left MFB to induce neuronal degeneration. Control animals were treated with vehicle. A week after toxin injection animals either received 0.5µg kg\(^{-1}\) i.p. dose of EX-4 alone, 0.5µg kg\(^{-1}\) dose of EX-4 preceded by a 1µg kg\(^{-1}\) dose of EX-9-39 or a 1µg kg\(^{-1}\) of EX-9-39 on its own. Injections were continued for one week as before and on day 14 animals were subjected to tests. Animals were tested as before, starting with the behavioural apomorphine followed by in vivo microdialysis to establish extracellular DA levels. Animals were killed after dialysis and neurochemical indicators were also measured as in the previous chapter followed by immunohistochemistry of SN region for TH expression. Like previously it was attempted to get as much data as possible from each animal and the numbers used were kept to a minimum. The total number used in this chapter was 67.
Chapter 4: The effect of exendin-4 is via GLP-1 receptor activation

4.1 GLP-1R receptor antagonist exendin-9-39 blocks the effect of exendin-4 in 6-OHDA treated rats.

4.1.1 Apomorphine induced circling

Here, apomorphine induced circling was tested in groups co-treated with a GLP-1R antagonist EX-9-39. Results are presented as numbers of full contralateral turns counted over 120 second period. Again, 6-OHDA only treated animals demonstrated very pronounced contralateral turning behaviour tested two weeks after toxin administration 27 ± 5 compared to sham surgery controlled animals 0.1 ± 0.1 turns. Contralateral circling was again significantly reduced to 4 ± 2 when animals were co-treated with EX-4 at the 0.5µg/kg dose. This was a reduction of 85%. Groups which were also given EX-9-39 at 1µg kg⁻¹ together with EX-4 showed 20 ± 4 turns on average which is significantly different compared to 6-OHDA and EX-4 only treated animals and not significantly different from 6-OHDA only groups. There was only a 27% reduction in the number of contralateral turns in these groups compared to 6-OHDA and EX-4 only treated animals. This means there is a 60% reduction in the protective effect of EX-4 in the presence of EX-9-39. Both EX-4 and EX-9-39 when administered to naïve animals separately did not have any effect on apomorphine induced turns 0.1 ± 0.1. Data are shown in Figure 4.1.1.
Chapter 4: The effect of exendin-4 is via GLP-1 receptor activation

Figure 4.1.1: Effect of EX-4 (0.5μg/kg) in the presence of EX-9-39 (1μg/kg) on apomorphine-induced rotational behaviour in 6-OHDA lesioned rats. EX-9-39 was co-administered with EX-4 twice daily for seven days, one week after toxin injection. Circling was measured for 120s 15 min after apomorphine injection. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare differences between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA only. ^indicates significant difference compared to 6-OHDA+EX4 group (ANOVA F[4,25] = 17.23, *p < 0.001, +p < 0.001, ^p < 0.01, n=6 per group, confidence interval set at 95%)
4.1.2 In vivo microdialysis

The effect of EX-9-39 in 6-OHDA and EX-4 treated animals was assessed by measuring the amount of both basal and K⁺ evoked extracellular DA using in vivo microdialysis. Figure 4.1.2 demonstrates DA release measured in the STR in 30 minute intervals over four hours. Data are plotted as amounts of DA in fmols moles per 10μl HPLC injection volume over time. Basal DA levels in the sham surgery control groups were approximately 39 ± 7 fmol/10μl, increasing almost 30 fold to 1108 ± 113 fmol when stimulated with 100mM KCl. DA levels returned to baseline on removal of the high K⁺ stimulus. In 6-OHDA only rats basal DA was reduced to 8 ± 3 which was an almost 5 fold reduction and K⁺ induced DA also decreased to 188 ± 34 fmol which was a 6 fold reduction compared to control groups. Animals lesioned with 6-OHDA and then co-treated with EX-4 0.5μg kg⁻¹ for one week show increased levels of DA both basal 28 ± 9 fmol which increased over 3 fold and K⁺ evoked 780 ± 87 which increased 4 fold compared to 6-OHDA lesioned animals. 6-OHDA and EX-4 rats co-treated with EX-9-39 at 1μg kg⁻¹ show reduced levels of extracellular DA both basal 6 ± 1 fmol which is an over 4 fold reduction and K⁺ induced 99 ± 17 fmol which is an almost 8 fold decrease compared to 6-OHDA and EX-4 only groups. In naïve animals given either EX-9-39 or EX-4 on their own, extracellular DA levels are essentially the same as controls 30 ± 6 fmol basal level and 922 ± 67 when K⁺ induced in EX-9-39 groups and 34 ± 5 fmol basal and 989 ± 75 when stimulated.

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Figure 4.1.2: Effect of EX-4 (0.5μg/kg) in the presence of EX-9-39 (1μg/kg) on amount of extracellular DA in 6-OHDA lesioned rats as measured by in vivo microdialysis. EX-9-39 was co-administered with EX-4 twice daily for seven days, one week after toxin injection. Perfusion rate was 1μl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K+. Results were analyzed using two way ANOVA and a post hoc Bonferroni multiple comparisons test. *indicates significant difference compared to 6-OHDA+EX4 group (ANOVA between treatments F[28,200] = 22.87 and time F[7,200] = 176.64, *p < 0.001, n=6 per group, confidence interval set at 95%).
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4.1.3 Striatal tissue DA levels

After dialysis experiments, striatal tissue DA levels were also estimated in the same treatment groups of animals including those co-treated with EX-9-39. Data demonstrated in Figure 4.1.3 shows that in sham surgery controlled groups the average amount DA was 6.8 ± 1.1 pg/g per gram of striatal tissue, which coincides well with previous findings for control groups. In 6-OHDA only treated groups tested after two weeks there is an expected reduction of DA levels to 0.77 ± 0.2 pg/g which was a near 90% reduction compared to control. In groups co-treated with of EX-4 0.5μg kg⁻¹ the total amount of DA was increased to 5.2 ± 0.33 pg/g which was a near 80% recovery and was significantly different from 6-OHDA only treated groups. In 0.5μg kg⁻¹ EX-4 and 1μg kg⁻¹ of EX-9-39 groups the amount of DA was decreased by 97% to 0.19 ± 0.1 pg/g which was significantly different from 6-OHDA and EX-4 treated groups. Groups receiving either EX-4 or EX-9-39 only did not show significant changes in their striatal DA compared to control groups and were 6.5 ± 0.9 pg/g in EX-4 only groups and 5.8 ± 0.6 pg/g in EX-9-39 groups.
Figure 4.1.3: Effect of EX-4 (0.5 μg/kg) in the presence of EX-9-39 (1 μg/kg) on amount of striatal DA in tissue homogenates of 6-OHDA lesioned rats. EX-9-39 was co-administered with EX-4 twice daily for seven days, one week after toxin injection. Striatal tissue DA was measured using HPLC with ED. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare differences between groups. * indicates significant differences compared to control. + indicates significant difference compared to 6-OHDA only. ^ indicates significant difference compared to 6-OHDA+EX4 (ANOVA F[4,25] = 26.87, *p < 0.001, +p < 0.001, ^p < 0.001, n=6 per group, confidence interval set at 95%).
4.1.4 TH enzyme assay

