THE CRF-LIKE PEPTIDE UROCORTIN REVERSES KEY INDICATORS OF NIGROSTRIATAL DAMAGE IN RODENT MODELS OF PARKINSON'S DISEASE

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Declaration

This thesis describes research conducted in the School of Pharmacy, University of London between October 2005 and July 2008 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 by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

Date 27.9.2008

Abstract

Parkinson's disease (PD) is an irreversible neurodegenerative disorder with no cure and current treatments have limited efficacy and unpleasant side effects. The aetiology of PD is complex involving apoptotic, excitotoxic, free radical mediated and inflammatory events. This work involved studying the effect of the corticotrophin releasing factor (CRF)-like peptide urocortin (UCN) on two distinct rodent models of PD, the well established 6-OHDA rat model and the more recently introduced LPS model. UCN's effects were examined in these models immediately after the lesion was induced and also after the lesion had significantly progressed. The mechanisms of action for UCN were examined through the use of selective CRF-R2 agonists UCNII and UCNIII to determine whether they exerted the same actions as UCN. The non selective antagonist α-helical CRF was also used in conjunction with UCN to examine whether reversed the effect of UCN. Another selective CRF-R1 antagonist, NBI-27914, was also used with UCN to confirm those findings.

Experiments were carried out in rats, and the effects of the different treatments used were evaluated through the use of multiple assessments. When UCN was given at the same time as 6-OHDA or LPS, this peptide reversed/restored key indicators of PD-Like damage. These included behavioral effects, loss of tissue and extracellular dopamine, loss of tyrosine hydroxylase (TH) activity and protein levels, in addition to the loss of TH immuno-reactivity in nigral sections.

The effects of reversing/restoring these key indicators of PD-like damage were also observed when UCN was given seven days after 6-OHDA or LPS treatment, when the lesion had significantly progressed.

When the pharmacology of the UCN phenomenon was studied using CRF receptor agonists and antagonists, it was determined that UCN was acting via CRF-R1 receptors, a finding which may prove significant for development of new medications and exploring CRF-R1 as a new target in PD treatment. It was concluded that an extension of these observations to the clinic could have potential benefits for PD patients.

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Dedication

FOR MY PARENTS

Quotation

"IF I DON'T DO IT SOMEBODY ELSE WILL"

PETER CLIFF   1946- 1992

Publications

The following publications have resulted from original work carried out during the course of this study:

Abuirmeileh A, Harkavyi A, Lever R, Biggs CS, Whitton PS (2007a) Urocortin, a CRF-like peptide, restores key indicators of damage in the substantia nigra in a neuroinflammatory model of PD. *J Neuroinflammation* 4(1): 19. (Appendix)

Abuirmeileh A, Lever R, Kingsbury AE, Lees AJ, Locke IC, Knight RA, Chowdrey HS, Biggs CS, Whitton PS (2007b) The corticotrophin-releasing factor-like peptide urocortin reverses key deficits in two rodent models of PD. *Eur J Neurosci* 26(2): 417-423. (Appendix)

One more paper is to be produced from this work. The expected tittle is: 'Urocortin arrests the development of parkinsonian-like features in rat via CRF-R1 receptors'

Published Abstracts

Biggs CS, **Abuirmeileh A**, Locke IC, Knight RA, Chowdrey RS and Whitton PS. Urocortin attenuates key indicators of nigrostriatal pathway destruction in rat hemiparkinsonian model. **Poster** presentation at the BPS winter meeting 2005.

Abuirmeileh A, Biggs CS, Lever R, Whitton PS. Urocortin reverses nigral DA cell loss in 6-OHDA and lipopolysaccharide treated rats. **Poster** presentation at the BPS 75th Anniversary Meeting 2006.

Abuirmeileh A, Biggs CS, Lever R, Whitton PS. Urocortin reverses parkinsonian like effects of intra-nigral lipopolysaccharide injection. **Poster** presentation at the BPS 75th Anniversary Meeting 2006.

Harkavyi A, **Abuirmeileh A**, Lever R, Biggs CS, Whitton PS. The CRF-like peptide urocortin restores striatal DA release in a neuro-inflammatory paradigm of Parkinson's disease. **Poster** presentation at the Life Sciences meeting 2007.

Abuirmeileh A, Harkavyi A, Biggs CS, Whitton PS. Urocortin restores striatal extracellular DA in the 6-hydroxydopamine hemiparkinsonian rat. **Poster** presentation at the Life Sciences meeting 2007.

A Harkavyi, **Abuirmeileh A**, Kingsbury AE, Lever R, Whitton PS.
The glucagon-like peptide 1 receptor agonist exendin-4 has neuroprotective properties in rat models of PD. **Poster** presentation at the BPS winter meetning 2007.