TH activity was measured in homogenates from all treatment groups including those treated with EX-9-39. It was found that in sham surgery controls the amount of L-DOPA was $67 \pm 6$ pmol min$^{-1}$mg$^{-1}$ compared to just $4 \pm 2$ pmol in 6-OHDA only treated groups after two weeks. This was a 16 fold reduction. Twice daily injections of EX-4 $0.5\mu g$ kg$^{-1}$ increased the amount striatal L-DOPA $59 \pm 8$ pmol min$^{-1}$mg$^{-1}$ which was a near 90% recovery compared to 6-OHDA only treated animals. In 6-OHDA and EX-4 groups co-treated with $1\mu g$ kg$^{-1}$ EX-9-39 the amount of striatal L-DOPA was decreased $6 \pm 2$ which was a near 90% decrease compared to 6-OHDA and EX-4 only groups. Groups treated with EX-4 or EX-9-39 only did not show any significant changes in their L-DOPA levels compared to controls $71 \pm 15$ pmol min$^{-1}$mg$^{-1}$ in EX-4 only groups and $73 \pm 12$ pmol min$^{-1}$mg$^{-1}$ in EX9-39 groups. Data are shown in Figure 4.1.4.
Figure 4.1.4: Effect of EX-4 (0.5μg/kg) in the presence of EX-9-39 on amount of striatal L-DOPA formation of 6-OHDA lesioned rats. EX-9-39 was co-administered with EX-4 twice daily for seven days, one week after toxin injection. Striatal L-DOPA was measured using HPLC with ECD. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare differences between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA only. ^indicates significant difference compared to 6-OHDA+EX4 (ANOVA F[4,25] = 23.11, *p < 0.001, +p < 0.001, ^p < 0.001, n=6 per group, confidence interval set at 95%)
4.1.5 *TH* immunohistochemistry

Remaining brain blocks were used to visualise the SN region using immunohistochemistry. Digital images which represented control groups had a relatively high level of *TH* expression in the region and therefore high intensity of DAB staining reflected by high colour intensities of approximately $21532 \pm 2853$ units on average when analyzed using the Lucia G image analysis software. Groups treated with 6-OHDA and tested after two weeks showed almost an 8 fold decrease in intensities $2721 \pm 1125$ units compared to control groups reflected by a decrease in DAB labelling on the microscope slides. Groups co-treated with EX-4 0.5 μg kg$^{-1}$ displayed an increase in DAB staining reflected by *TH* expression and therefore also higher intensity values of relevant images $18293 \pm 2620$ which indicated an over 80% recovery and was significantly different from 6-OHDA only treated animals. In 6-OHDA and EX-4 groups which also received EX-9-39 at a dose of 1 μg kg$^{-1}$, the intensities were decreased to $3225 \pm 1893$ units on average reflecting a decrease in *TH* staining. Groups which were treated with either EX-4 or EX-9-39 only are visually indistinguishable from controls and their intensities of $23569 \pm 4126$ units and $22472 \pm 3282$ units respectively are insignificant from control groups statistically. Photomicrograph examples are presented in Figure 4.1.5(a) and data are shown in Figure 4.1.5(b).
Figure 4.1.5(a): Photomicrographs of selected 12µm cryostat sections through the rat SN immunoassayed for TH. Nigral TH cell bodies appear dark brown in colour visualised using DAB staining. The right side is ipsilateral i.e. treated and left is contralateral i.e. untreated. (A) and (B) represent an EX-4 only treated group. (D) represents 6-OHDA lesion assessed after two weeks compared to control (C). (F) shows the effect of 0.5µg/kg exendin-4 in 6-OHDA treated animals one week post lesion compared to control (E). (H) demonstrates the effect of EX-9-39 co-administration in 6-OHDA+EX-4 treated animals compared to control (G). Scale bars – 100µm.
Chapter 4: The effect of exendin-4 is via GLP-1 receptor activation

Figure 4.1.5(b): Effect of EX-9-39 (1µg/kg) on TH expression in the SN visualised using DAB in 6-OHDA and EX-4 treated rats. EX-9-39 was administered twice daily for seven days together with EX-4, one week after 6-OHDA injection. TH immunostaining was quantified using Lucia G image analysis software based on colour intensity. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare difference between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA only group. ^indicates significant difference compared to 6-OHDA + EX-4 group (ANOVA F[5,30] = 11.88, *p < 0.001, +p < 0.01, ^p < 0.05 n=6 per group, confidence interval set at 95%)
4.2 Summary of findings

1. Co-administration of GLP-1R antagonist EX-9-39 increased the numbers of apomorphine induced contralateral turns in animals treated with 6-OHDA and EX-4 but had not effect on untreated groups.

2. EX-9-39 treatment also decreased the amount of extracellular DA in 6-OHDA and EX-4 groups of animals measured using *in vivo* microdialysis. EX-9-39 groups were comparable to 6-OHDA only treated groups, but there was no change when antagonist was given to untreated animals.

3. Striatal tissue DA was decreased in groups co-treated with EX-9-39 compared to 6-OHDA and EX-4 only treated animals. Again, EX-9-39 groups displayed unaltered levels of striatal DA and were comparable to controls.

4. Amounts of L-DOPA measured in striatal homogenates were also significantly reduced in 6-OHDA and EX-4 treated animals also given EX-9-39 which indicates reduced TH activity compared to 6-OHDA and EX-4 only animals. There was no significant change in TH activity in EX-9-39 only treated groups.

5. Finally, TH immunohistochemistry shows the same pattern. 6-OHDA and EX-4 only treated groups are comparable to controls whereas when co-treated with EX-9-39 the level of TH expression in the SN was reduced. Animals given EX-9-39 only had no change in their TH expression in SN compared to controls.
Chapter 5

GLP-1R expression and neurogenesis
Introduction and experimental details

GLP-1 receptors were recently linked to neurogenesis. It was also reported that they might be present in the SVZ which is a neurogenic region of the brain. It was proposed that EX-4 by activating these receptors may influence the fate of neural stem cells in vivo. The main objective of this chapter was to test cellular proliferation in the SVZ using BrdU. The experimental protocol was similar to previous chapters. Rats were divided into different treatment groups. All animals except for controls were injected with 6-OHDA stereotaxically to induce Parkinsonism. One week later animals were started on different drug treatments. Some received saline injections, while others had EX-4 at 0.5μg kg⁻¹ dose. All animals received BrdU 50mg kg⁻¹ dose i.p. twice daily. Another group also received a 1mg kg⁻¹ dose of selective D₃ receptor antagonist nafadotride. Since D₃ receptors are thought to be involved regulation of neurogenesis we were interested to see if this antagonist would cause changes in BrdU positive cells in the SVZ of EX-4 treated groups. In this chapter the only test performed was immunohistochemistry. Animals were injected with the toxin on day 0 and sacrificed on day 14. Care was always taken to minimize the numbers of animals used. In total 30 animals were used in this chapter.

5.1 GLP-1R expression in rat brain LVW

Using a specific antibody directed towards rat GLP-1R it was confirmed that the rat lateral ventricular wall (LVW) expresses the receptors. Data is presented as numerical
density in cell numbers per 90\(\mu\)m of DAB labelled cells along the wall of ventricles of different treatment groups. Cell counts were carried out starting in the dorsolateral corner of the ventricle. Blank system containing no primary antibody was used as control in these experiments.

Each photomicrograph represents ipsilateral i.e. treated side only. One slide from each of the six brains in each treatment group was used for analysis. The numerical density calculated for slides from sham surgery controlled groups of rats had on average 0.12 ± 0.0016 DAB labelled cells per unit distance compared to blanks which only had 0.033 ± 0.0055 cells. 6-OHDA treated groups had on average 0.13 ± 0.0087 cells which was significantly different from blank but not significantly different from sham groups. 6-OHDA and EX-4 treated groups were essentially the same 0.12 ± 0.0078 cells compared to both shams and 6-OHDA only treated groups as were EX-4 only and groups which received 6-OHDA, EX-4 and a DA D\(_3\)-receptor antagonist nafadotride.

According to our data, GLP-1R expression in the LVW does not change with administration any of the drugs. Immunoassaying for GLP-1R was also carried out in the STR and the SN, but with no significant difference between any of the groups including the blank control suggesting that these areas do not express the receptors or the density is not detectable by the technique used. Photomicrograph examples are presented in Figure 5.1(a) and data are shown in Fig 5.1(b).
Figure 5.1(a): Photomicrographs of selected 12μm cryostat sections through the rat LVW immunoassayed for GLP-1R. Cells along the LVW appear dark brown in colour visualised using DAB staining. Cell counts were carried out starting in the dorsolateral corner of the ventricle. All micrographs represent ipsilateral i.e. treated side (A) Sham injected, compared to blank control (B), (C) 6-OHDA only group compared to 6-OHDA blank control (D), (E) 6-OHDA+EX4 group compared to its blank control (F). Scale bars = 30μm.
Figure 5.1(b): GLP-1R expression in the LVW of the rat brain. DAB cell count along the wall of the ventricle was carried out manually along the wall of the ventricle and expressed as numerical density. Results were analyzed using one way ANOVA and a post hoc Bonferroni's test to compare each group to each group. *indicates significant difference compared to control (ANOVA F[3,20] = 48.71, *p < 0.001, n=6 per group, confidence interval set at 95%)
5.2 BrdU uptake in the LVW of rats

BrdU was co-administered to different treatment groups to investigate neurogenesis in the rat SVZ. DAB staining was used to visualise BrdU antibody binding. Results were expressed as numerical density calculated as DAB positive cells per 300μm distance each time starting in the dorsolateral corner of the ventricle. Blank system containing no primary antibody was used as control in these experiments.

According to our findings the blank control groups containing no primary antibody had the lowest number of BrdU positive cells in the rat LVW 0.0016 ± 0.0016 cells. In sham surgery controlled animals the density was 0.188 ± 0.018 cells which was more than 100 fold higher compared to blanks. In 6-OHDA only treated animals the density was 0.24 ± 0.01 and was significantly higher than in sham groups. 6-OHDA and EX-4 only treated animals had an almost 2 fold decrease in BrdU positive cells compared to 6-OHDA only treated animals. Treatment with a DA D3-receptor antagonist nafadotride caused a significant increase of BrdU positive cells in 6-OHDA and EX-4 groups co-treated with this antagonist. Interestingly, groups receiving EX-4 on its own had the lowest numbers of BrdU positive cells 0.055 ± 0.007 cells compared to all groups except the blank control. According to our data treatment with 6-OHDA caused an increase in BrdU positive cells in the LVW of rats and EX-4 caused a decrease which was reduced when animals were also given nafadotride. Photomicrographs are shown in 5.2(a) and data is demonstrated in 5.2(b).
Figure 5.2(a): Photomicrographs of selected 12μm cryostat sections through the rat LVW immunoassayed for BrdU. Cells along the LVW appear dark brown in colour visualised using DAB staining. All micrographs represent ipsilateral i.e. treated side; (A) Blank compared to (B) sham injected group. (C) 6-OHDA only compared to (D) 6-OHDA and EX4 group. (E) EX4 only treated group. (D) 6-OHDA and EX4 treated group compared to 6-OHDA and EX4 in the presence of D3-receptor (F) antagonist nafadotride. Scale bars = 100μm.
Figure 5.2(b): Effect of 0.5μg/kg of EX-4 on BrdU positive cells in the LVW of 12μm coronal sections through rat brain. DAB cell count along the wall of the ventricle was carried out manually.

Results were analyzed using one way ANOVA (F value = 445.3) and a post hoc Bonferroni test to compare relevant treatment groups.

*indicates significant difference compared to blank. +indicates significant difference compared to sham. ^indicates significant difference compared to 6-OHDA only treated group. ***indicates significant difference compared to all other groups. “indicates significant difference compared to 6-OHDA and EX4 only treated group (ANOVA F[5,30] = 445.3, *p < 0.001, +p < 0.001, ^p < 0.001, ***p < 0.001, “p < 0.001, n=6 per group, confidence interval set at 95%)
5.3 Summary of findings

1. Using immunohistochemistry GLP-1Rs were shown to be expressed in the LVW of rats. Cell staining remained unchanged with different treatments including shams, 6-OHDA only and 6-OHDA and EX-4 groups suggesting receptor expression is not affected by any of the treatments.