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Abbreviations

AADC	Aromatic acid decarboxylase
AC	Adenylate cyclase
ACSF	Artificial cerebrospinal fluid
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BH_4	Tetrahydrobiopterin
cAMP	3', 5'- cyclic adenosine monophosphate
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CRF	Corticotropin releasing factor
CRF-R	Corticotropin releasing factor receptor
DA	Dopamine
DAB	3, 3'-Diaminobenzidine
DAT	DA transporter
DDC	Dopa decarboxylase
DOPAC	3,4-Dihydroxyphenylacetic acid
GABA	Gamma aminobutyric acid
GDNF	Glial cell-derived neurotrophic factor
HVA	Homovanillic acid
i.c.	Intracerebral (injection)

i.p.	Intraperitoneal (injection)
LPS	Lipopolysaccharide
L-DOPA	L-Dihydroxyphenylalanine
MAO	Monoamine oxidase
MAPK	Mitogen activated protein kinase
MFB	Medial forebrain bundle
MPP+	1-Methyl-4-phenyl pyridinium
MPTP	Methyl-4-phenyl-1,2,3,6 tetrahydropyridine
NMDA	N-methyl d-aspartate
6-OHDA	6-Hydroxydopamine
PA	Peak area
PAR	Peak area ratio
PC12	Rat Phaeochromocytoma cell line
PD	Parkinson's disease
PFA	Paraformaldehyde
PKA	Protein kinase A
ROS	Reactive oxygen species
s.c.	Subcutaneous (injection)
SNc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
UCN	Urocortin

Chapter 1

General Introduction

1 Parkinson's disease (PD)

1.1 History

PD was first described in 1817 by James Parkinson, today it is recognized as being the second most common neurodegenerative disorder affecting 0.3% of the entire industrialized country population (de Lau *et al.*, 2006). When PD was first noted it was known as paralysis agitans or *Shaking Palsy*, characterized by muscle rigidity, tremor, and slowing of physical movement (Parkinson, 2002). Until the 1950's the underlying biochemical changes in the brain were still unidentified, these were then uncovered mostly due to the work of the Swedish scientist Arvid Carlsson who later went on to win a Nobel Prize. Unfortunately, the disease is progressive and the symptoms start to arise only when about 70% of the DA neurons in the substantia nirga have already died (Fearnley *et al.*, 1991). Although there is no current cure for the disease; there are a number of effective symptomatic therapies available (Poewe, 2006). The first specific treatment to be used for PD was L-dihydroxyphenylalanine (L-DOPA), this entered clinical practice in 1967, and the first study reporting improvements in patients with PD through the use of L-DOPA as treatment was published in 1968 (Cotzias, 1968; Cotzias *et al.*, 1967).

1.2 Prevalence

PD is a movement disorder occurring throughout the world and affecting all ethnic groups and both genders. In industrialized countries it is estimated that about 0.3% of the whole population and 1% of the population above the age of 60 suffer from PD (de Lau *et al.*, 2006). Worldwide, PD affects around 0.1% of the global population; it is an age related disorder which affects about 1-3% of people above the age of 65 and 4-5% of people above the age of 85 years, only 5-10% of PD patients are below the

age of 40 (early-onset PD) (Tanner *et al.*, 1999). In the U.S.A, the prevalence of PD rises from 0.3% in the general population to 1% to 2% in persons whose age is 65 years or older, with some data indicating a prevalence of 4% to 5% in individuals above the age of 85 years (Weintraub *et al.*, 2008).

There are cross-cultural variations in the occurrence of PD, these are possibly a consequence of the dissimilarity in environmental exposures or the difference in susceptibility gene distribution (Van Den Eeden *et al.*, 2003). For example it has been established that there is a 5 to 10 fold decrease in the prevalence of age specific PD in mainland China compared with Europe (Zhang *et al.*, 1993). PD exists both as an idiopathic and a familial disorder, and the contribution of environmental factors and genetic factors in the genesis of the disease is still not completely clear. Although many causative monogenetic mutations have been identified in recent years, they only account for a small proportion of the disease leaving about 90% of cases of sporadic origin (de Lau *et al.*, 2006). Many studies have shown that PD is more common in men than woman, while others found that there is no significant difference between the two genders (de Lau *et al.*, 2006). Also, PD is one of the few diseases that appear to occur less amongst the population who smoke tobacco (Veldman *et al.*, 1998).

1.3   Types of PD

As described above, PD exists in two forms: familial or idiopathic. In familial PD some genetic linkage has been found between the occurrence of the disease and a form of genetic alteration that is passed onto offspring, idiopathic PD however occurs sporadically with no well known cause.

Genetic implications in the aetiology of PD have been debated since both Gowers (1902) and Leroux (1880) noted that 15% of their PD patients reported an affected family member (Gowers, 1902; Pankratz *et al.*, 2004). A large sample of World War II veterans who were twins was analyzed and evidence was found for a genetic susceptibility to PD only among twins with early-onset PD (Tanner *et al.*, 1999).

Ideas on the genetic contribution to familial cases of PD have mostly been by the identification of mutations or over expression of genes encoding for various proteins. Due to the renewed interest in familial PD many genetic loci have been uncovered, namely PARK 1 to PARK 11 (McInerney-Leo, 2005), these encode proteins such as parkin, α-synuclein, ubiquitin carboxy hydrolase L1, PINK1 and DJ-1 (Di Monte, 2003; Lai *et al.*, 2002). One example is the alpha-synuclein gene mutations which are mostly point mutations and were found to be autosomal dominant (Leung *et al.*, 2005; Mouradian, 2002).

It has been shown in more than 10 studies that the risk of PD is 3 to 14 times higher in first-degree relatives of an individual suffering from PD when compared to risk in members of unaffected families (Pankratz *et al.*, 2003). The table below summerises the genes that have been linked with PD (table 1.1).