2. 6-OHDA treatment increased the numbers of BrdU positive cells found in the LVW of rats. This change was reduced when animals were given EX-4 and increased again when co-treated with DA D3-receptor antagonist nafadotride.
Chapter 6

$D_3$-receptor is involved in the mechanism of action of EX-4
Introduction and experimental details

Previous chapters demonstrate evidence of neuroprotective effects of EX-4 in two rodent models of PD. There is also accumulating literature evidence suggesting that stimulation of D_3 receptors provides neuroprotection in the same animals models PD. This subtype of DA receptors is also linked to proliferation of neural stem cells. Data from our own experiments presented in the previous chapter looking at BrdU uptake in the SVZ demonstrated that BrdU uptake decreased in the presence of EX-4 but increased in the presence of nafadotride which selectively block D_3 receptors. The aim of this chapter was to look at protective effects of EX-4 with and without co-administration of a selective D_3 receptor blocker nafadotride. The experimental design was the same as in the case of GLP-1 receptor antagonist EX-9-39. Rats were initially lesioned with 6-OHDA to induce Parkinsonism. One week later animals were given either EX-4 on its own or EX-4 preceded by 1mg kg^{-1} dose of nafadotride. Treatment lasted for one week, twice daily as before and the animals were assessed using the same behavioural, neurochemical and histological tests described in previous chapters. Care was taken to minimize the use of experimental animals and in this chapter 45 animals were used in total.
Chapter 6: \textit{D}_3\text{-receptor is involved in the mechanism of action of EX-4}

6.1 Effects of \textit{D}_3\text{-receptor antagonist nafadotride in 6-OHDA and EX-4 treated rats}

6.1.1 Apomorphine induced circling

Here, apomorphine induced circling was tested in groups co-treated with a \textit{D}_3\text{-receptor antagonist nafadotride as well as shams and toxin only treated rats. Results were presented as numbers of full contralateral turns counted over 120 second period. Again, 6-OHDA only treated animals demonstrated very pronounced contralateral turning behaviour tested two weeks after toxin administration 23 ± 3 compared to sham surgery controlled animals 0.1 ± 0.1 turns. This was again significantly reduced when animals were co-treated with EX-4 at 0.5µg/kg\textsuperscript{-1} dose 3 ± 1 which was a reduction of almost 90%. Groups co-treated with nafadotride 1mg kg\textsuperscript{-1} together with EX-4 showed 17 ± 2 turns on average which was almost the same as 6-OHDA only treated groups and there was no significant difference between these statistically. There was only a 26% reduction in the numbers of contralateral turns in these nafadotride co-treated groups compared to 6-OHDA and EX-4 only treated animals which had almost 90% indicating that the protective effect of EX-4 was reduced in the presence of the antagonist. Nafadotride administered to naïve animals did not have any effect on apomorphine induced turns 0.1 ± 0.1. Data are shown in Figure 6.1.1
Figure 6.1.1: Effect of EX-4 (0.5μg/kg) in the presence of nafadotride (1mg/kg) on apomorphine-induced rotational behaviour in 6-OHDA lesioned rats. Nafadotride was co-administered with EX-4 twice daily for seven days, one week after toxin injection. Circling was measured for 120s 15 min after apomorphine injection. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare differences between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA only. ^indicates significant difference compared to 6-OHDA+EX4 group (ANOVA F[4,25] = 40.46, *p < 0.001, +p < 0.001, ^p < 0.001, n=6 per group, confidence interval set at 95%)
6.1.2 In vivo microdialysis

Here the effect of nafadotride in 6-OHDA and EX-4 treated animals was assessed by measuring the amount of both basal and K⁺ evoked extracellular DA. Figure 6.1.2 demonstrates DA levels measured in the STR in 30 minute intervals over four hours. Data were plotted as amounts of DA in fmols per 10μl HPLC injection volumes over time as before. This time basal DA levels in the sham surgery control groups were approximately 40 ± 8 fmols/10μl injection samples, increasing 25 fold to 1015 ± 122 fmol when stimulated with 100mM KCl. DA levels returned to baseline on removal of high K⁺ stimulus. In 6-OHDA only rats basal DA was reduced to 8.5 ± 2.5 which was an almost 5 fold reduction and K⁺ induced DA also decreased to 75 ± 26 fmol which was a 13 fold reduction compared to control groups. Animals lesioned with 6-OHDA and then co-treated with EX-4 0.5μg kg⁻¹ for one week show recovered levels of DA both basal 33 ± 10 fmol which increased almost 4 fold and K⁺ evoked 970 ± 94 which increased over 10 fold compared to 6-OHDA lesioned animals. 6-OHDA and EX-4 rats co-treated with nafadotride at 1mg kg⁻¹ show a decrease in extracellular DA both basal 5 ± 1 fmol which is an over 6 fold reduction and K⁺ induced 99 ± 17 fmol which is almost a 10 fold decrease compared to 6-OHDA and EX-4 only groups. In naïve animals given nafadotride extracellular DA levels are not significantly different from shams at both basal 29 ± 6 fmol and K⁺ induced 983 ± 67 fmol.
Figure 6.1.2: Effect of EX-4 (0.5μg/kg) in the presence of Nafadotride (1mg/kg) on amount of extracellular DA in 6-OHDA lesioned rats as measured by in vivo microdialysis. Nafadotride was co-administered with EX-4 twice daily for seven days, one week after toxin injection. Perfusion rate was 1μl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K⁺. Results were analyzed using two way ANOVA and a post hoc Bonferroni multiple comparisons test. *indicates significant difference compared to 6-OHDA+EX4 group (ANOVA between treatments $F[28,200] = 20.22$ and time $F[7,200] = 223.29$, *$p < 0.001$, n=6 per group, confidence interval set at 95%).
6.1.3 Striatal tissue DA levels

Striatal tissue DA levels were estimated in groups co-treated with nafadotride. Data demonstrated in Figure 6.1.3 shows that in sham surgery controlled groups the average amount of DA was 7.3 ± 1.7 pg/g of striatal tissue. In 6-OHDA only treated groups tested after two weeks the amount of DA decreased to 0.5 ± 0.4 pg/g which was an over 90% reduction compared to control. In groups co-treated with EX-4 at 0.5μg kg⁻¹ the total amount of DA was increased to 6.4 ± 0.47 pg/g which was a near 90% recovery and was significantly different from 6-OHDA only treated groups. In 0.5μg kg⁻¹ EX-4 and 1mg kg⁻¹ nafadotride groups the amount of DA was decreased by 60% to 2.6 ± 0.53 pg/g which was significantly different from 6-OHDA and EX-4 treated groups. Groups which were treated with nafadotride only did not show significant changes in their striatal DA which was 7.5 ± 0.9 pg/g and were comparable to controls.
Chapter 6: D₁-receptor is involved in the mechanism of action of EX-4

Figure 6.1.3: Effect of EX-4 (0.5 µg/kg) in the presence of Nafadotride (1 mg/kg) on amount of striatal DA in tissue homogenates of 6-OHDA lesioned rats. Nafadotride was co-administered with EX-4 twice daily for seven days, one week after toxin injection. Striatal tissue DA was measured using HPLC with ECD. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare differences between groups. * indicates significant differences compared to control. + indicates significant difference compared to 6-OHDA only. ^ indicates significant difference compared to 6-OHDA + EX4 (ANOVA F[4,25] = 11.29, *p < 0.001, +p < 0.001, ^p < 0.05, n=6 per group, confidence interval set at 95%)
6.1.4 TH enzyme assay