Locus	Gene	Chromosome	Inheritance	Probable function
PARK1 and PARK4	a-Synuclein	4q21	AD	Presynaptic protein, Lewy body
PARK2	Parkin	6q25.2-27	AR	Ubiquitin E3 ligase
PARK3	Unknown	2p13	AD	Unknown
PARK4	Unknown	4p14	AD	Unknown
PARK5	UCH-L1	4p14	AD	Ubiquitin C-terminal hydrolase
PARK6	PINK1	1p35-36	AR	Mitochondrial kinase
PARK7	DJ-1	1p36	AR	Chaperone, Antioxidant
PARK8	LRRK2	12p11.2	AD	Mixed lineage kinase
PARK9	ATP13A2	1p36	AR	Unknown
PARK10	Unknown	1p32	AD	Unknown
PARK11	Unknown	2q36-37	AD	Unknown
PARK12	Unknown	Xq21-q25	Unknown	Unknown
PARK13	HTRA2	2p12	Unknown	Mitochondrial serine protease

Table 1.1 Summary of genes found to be linked to PD
AD, autosomal dominant; AR, autosomal recessive. Table adapted from Thomas *et al.*, 2007.

1.4 Symptoms

There are many symptoms of PD, perhaps the most pronounced are the motor symptoms which include: resting tremor, skeletal muscle rigidity, akinesia/ bradykinesia and postural instability. These motor symptoms usually develop over many years as the disease progresses. The first components of the symptomatic profile are the resting tremor, muscle rigidity and akinesia, while postural instability occurs at a later stage of the disease (Obeso *et al.*, 2000; Quinn, 1997). Although the most pronounced characteristics of PD affect movement (motor symptoms), there are other typical symptoms which include disorders of mood, behaviour, thinking, and sensation (non-motor symptoms). Individual patients' symptoms may be quite dissimilar; progression is also distinctly individual (Aarsland *et al.*, 1999; Schrag, 2004). Mood and anxiety disorders, fatigue and apathy, psychosis, cognitive impairments and sleep disorders are neuropsychiatric disturbances which are either a part of the process of PD itself or could be a result of the multifaceted interactions between the progressive and broad pathologic changes of the disease, emotional response to Parkinsonism and treatment-related side-effects (Ferreri *et al.*, 2006). One or more of the psychiatric symptoms are reported by more than 60% of patients with PD at some point in the course of their disease (Aarsland *et al.*, 1999).

1.5 Diagnosis

Unfortunately, until now the main method for diagnosing idiopathic PD is the presentation of clinical symptoms (Rao *et al.*, 2006). No blood or laboratory tests that have proved to be useful in diagnosing PD are currently available and thus medical history and neurological examination serve as the chief means for diagnosis, consequently the disease can be difficult to diagnose accurately (Gelb *et al.*, 1999).

The existing criteria for diagnosing PD requires the presence of at least two of the following cardinal symptoms of the disease: resting tremor, bradykinesia, rigidity, or postural imbalance, with the exclusion of other potential causes of secondary

parkinsonism (de Lau *et al.*, 2006). So the diagnosis of PD is established when at least two motor symptoms are found, at least one being tremor or bradykinesia, and when patients respond positively to L-DOPA therapy (Gelb *et al.*, 1999). Post mortem autopsy is used to confirm the clinical diagnosis, and only then is there a definite diagnosis of PD, as only 80-90% of clinical diagnoses of probable PD are confirmed by post mortem examination (Litvan *et al.*, 2003). Early stage diagnosis of PD is especially difficult, particularly when only assessed at a single time point (de Rijk *et al.*, 1997). Long term follow-up of patients, however, does improve the accuracy of diagnosis because with long term observation new information on disease course and progression can be evaluated, such as the appearance of additional symptoms and responsiveness to L-DOPA therapy (de Lau *et al.*, 2006).

1.6 Dopamine (DA)

DA is a neurotransmitter related to adrenaline in structure, it functions as a chemical messenger in the brain which plays an important role in controlling movement, emotional response and cognition, the ability to feel pleasure and pain, control of food intake and endocrine regulation. DA is essential for performing balanced and controlled movements, and its deficiency in certain pathways in the brain can cause a lack of controlled movements as experienced in PD (Missale *et al.*, 1998). DA also plays numerous roles in the periphery in modulating cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function and gastrointestinal motility (Missale *et al.*, 1998).

A shortage of DA in the frontal lobe may lead to memory decline. On the other hand increased levels of DA in the limbic system and not enough in the cortex may lead to schizophrenia and possible paranoia (Seutin, 2005). Pain relief and a boost in the feeling of pleasure is experienced when DA levels increase in the frontal lobes (Van Der Borne, 2004). DA is the major neurotransmitter in the nigrostriatal pathway in

the midbrain, it originates from DA-synthesizing neurons of the midbrain substantia nigra pars compacta (SNc) complex and innervates the striatum (Moore *et al.*, 1978).

1.6.1 DA system

DA plays many important and different roles in the brain; these roles are presented in the form of different neuronal pathways which use DA as a neurotransmitter. There are four pathways in the brain that involve DA.

1.6.1.1 DA Pathways

The mesocortical pathway is a neural tract connecting the ventral tegmentum in the midbrain to the frontal lobes of the cortex through DA neurons. This pathway is thought to be involved in motivation and in emotional responses and consequently is associated with the negative symptoms accompanying schizophrenia, such as lack of desire, motivation or emotional response. The mesolimbic pathway connects the ventral tegmentum to the neucleus accumbens located in the striatum. This pathway is thought to be involved in the feeling of pleasure, reward and desire; and consequently is thought to be associated in the neurobiological theories of addiction. The third DA pathway is the tuberoinfundibular pathway. Here, DA neurons in the arcuate nucleus of tuberal region in the hypothalamus project to the median eminence, and when DA is released at the latter site it regulates the secretion of prolactin from the anterior pituitary gland. The fourth, nigrostriatal pathway consists of DA neurons extending from the SNc to the striatum, and as a part of the basal ganglia this pathway is mainly involved in movement, and its degeneration leads to PD (Crocker, 1994; Dahlstrom *et al.*, 1964).
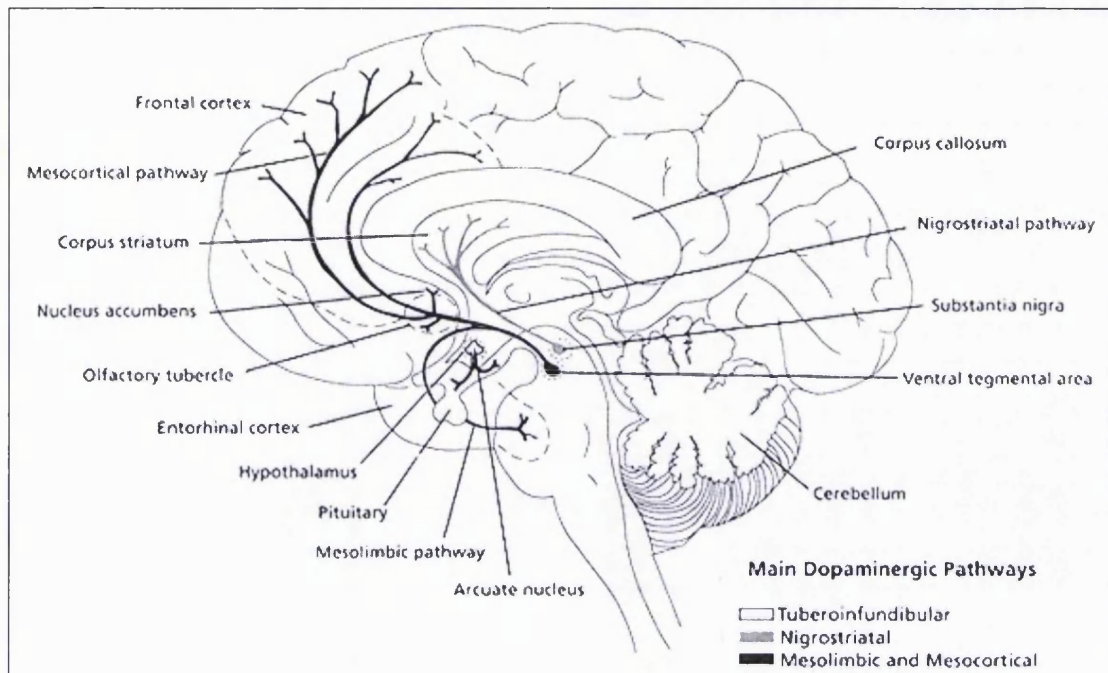
Figure 1.6.1 Sagital section of human brain showing the DA pathways.
Figure from Crocker, 1994

1.6.1.2 DA Synthesis

DA is one of the catecholamine neurotransmitters, it is predominately found in the central nervous system. DA is synthesized from the amino acid tyrosine after its metabolism into L-DOPA by the enzyme TH. L-DOPA is then converted to DA by the enzyme aromatic amino acid decarboxylase (AADC) (Foye, 1995).
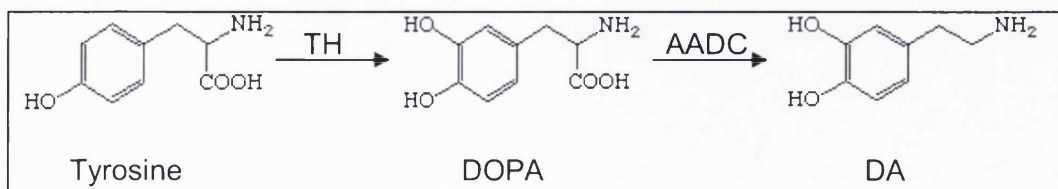
Figure 1.6.2 Synthesis of DA.
Pathway illustrating the enzymatic conversion of tyrosine into DOPA and finally into DA. Adapted from (Foye, 1995).

DA can also be further metabolized by the enzyme DA-β-hydroxylase to form noradrenaline which can subsequently turn into adrenaline. The rate limiting step in the synthesis of DA is through the TH enzyme.

DA is synthesized in the presynaptic terminal and is regulated by the TH enzyme (Figure 1.6.2). Once synthesized, DA is taken up and stored in synaptic vesicles in the presynaptic terminal by the vesicular monoamine transporter. DA mediates its effects by interacting with D_1 and D_2 receptors located on post-synaptic neurons but also, binds to auto receptors modulating DA cell function. Catecholamines can go through reuptake into neurons after their release via the DA transporter. DA can also be metabolized by monoamine oxidase (MAO) to 3,4,-dihydroxyphenylacetic acid (DOPAC) or by the enzyme catechol-O-methyltransferase (COMT) to 3-methoxytyramine (Figure 1.6.3). These two enzymes are considered to be major mechanisms for inactivation of catecholamines, and the action by both enzymes results in the formation of homovanillic acid (HVA)(Moore *et al.*, 1978).
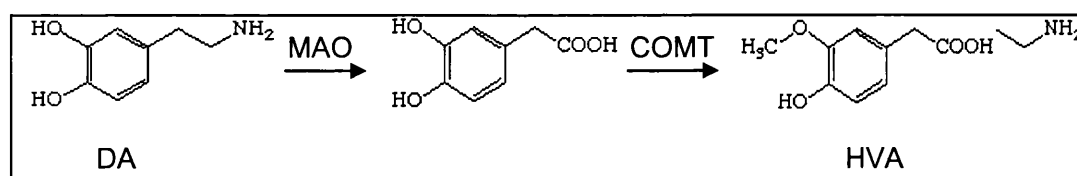
Figure 1.6.3 Metabolic inactivation of DA.
DA is metabolized through MAO enzyme into DOPAC then through COMT to finally form HVA (Foye, 1995).