TH enzyme activity was measured in 6-OHDA, EX-4 and nafadotride groups. Data were plotted as amounts of striatal L-DOPA in pmol min\(^{-1}\) mg\(^{-1}\) protein. In sham surgery controls the amount of L-DOPA was 81 ± 9 pmol min\(^{-1}\) mg\(^{-1}\) compared to just 6 ± 2 pmol in 6-OHDA only treated groups after two weeks. This was an over 13 fold reduction. Twice daily injections of EX-4 0.5µg kg\(^{-1}\) increased the amount striatal L-DOPA to 63 ± 9 pmol which was a near 80% recovery compared to 6-OHDA only lesioned animals. In 6-OHDA and EX-4 groups also co-treated with nafadotride the amount of striatal L-DOPA was decreased 27 ± 7 pmol min\(^{-1}\) mg\(^{-1}\) which was a near 60% reduction and was significantly different compared to 6-OHDA and EX-4 only groups. Groups treated with nafadotride alone did not show any significant changes in their L-DOPA levels 77 ± 16 pmol min\(^{-1}\) mg\(^{-1}\) compared to controls. Data are shown in Figure 6.1.4
Figure 6.1.4: Effect of EX-4 (0.5μg/kg) in the presence of nafadotride (1mg/kg) on amount of striatal L-DOPA formation of 6-OHDA lesioned rats. Nafadotride was co-administered with EX-4 twice daily for seven days, one week after toxin injection. Striatal L-DOPA was measured using HPLC with ED. Results analyzed using one way ANOVA (f value = 11.49) and a post hoc Bonferroni’s test to compare differences between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA only. ^indicates significant difference compared to 6-OHDA+EX4 (ANOVA F[4,25] = 11.49, *p < 0.001, +p < 0.001, ^p < 0.05, n=6 per group, confidence interval set at 95%)
6.1.5 TH immunohistochemistry

TH immunostaining was measured in groups co-treated with the D₃ receptor antagonist. Images which represented control relatively high TH immunostaining represented by high numbers of DAB labelling in the SN region. This was reflected by relatively high colour intensity values 27385 ± 4735 units on average assigned by the software when these images were analyzed. Groups treated with 6-OHDA and tested after two weeks showed an over 6 fold decrease in intensity 4372 ± 2096 compared to control groups reflected by a decrease in DAB staining on the slides. Groups co-treated with EX-4 0.5μg kg⁻¹ displayed an increase in DAB labelling reflected by the intensity of relevant images 21642 ± 3975 units which indicated a near 80% recovery in TH expression and was significantly different from 6-OHDA only treated animals. In 6-OHDA and EX-4 groups which also received nafadotride at 1mg kg⁻¹, the intensities were decreased to 5680 ± 2854 units concomitant with TH staining. Groups which were treated with nafadotride alone are indistinguishable from controls and their intensity values are insignificant from control groups statistically 26312 ± 5283 units. Photomicrograph examples are presented in Figure 6.1.5(a) and data are shown in Figure 6.1.5(b).
Figure 6.1.5(a): Photomicrographs of selected 12μm cryostat sections through the rat SN immunoassayed for TH. Nigral cell bodies appear dark brown in colour visualised using DAB staining. The right side is ipsilateral i.e. treated and left is contralateral i.e. untreated. (A) and (B) represent a control group. (D) represents 6-OHDA lesion assessed after two weeks compared to control (C). (F) shows the effect of 0.5μg/kg exendin-4 in 6-OHDA treated animals one week post lesion compared to control (E). (H) demonstrates the effect of nafadotride co-administration in 6-OHDA+EX-4 treated animals compared to control (G). Scale bars – 100μm.
Figure 6.1.5(b): Effect of nafadotide (1mg/kg) on TH expression in the SN visualised using DAB in 6-OHDA and EX-4 treated rats. Nafadotide was administered twice daily for seven days together with EX-4, one week after 6-OHDA injection. TH immunostaining was quantified using Lucia G image analysis software based on colour intensity. Results analyzed using one way ANOVA (f value = 8.00) and a post hoc Bonferroni’s test to compare difference between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA only group. ^indicates significant difference compared to 6-OHDA + EX-4 group (ANOVA F[4,25] = 8.00, *p < 0.01, +p < 0.05, ^p < 0.05 n=6 per group, confidence interval set at 95%)
Chapter 6: **D<sub>1</sub>-receptor is involved in the mechanism of action of EX-4**

6.2 *Summary of findings*

1. 6-OHDA and EX-4 groups given DA D<sub>1</sub>-receptor antagonist nafadotride displayed increased numbers of apomorphine induced contralateral turns compared to 6-OHDA and EX-4 only treated animals.

2. Nafadotride treatment decreased the amount of extracellular DA in 6-OHDA and EX-4 groups of animals measured using *in vivo* microdialysis. There was no change in DA levels when antagonist was given to untreated animals.

3. Striatal tissue DA was also decreased in groups co-treated with nafadotride compared to 6-OHDA and EX-4 only treated animals. Again, nafadotride only groups displayed unaltered levels of striatal DA.

4. Amounts of L-DOPA measured in striatal homogenates were also significantly reduced in 6-OHDA and EX-4 treated animals co-treated with nafadotride but not the vehicle only treated groups. There was no significant change in TH activity in nafadotride only treated animals.

5. Finally, TH immunohistochemistry shows the same pattern. 6-OHDA and EX-4 only treated groups are comparable to controls whereas when co-treated with nafadotride the level of TH expression in the SN was reduced. Animals given nafadotride only had no change in their TH expression in SN compared to controls.
Chapter 7

Discussion and future work
Chapter 7: Discussion and future work

7.1 Discussion

The aim of this study was to investigate whether EX-4 was neuroprotective when given one week after toxin injection in two distinct in vivo rodent models of PD based upon accumulating literature evidence of its neuroprotective properties both in vitro and in vivo and its potential to treat neurodegenerative disorders (Bertilsson et al., 2008; During et al., 2003; Kim et al., 2009; Li et al., 2009; Perry et al., 2002a; Perry et al., 2002b; Perry et al., 2007). The results of this work clearly demonstrate that EX-4 has a potent neuroprotective effect in both the 6-OHDA and the LPS models of PD. The important factor to note about this work is that here we have only started treatment with EX-4 one week after toxin administration unlike most neuroprotective therapies previously and currently researched which have to be given either at the same time or in some cases prior to the toxin. This is very important as obviously when PD patients are present into the clinic most of the DA innervation is already lost and drugs which provide some degree of neuroprotection in animal models after pre-treatment would be of little benefit clinically. As first demonstrated in chapter 3, treatment with both doses of EX-4 seven days after toxin administration resulted in decreased apomorphine induced contralateral turning behaviour. Such turning indicates a DA imbalance between two sides of the brain. The drug appears to be very potent as the doses given to these hemiparkinsonian 6-OHDA and LPS lesioned rats were in μg quantities. These were chosen based on doses used in animals models of type 2 diabetes and are comparable to the ones used in human diabetic patients for treatment clinically (Drucker et al., 2008). In the present study rats were given one week for the lesion to develop following toxin administration in both models. This resulted in approximately
60-70% loss of striatal DA and was followed by administration of EX-4 twice daily for another week after which animals were assessed using various paradigms. Other groups which carried out similar behavioural tests with EX-4 left animals to develop 6-OHDA lesions for five weeks after which EX-4 was injected for the duration of 3 weeks (Bertilsson et al., 2008). The results from these experiments also demonstrated a reduction but in amphetamine induced turning behaviour with EX-4 compared to vehicle only treated animals in which the circling persisted. Amphetamine causes DA release from the nerve terminals unlike apomorphine which directly stimulates DA receptors, therefore in the case of amphetamine, lesioned animals display ipsiversive turning behaviour i.e. towards the side of the lesion. But it still demonstrates an imbalance of DA innervation and this behaviour when reduced suggests normalisation of DA balance. Although apomorphine and amphetamine induced rotation tests are often used in unilateral lesion models of PD, some authors argue that these are not reliable indicators of nigrostriatal DA depletion (Chang et al., 1999). As described by Chang and colleagues the adjusting steps test which is an indicator of akinesia is able to pick up smaller lesions where DA content of the STR is reduced by 60-80% in contrast to apomorphine rotations which are only evident when there is > 90% DA depletion in the STR. This fits nicely with the situation in the clinic where patients only start experiencing symptoms when striatal DA content drops below approximately 60%. Also, adjusting steps task may be especially useful in an experiment where there is a possibility of physically damaging the STR like in transplant studies. Such damage would further exacerbate stepping test deficits but will reduce drug induced rotations due to damage of postsynaptic receptors in the STR (Barker et al., 1994).
Chapter 7: Discussion and future work

In addition to our data with apomorphine which indicated a behavioural recovery in groups co-treated with EX-4, measuring amounts of extracellular DA in the STR using \textit{in vivo} microdialysis indicated increased DA levels both basal and K$^+$ evoked in the striata of rats co-treated with EX-4 compared to 6-OHDA or LPS only treated groups. Restoration of extracellular DA indicates a potential anti parkinsonian effect of EX-4 since PD is characterised by loss of DA in the STR. The fact that both basal and K$^+$ evoked levels of DA are recovered with EX-4 treatment is interesting and may suggest that there is an increase in a number of functional DA neurones which release DA in the STR. The whole tissue DA levels estimated in striatal homogenates from these groups of animals indicate an increase in total striatal DA in 6-OHDA groups receiving EX-4 but not the vehicle groups. The amounts of L-DOPA measured in striatal homogenates pre-incubated with L-tyrosine indicates the activity of TH enzyme which is the rate limiting step in DA synthesis and is an important measure of functional capacity of DA neurons. Groups co-treated with EX-4 showed increased amounts of TH activity compared to 6-OHDA only treated groups, supporting previous behavioural and dialysis data. When investigating the SN using immunohistochemistry it was found that groups co-treated with EX-4 had increased levels of TH expression in the region compared to 6-OHDA or LPS only treated animals. All of these findings cumulatively suggest that EX-4 may be a useful compound for treating neurodegenerative disorders such as PD and Alzheimer’s disease. Although TH expression studies fit well with behavioural and neurochemical indicators it is unclear what exactly happens with SN neurons in our studies. EX-4 treatment is started 7 days after the initial toxin administration at a point where the level of TH expression is approximately 30% compared to that of controls. However the data does not inform if the cells are killed by
the toxin. There is evidence to suggest that in the 6-OHDA MFB model of PD, the injection of the toxin results in rapid loss of DA phenotype with actual cell loss occurring over a much longer time period (Bowenkamp et al., 1996; Jeon et al., 1995). For example Bowenkamp and colleagues used both TH immunolabelling and a retrograde neuronal tracer fluorogold to label neurons of the SN after 6-OHDA exposure. It was shown that when there was 85% loss of TH expression in the SN, the actual cell loss demonstrated via fluorogold staining was in fact only 50%. Therefore it likely that 6-OHDA instead of killing cells causes a phenotypic shift meaning cells are still alive but not producing TH whereas EX-4 may reverse this especially taking into account evidence that EX-4 is able to cause rapid TH expression in catecholamine neurons of the area postrema (Yamamoto et al., 2003). To address this it would be interesting to immunostain adjacent sections of SN for a suitable neuronal marker e.g. NeuN in both 6-OHDA only and EX-4 co-treated groups. This would help clarifying what happens both after toxin administration at different time points and EX-4 mediated recovery of TH expression. Alternatively, instead of injecting 6-OHDA in the MFB it could be injected in the STR. The striatal 6-OHDA model of PD is widely used to assess neuroprotective therapies and it has been shown that in this model albeit at a slower rate it is cell death that occurs rather than simply relatively rapid loss of TH expression (Sauer et al., 1994).

Before its FDA approval in 2005 and introduction into the clinic for diabetes, EX-4 was extensively studied in animal models of the condition. Its mechanism is thought to be via the activation of GLP-1R on the surface of β-islets of the pancreas resulting in glucose dependent insulin secretion. The next chapter addressed the second question
raised in the aims section of this thesis where it was investigated if GLP-1R are responsible for EX-4’s neuroprotective properties. The same experiments were performed, but this time in the presence of a specific GLP-1R antagonist EX-9-39. The antagonist experiments were carried out in the MFB 6-OHDA model of PD. It was chosen because it is a harsher model compared to LPS and mimics end stage of the human condition. The dose of antagonist was chosen based on research in diabetes models (Goke et al., 1993). Treatment groups receiving both EX-4 and EX-9-39 did not show a reduction in apomorphine induced turns unlike groups treated with 6-OHDA and EX-4 only. This was also reflected by the amount of extracellular DA which was reduced in groups co-treated with EX-9-39 but not in 6-OHDA and EX-4 only groups. The same pattern was seen when estimating total striatal DA in brain homogenates of these groups. Also when incubating L-tyrosine with these brain homogenates, L-DOPA levels were reduced in groups co-treated with antagonist suggesting that the neuroprotective effects of EX-4 were GLP-1R dependent.