1.6.1.2.1 Tyrosine hydroxylase (TH)

TH is a mixed-function monooxygenase that catalyses the enzymatic conversion of L-tyrosine, in peripheral and central catecholaminergic neurons and in chromaffin cells of adrenal medulla, to L-DOPA (Nagatsu *et al.*, 1964; Swaans *et al.*, 2000). This TH

enzymatic reaction is the initial and rate-limiting step in the biosynthesis of catecholamines; DA, adrenaline, noradrenaline as shown in figure 1.6.4 (Anagnoste *et al.*, 1974; Goldstein *et al.*, 1974). Hence, TH which is primarily expressed in the brain and adrenal glands plays a key role in the physiology of DA as well as adrenergic neurons (Swaans *et al.*, 2000). TH exists as a tetramer of four identical subunits and requires (6R)-L-erythro-tetrahydro-pteridine (BH4) as a cofactor (Nagatsu *et al.*, 1991). The enzyme uses tyrosine as the substrate, molecular oxygen and BH4 to generate L-DOPA (Kumer *et al.*, 1996). The enzyme acts when the pterin cofactor, oxygen and tyrosine bind to the enzyme before the hydroxylation reaction.
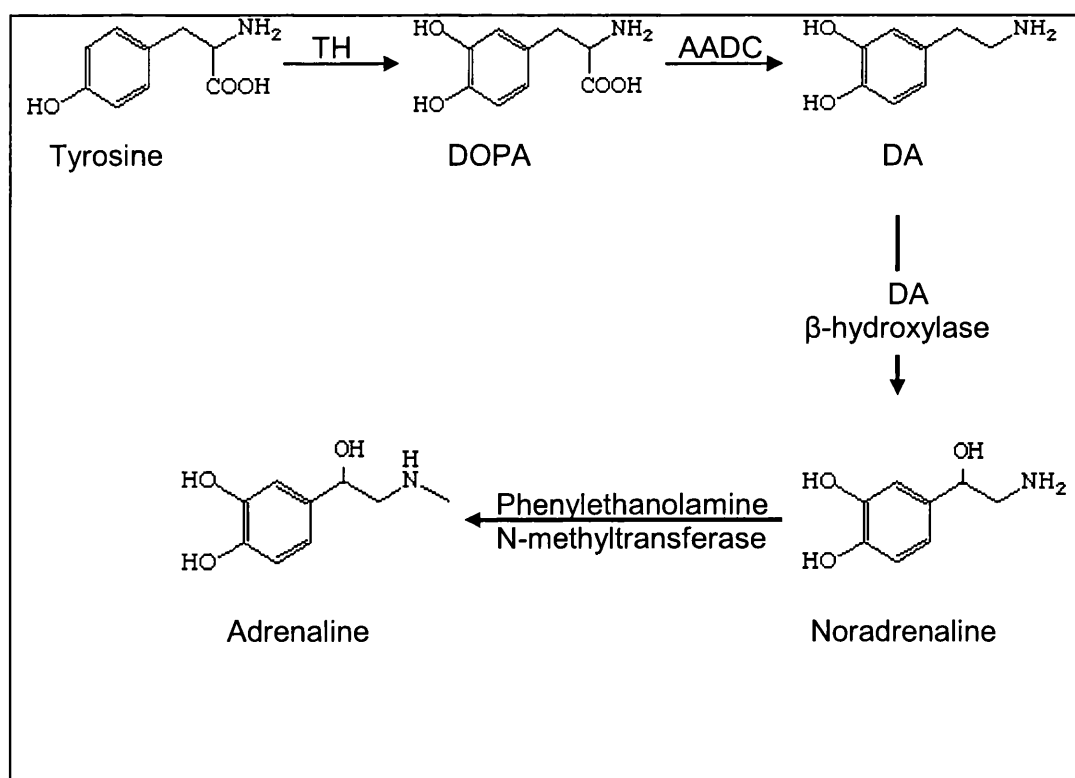
Figure 1.6.4 Biosynthesis of catecholamines.
Adapted from (King, 2007).

Short-term regulation of TH activity occurs at the post-transcriptional level, mainly activated via phosphorylation of the TH molecule; TH is phosphorylated and activated by different protein kinases including cAMP dependent protein kinase A

(PKA)(Campbell *et al.*, 1986). Long-term regulation has been exerted at the TH-protein synthesis level following TH-gene transcription (Kumer *et al.*, 1996). Interestingly, estrogens differentially regulate the expression of TH (Maharjan *et al.*, 2005), which could underlie the lower incidence of PD in women.