The third question raised in the aims section concerns the fact that EX-4 was recently proposed to promote neurogenesis in the rat SVZ via GLP-1R activation. Authors suggested the presence of GLP-1Rs in the wall of the rat lateral ventricles (Bertilsson et al., 2008). Using immunochistochemistry the presence of GLP-1Rs in the rat lateral ventricular wall in different treatment groups of rats was investigated. Immunostaining indicated presence of GLP-1Rs in the LVW of the rat brain. Also, according to our data the numbers of antibody labelled cells did not change in any of the treatment groups suggesting receptor expression is not affected by any of the drugs that were administered. On the other hand, when these treatment groups were investigated using
an anti BrdU antibody for cell proliferation in the LVW, an interesting pattern was evident. 6-OHDA treatment resulted in an increase of BrdU labelled cells in the LVW of rats whereas EX-4 treatment caused a decrease which was blocked by co-administration of a DA D3-receptor antagonist nafadotride. Taking into account the findings from previous chapters demonstrating EX-4 neuroprotective effects and reports that it might promote neurogenesis, these data suggest that EX-4 could cause neural stem cell (NSC) migration, possibly to repopulate the damaged SN. It seems extremely unlikely but would be remarkable. The proliferation of NSCs in neurogenic regions of the brain is an important process which appears to be affected by neurodegenerative disorders and in future may offer an attractive approach for therapeutic intervention. There is accumulating evidence in the literature that NSCs are able to migrate great distances in the adult rodent brain, in addition this migration appears to be directed towards CNS injury (Belmadani et al., 2006; Imitola et al., 2004) however at the moment the idea that NSCs become functional neurones is still debatable. In the 6-OHDA model for example, there is a transient decrease in neurogenesis in the granule cell layer of the olfactory bulb but in contrast there is an increase in the glomerular cell layer. The increased neurogenesis in the glomerular layer is characterized by more newly generated neurones co-expressing NeuN and TH (Mohapel et al., 2005). Other authors detected increases in cell proliferation in the rat SN which had their MFB lesioned with 6-OHDA (Frielingsdorf et al., 2004) however none of those cells displayed a dopaminergic phenotype and there is no evidence for NSCs migrating from the SVZ to the SN. Growth factors like platelet-derived growth factor (PDGF) and BDNF can induce striatal neurogenesis in 6-OHDA treated rats, but again there is no evidence that these newly generated cells differentiate into
dopaminergic neurones (Mohapel et al., 2005). Interestingly, it was recently demonstrated that in a salamander 6-OHDA model of PD there was robust and complete regeneration of midbrain DA system. Authors also specifically demonstrated that 6-OHDA administration eliminates DA neurones and not just turns off their phenotype (Parish et al., 2007). Regeneration was demonstrated histologically and also lead to a full behavioural recovery. This 6-OHDA salamander model gives new insight into animal models of PD and may help understand mechanisms of DA cell neurogenesis. Going back to our own data, an interesting fact is that a decrease in BrdU uptake in the LVW caused by EX-4 was reduced by a selective D3-receptor antagonist nafadotride. Accumulating literature evidence suggests that DA regulates adult neurogenesis through D3-receptors and these are also expressed in early brain development. Moreover D3-receptor agonists were found to be neuroprotective in animal models of neurodegenerative disorders (Borta et al., 2007; Carvey et al., 2001; Van Kampen et al., 2006; Van Kampen et al., 2004). There are of course controversies regarding using BrdU to demonstrate neurogenesis. Because it is a toxic and mutagenic substance there are pitfalls to consider. For example it triggers cell death, formation of teratomes, alters DNA stability, lengthens the cell cycle and has mitogenic, transcriptional and translational effects on the cells that incorporate it. BrdU is not in fact a marker of cell proliferation but of DNA synthesis (Taupin, 2007). To get more conclusive results other neuronal markers such as proliferating cell nuclear antigen (PCNA) and a marker for immature neurons doublecortin (DCX) could be used in conjunction with BrdU to demonstrate changes in proliferating cells.
Chapter 7: Discussion and future work

The next rational step was to address the last question raised in the aims sections of this thesis and test if the D₃-receptor antagonist nafadotride blocks the neuroprotective effects of EX-4 in 6-OHDA model of PD. Groups of animals co-treated with nafadotride showed increased apomorphine induced turns compared to 6-OHDA and EX-4 only groups. Nafadotride co-treated groups also had reduced levels of extracellular DA compared to 6-OHDA and EX-4 only treated groups. The same pattern was seen with total striatal DA estimated in brain homogenates. Also amounts of L-DOPA estimated in striatal homogenates after incubation with L-tyrosine was reduced in groups co-treated with nafadotride as well as levels of TH expression in the SN of these groups compared to 6-OHDA and EX-4 only treated animals. All of these findings strongly suggest that D₃-receptor activation is needed for neuroprotective effects of EX-4 in the 6-OHDA model of PD. This coincides well with accumulating evidence that activation of D₂-receptors is neuroprotective in animal models of neurodegenerative disorders. Overall these experiments demonstrate that EX-4 was neuroprotective in two distinct models of PD and the mechanism of action of EX-4 does not only depend on GLP-1R activation but also D₃-receptors.

It is believed that EX-4’s mechanism of action is not simply the activation of one or two receptor subtypes. It is probably complex and multimodal. Preliminary data shown in Figure 7.1.1 suggest that the mechanism of action of EX-4 may also involve activation of corticotrophin-releasing factor-1 receptor (CRF-R₁) since administration of a selective antagonist to 6-OHDA and EX-4 groups reduced the neuroprotective effect of EX-4 demonstrated by an increase in apomorphine induced turns in groups receiving 6-OHDA, EX-4 and the selective CRF-R₁ antagonist NBI-27914 compared to
6-OHDA and EX-4 only treated animals. These data are interesting because of recent findings that urocortin (UCN) an endogenous peptide which is a non selective CRF receptor agonist, was neuroprotective in the same rodent models of PD and its mechanism was CRF-R₁ receptor dependent (Abuirmeileh et al., 2008; Abuirmeileh et al., 2007). Data shown here suggest that both EX-4 and UCN may utilise a common mechanism involving CRF-R₁ receptors.
Figure 7.1.1: Effect of EX-4 (0.5μg/kg) in the presence of NBI-27924 (1mg/kg) on apomorphine-induced rotational behaviour in 6-OHDA lesioned rats. NBI-27914 was co-administered with EX-4 twice daily for seven days, one week after toxin injection. Circling was measured for 120s 30 min after apomorphine injection. Results analyzed using one way ANOVA and a post hoc Bonferroni’s test to compare differences between groups. *indicates significant differences compared to control. +indicates significant difference compared to 6-OHDA only. ^indicates significant difference compared to 6-OHDA+EX4 group (n=6 per group, confidence interval set at 95%)
GLP-1Rs are expressed in both rodent and human CNS, but are especially abundant in the hypothalamus where they are thought to regulate food intake (During et al., 2003). Taking into account the current findings it is possible that EX-4 might work by activating GLP-1Rs in the hypothalamus which in turn would cause a release of CRF activating CRF-R1 receptors on nigral neurones promoting their survival. The release of CRF would also cause stress-induced DA release due to the stimulation of hypothalamic-pituitary-adrenal axis (Sarkar et al., 2003) which might lead to increased DA receptor stimulation including the D3-type which is involved in neuroprotection. As mentioned previously, EX-4 was shown to reduce food intake via hypothalamic GLP-1R stimulation. This is interesting because there is accumulating evidence in the literature suggesting that diet restriction in laboratory animals is neuroprotective (Mattson, 2000; Plunet et al., 2008; Sharma et al., 2005). Authors report an upregulation of oxyradical defences such as superoxide dismutase, glutathione peroxidase and increased expression of heat shock protein 70 in animals kept on restricted diets and also an increase in brain derived neurotrophic factor (BDNF) expression which promotes neuronal survival via TrkB receptor activation (Lee et al., 2000). It is possible that another part of EX-4’s neuroprotective mechanism is through reducing food intake in animals leading to metabolic changes similar to animals kept on restricted diets. Therefore EX-4 might work through GLP-1Rs present in the SVZ, where it was proposed to stimulate neurogenesis (Bertilsson et al., 2008) as well as through multiple metabolic changes induced by reduced food intake. The diet restriction hypothesis ties in well with the preliminary data shown in Figure 7.1.1 suggesting that EX-4’s protective mechanism involves CRF-R1 receptors. BDNF
expression associated with reduced food intake would also benefit neuronal survival. Possible mechanisms of EX-4 are summarised in Figure 7.1.2.

Figure 7.1.2: Proposed mechanism of action of exendin-4 in the adult rat brain. EX-4 directly activates GLP-1Rs in the SVZ promoting neurogenesis. EX-4 may also cause CRF release from paraventricular nucleus (PVN) of the hypothalamus (HT) via GLP-1R, CRF protects SN neurones via CRF-R1. Stress-induced release of DA from SN neurons and other parts of CNS activates all DA receptor types. Food intake is reduced via hypothalamus simulating dietary restriction (DR), this leads to upregulated BDNF expression. BDNF promotes neuronal growth and survival via TrkB receptor activation. This also leads to more DA release activating all receptor subtypes including D3-receptor and promotes neuronal differentiation.
7.2 Future work

The obvious question after looking at these findings is how exactly does the drug work? The present studies have addressed some aspects of the mechanism of action but there are still questions that remain unanswered. For example, in the present series of experiments the GLP-1R antagonist was injected systemically, so in theory it would block all the GLP-1Rs both central and peripheral. Our findings demonstrate that administration of EX-9-39 blocks the protective effects of EX-4. The question arises, which receptors are involved in the mechanism of action of EX-4? Is it the pancreatic receptors, the central hypothalamic receptors or GLP-1Rs expressed in the LVW or all of them? To address this question, EX-4 could be injected stereotaxically directly into the STR and/or the SN and its protective potential investigated using this experimental protocol. Another approach to distinguish between central and peripheral GLP-1R activation would be to use an antagonist which does not cross the BBB and assess the protective properties of EX-4 in the presence of such antagonist. Alternatively it would also be interesting to use a GLP-1R agonist which does not cross the BBB and investigate if it has similar neuroprotective properties to EX-4.

Our data suggest that nigrostriatal system integrity may be restored when EX-4 is administered to 6-OHDA treated animals. Work using BrdU labelling of newly divided cells demonstrated that EX-4 reduced these in the SVZ of rats. Assuming the reduction is due to EX-4 the question is where do these cells go? Could they migrate to repopulate the damaged SN? This question is not easily addressed using BrdU labelling, since there is no way of tracking migrating neurons using this method and our
obvious approach to look at BrdU changes in the STR and SN of EX-4 treated animals did not give a positive result. When treating animals with BrdU systemically, all newly divided cells will take up the marker. Therefore it was hypothesised that 6-OHDA and EX-4 groups in which there was recovery of DA imbalance would have higher numbers of BrdU labelled cells in the SN. Clearly, our data do not support the theory in this case as no BrdU changes were observed in the SN in these groups. This gives further support to the idea that EX-4 in fact just rescues existing neuronal populations in the SN which were "stunned" by the neurotoxin. This would explain why there was no BrdU labelling. The idea that increased TH expression in the SN of 6-OHDA and EX-4 treated animals is due to de novo neurogenesis, is of course extremely unlikely considering the time course of experimental design in this study and evidence suggesting 6-OHDA causes phenotypic shift rather than actual cell loss in such short time periods. Therefore it is more likely that the neuroprotective effect of EX-4 is attributable to its anti-inflammatory properties (Li et al., 2003).

Although from our results it is unclear if EX-4 has any neurogenic effects it is interesting that BrdU uptake in the LVW appears to change with EX-4 in a D<sub>1</sub>-receptor dependent manner. One possibility of course is that this increase is just a CNS response to injury since groups treated with 6-OHDA only had the highest numbers. In groups which were co-treated with EX-4 a reduction may just indicate that EX-4 repairs some of the 6-OHDA induced damage and hence a decrease in BrdU uptake in the SVZ is seen. As mentioned already BrdU is far from an ideal method to measure neurogenesis and results should be interpreted with care. Other cell proliferation markers should be used alongside BrdU to back up the data e.g. PCNA and DCX.
Chapter 7: Discussion and future work

It was found that blocking D3-receptors reduced the neuroprotective effects of EX-4. Similar to experiments with the GLP-1R antagonist, nafadotride was also injected systemically and although it is more intuitive that the central receptors are involved in the mechanism, more work needs to be done to establish the precise location of D3-receptors involved in the mechanism of action of EX-4. This could be resolved by trying approaches described in this section earlier for GLP-1Rs. Another interesting avenue one could pursue is to test the effect of EX-4 in aged animals which would mimic the situation of PD in the clinic slightly better.

7.3 Final remarks

The major limitation of this work is that it fails to demonstrate exactly how EX-4 produces its effects and whether it works via neuroprotection or neurogenesis. However the body of data demonstrating its remarkable effects in different models of PD is growing and cannot be ignored. Finally, results presented in this thesis combined with studies of Bertilsson et al., 2008 based in Sweeden, work of Perry et al., in the US over the past 7 years and more recently studies of Kim et al., 2009 have led to the first EX-4 clinical trial with support of The Cure Parkinson’s Trust, UK and the Michael J. Fox Foundation. Trial is currently being set up and is scheduled to start before the end of this year. It will be exciting to see if EX-4 has any therapeutic benefits in human PD subjects however if there are no benefits with EX-4, serious questions will be raised regarding the validity of current animal models.
Reference list


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