1.6.2   DA receptors

In 1972, the first evidence for the existence of DA receptors in the central nervous system was uncovered through biochemical studies, which demonstrated that DA had the ability to stimulate adenylyl cyclase (Kebabian *et al.*, 1972). In 1978, DA receptors were found to exist as two discrete populations; one positively coupled to adenylyl cyclase (AC) and the other independent of the adenosine 3',5'-cyclic monophosphate (cAMP)-generating system (Spano *et al.*, 1978). Kebabian and Calne classified the D1 receptor as the one that activates AC, via a Gs alpha-subunit (a heterotrimeric G protein subunit), leading to an increase in cAMP concentration, and the D2 receptor as the receptor that inhibits AC (Kebabian *et al.*, 1979).

After the first firm proposal for the presence of at least two distinct DA receptors by Kebabian and Calne in 1979, and with the introduction of gene cloning procedures, five subtypes of DA receptor have been identified. These receptors have been subdivided, according to their primary structure, gene organization, signaling systems and pharmacology, into D1-like (D1 and D5) or D2-like (D2, D3 and D4). Through the interaction with these five different subtypes, DA seems to mediate its different functions (Schwartz *et al.*, 1992). The D1 and D5 receptors are closely related and stimulate AC activity. On the contrary, D2, D3 and D4 receptors seem to inhibit the formation of cAMP (McAllister *et al.*, 1995; Sokoloff *et al.*, 1995).

Clinically, anti psychotic drugs used for the treatment of schizophrenia were believed to work through blocking cerebral DA receptors; the exact target on which these drugs give their effects are still not recognized (Griffon *et al.*, 1995). Almost all

clinically active compounds used for antipsychotic drug therapy are mainly antagonists of DA D2-like (D2, D3 and D4) receptors (Griffon *et al.*, 1995; Sokoloff *et al.*, 1995). These are mainly devided into typical and atypical antipsychotic drugs, with typicals producing extrapyramidal signs and symptoms. Atypicals however, clinically help patients by transiently occupying D2 receptors and then rapidly dissociating to allow normal dopamine neurotransmission which prevents the occurance of extrapyramidal signs (Seeman, 2002).

The case in PD however is different, as there is a shortage of DA in the striatum due to the loss of nigrostriatal DA neurons. DA here acts on both D1 and D2 receptors and thus non selective DA agonists, and more recently some selective DA agonists, are used for treatment.

1.7 Functional neuroanatomy of the basal ganglia

PD is a chronic, progressive neurological disease characterized by the degeneration of neurons that normally produce the neurotransmitter DA. This leads to a DA deficiency in the basal ganglia and finally results in loss of striatal DA concentrations (Rothblat *et al.*, 2001).

To understand the pathology of PD, one has first to evaluate the functional neuroanatomy of the basal ganglia and its connections. Although the striatum is not the only zone where other brain areas connect to the basal ganglia, it is considered to be the major one. Here, the basal ganglia receive input through the striatum from the cortex, mainly from the motor and prefrontal cortices (Sabatini *et al.*, 2000; Wichmann *et al.*, 2003).

The way the basal ganglia are connected is mainly divided into two main pathways, the direct pathway, and the indirect pathway. Through these two pathways the striatum regulates the voluntary movements of the body. In the direct pathway striatal excitation due to cortical activity leads to the inhibition of the globus pallidus internus (GPi) and the SNr. The inhibition of these, which are tonically inhibiting the thalamus

(in charge of activating the pre motor cortex), leads to the removal of the continuous inhibition, i.e. disinhibition. In the indirect pathway however, the excitation in the striatum due to the cortical activity seems to inhibit the thalamus, i.e. inhibition of the disinhibition. The overall outcome from the direct and indirect pathway is a disinhibition effect; this is because DA from the SNc activates the direct pathway over the indirect pathway, leading to increased signaling to the thalamus and thus allowing the ability to practice movement (Albin *et al.*, 1989; Alm, 2004; Wichmann *et al.*, 2003).

In the basal ganglia there are three primary types of neurons categorised according to the type of neurotransmitter they release at their terminals. The first neurons are glutamatergic neurons which release glutamate, an amino acid neurotransmitter which is excitatory in nature, acts on post synaptic neurons through binding glutamate receptors such as NMDA (N-methyl d-aspartate) receptors. These receptors confer a positive or excitatory response. The second neurons are GABAergic and release gamma aminobutyric acid (GABA) from their terminals; these neurons are inhibitory in nature and inhibit the post synaptic motor neurons through binding GABA receptors. The third type of neurons are dopaminergic; these are responsible for voluntary movement refinement through reducing the influence of the indirect pathway, and increasing the actions of the direct pathway within the basal ganglia (Alm, 2004). These Events are illustrated in Figure 1.7.1.
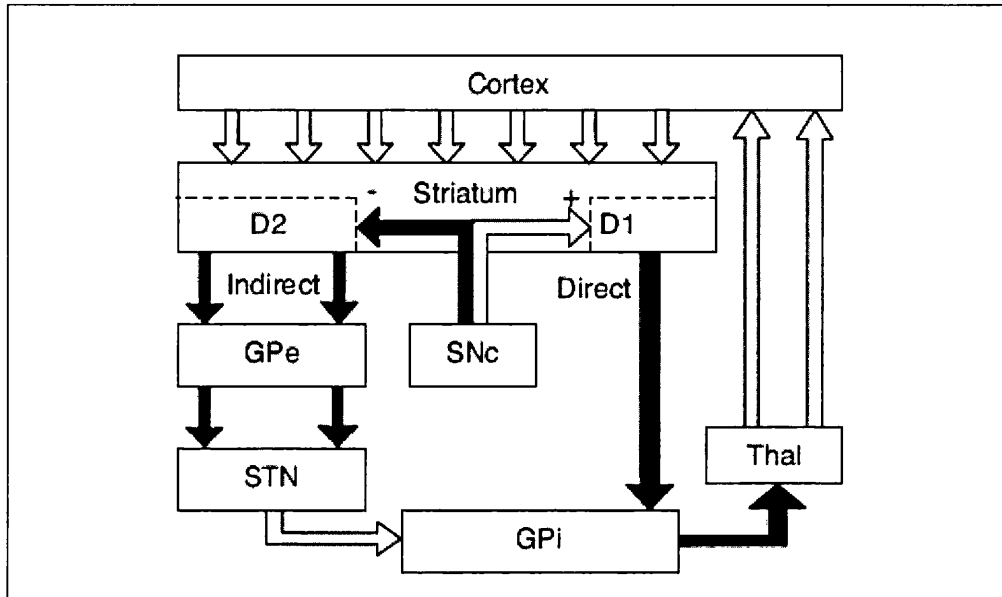
Figure 1.7.1 Neuronal pathways in the basal ganglia.
White arrows are excitatory and black arrows are inhibitory. Gpe: external segment of globus pallidus, Gpi: internal segment of globus pallidus, STN: subthalamic nucleus, SNc substantia pars compacta, Thal: thalamus. The striatum projects to the Gpi by both the direct and indirect pathways. The activity in these pathways is modulated by DA from the SNc: D1-receptors activate the striatal neurons forming the direct pathway while D2-receptors inhibit the striatal neurons in the indirect pathway. (Alm, 2004).

1.8 Pathology

1.8.1 Behavioural / Movement disorder

In PD the nigrostriatal pathway degenerates, and due to this disruption the major motor symptoms arise. There are also some non-motor symptoms that are associated with PD including apathy, mood disturbances, depression, cognitive disturbances, and sleep disturbances. These non-motor symptoms may be associated with the other DA pathways. Disruption of DA transmission along the non-striatal pathways explains some of the associated neuropsychiatric pathology, this however happens at later stages of the disease (Aarsland et al., 2001). Apathy for example is one of the behavioural symptoms in PD, and it is caused by dysfunctions within the prefrontal

cortex and the basal ganglia system (Aarsland et al., 2003; Pluck et al., 2002). Apathy can also occur after focal lesions of specific structures of the basal ganglia such as the caudate nuclei, the internal pallidum and the medial-dorsal thalamic nuclei (Bhatia et al., 1994; Laplane et al., 1989). Other non-motor symptoms, such as depression, could develop as a consequence of damage to serotonergic neurotransmission, as well as limbic noradrenergic and dopaminergic mechanisms (Remy *et al.*, 2005).

Also, noradrenaline deficiency due to the degeneration of the locus coeruleus has been linked to neuropsychiatric symptoms seen in PD, such as depression and sleep disturbances (Chaudhuri *et al.*, 2006).

The major or cardinal symptoms in PD however are the motor symptoms. These include resting tremor, muscle rigidity, hypokinesia and postural instability (Rao *et al.*, 2006; Zigmond, 2002). These symptoms arise early in the disease and are due to disruption in the nigrostrial DA pathway which is responsible for mediating movement (Herrero *et al.*, 2002). As mentioned before there are two key pathways in the basal ganglia involved in the voluntary motor movements, the direct and the indirect pathways. While the direct pathway facilitates movement the indirect pathway inhibits this movement. The lack of DA leads to an increased inhibition of the ventral lateral nucleus of the thalamus, which sends excitatory projections to the motor cortex, consequently leading to hypokinesia (Albin *et al.*, 1989; Sabatini *et al.*, 2000). The muscle rigidity and the resting tremor in PD are caused by abnormal synchronous oscillating neuronal activity within the basal ganglia (Jankovic, 2006).

1.8.2 Neuronal pathology

Pathologic features of PD include the loss of DA neurons in the SNc. This nucleus, which is located in the ventral midbrain, contains high amounts of neuromelanin pigment and plays a significant role in motor movements (Zigmond, 2002).

Another pathologic hallmark of PD is the presence of Lewy bodies (Gwinn-Hardy, 2002). These are identified as eosinophilic inclusions within neurons that consist of intracytoplasmic aggregates of α-synuclein (Gibb *et al.*, 1988). Lewy bodies usually present as rounded aggregates with an eosinophilic core surrounded by a pale halo (Gwinn-Hardy, 2002; Whitton, 2007). Lewy bodies are commonly observed in the brain regions demonstrating the most neuronal loss in PD, these include the SNc, the locus coeruleus, the dorsal motor nucleus of the vagus, and the nucleus basalis of Meynert, but they are also found in the neocortex, diencephalons, spinal cord and peripheral autonomic ganglia (Gibb *et al.*, 1988; Gibb *et al.*, 1991). Lewy bodies are also encountered in the normal ageing brain; they are also present in the brains of patients suffering from other neurodegenerative diseases such as Alzheimer's disease and dementia (Goedert, 2001). Whether Lewy bodies are symptomatic or causative in PD is not clear (Whitton, 2007).

In PD, the DA neurons die and thus lose their nerve terminals in the basal ganglia, mainly the striatum (Gelb *et al.*, 1999). The reason behind the death of these neurons is still not completely understood, although some aetiopathogenic processes are suspected to stimulate or bring about PD, these include; oxidative stress, mitochondrial dysfunction, proteasome dysfunction and protein aggregation, all of which can lead to nigrostriatal cell dysfunction and death (Dawson *et al.*, 2003). Regardless of the cause, one very important feature of PD is that the only affected cells are the DA neurons in the SNc, while other neighboring DA neurons are unaffected (Greene *et al.*, 2005).

1.8.3 Molecular pathology

There is evidence that genetic factors can play a role in the pathogenesis of PD. In large families with early onset or juvenile PD, the disease is transmitted as an autosomal dominant or recessive trait resulting from mutations in the genes encoding

various proteins (Zigmond, 2002). Although the majority of PD cases cannot be directly linked to a definite causative factor, familial PD has been linked with mutations in several genes such as parkin, ubiquitin C-terminal hydrolase L1 and α-synuclein (Gwinn-Hardy, 2002).

1.9 Causative factors

The aetiology of PD is still unknown, and most cases are sporadic, the cause of the disease is believed to be a result of a complex mix of environmental factors acting on genetically predisposed individuals with aging (Riess *et al.*, 1999). Many theories have been proposed to explain the reason for the neuronal degeneration that takes place in PD, these include genetic factors, mitochondrial dysfunction, free radical mediated cell death, apoptosis, oxidative stress and neuroinflammation (Whitton, 2007).

1.9.1 Internal predisposing factors

PD may occur as a result of internal factors that may lead to the generation of endogenous toxins, these endogenous chemicals contain the methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) moiety and are substrates for mono amino oxidase type B (MAO B; Schapira *et al.*, 1998), examples of these are some tetrahydroisoquinolines and β-carbolines, these chemicals show neurotoxicity, and inhibit mitochondrial complex I, which then will lead to disruption in cell respiration and eventually cell death. Reduced activity of complex I has been demonstrated consistently in patients with PD (Schapira *et al.*, 1990; Schapira *et al.*, 1998). The normal metabolism of DA via MAO B in the brain may also lead to the production of compounds like HVA and

DOPAC. Neuromelanin can also be produced as a result DA auto-oxidation. All of these products may induce oxidative stress through the generation of free radicals and other reactive oxygen species (Jenner *et al.*, 1992). MAO B has been demonstrated to increase its activity with increased age (Shih, 1979), so this could partly explain why PD is common in the elderly, as more MAO B leads to higher levels of DA metabolites and thus might produce a higher toxic load in the brain or possibly impair the brain's defense mechanisms for dealing with the toxic metabolites generated from normal metabolism (Jenner *et al.*, 1992).

Other factors that suggest the implication of oxidative stress in nigral cell death are: decreased levels of reduced glutathione enzyme (Perry *et al.*, 1986; Sofic *et al.*, 1992), an increase in the levels of iron (Dexter *et al.*, 1991), alteration in the functions of the mitochondria (Schapira *et al.*, 1990), and an increased level of lipid peroxidation (Dexter *et al.*, 1994; Jenner *et al.*, 1992).

Two other linked endogenous factors that can lead to PD are neuroinflammation and the immune response; these can be either activated through an external exposure or possibly stimulated endogenously. It is becoming more and more apparent that neuro inflammation does play an important role in the aetiology of PD especially in association with the microglia which highly populate the SNc (McGeer *et al.*, 1988; McGeer *et al.*, 2001). Some studies have reported inflammation and increased microglial activation in PD patients and in animal models of the disease (Czlonkowska *et al.*, 2002; McGeer *et al.*, 2004). It has also been noted that the inflammation resulting from neuronal damage and aggregated proteins in the striatum and the SNc could exacerbate the course of the disease or be a causative factor (Czlonkowska *et al.*, 2002). Activated microglia have also been reported in the SNc of PD sufferers, and the fact that anti-inflammatory agents can inhibit DA cell death in animal models of PD supports the role of inflammation in the disease (Bonifati *et al.*, 2007). However, the implication of the immune response in PD was considered when antibodies to DA neurons were found in the cerebrospinal fluid of PD patients (McRae-Degueurce *et al.*, 1988). And thus it was speculated that immune

mechanisms do play a role in initiating or maybe even amplifying a cascade of neuronal injury. This was demonstrated when IgG isolated from the serum of patients with PD caused damage to DA neurons in the SNc of rats *in vivo* (Chen *et al.*, 1998). Another factor to be considered in the aetiology of the disease is the existence of a genetic vulnerability that could lead to either irregular metabolism of endogenous molecules in the brain or the inability of these enzymes to function properly rendering them less effective in detoxifying exogenous compounds that in normal conditions would be harmless (Jenner *et al.*, 1992).

1.9.2 External (Environmental)

PD has been considered an idiopathic disorder ever since James Parkinson first described it in the early nineteenth century. It was first realized that PD can be produced from external or environmental factors when an acute intravenous dose of the compound MPTP caused parkinsonism in a group of drug users in 1983. This incident suggested for the first time that environmental factors could be a major contributor to the aetiology of the disease (Langston *et al.*, 1983). MPTP leads to neuronal cell injury in the SNc through its toxic metabolite 1-methyl-4-phenylpyridinium+ (MPP+) which causes oxidative stress through inhibiting mitochondrial complex I (Langston *et al.*, 1983)

Pesticides are believed to play a role in causing PD, some of these include pyridinium (paraquat), dithiocarbamate (maneb), complex inhibitor rotenone and organochlorine (dieldrin) (Liu *et al.*, 2003). All of these molecules lead to DA depletion when animals are systemically exposed to them, and most of them cause a decline in the number of TH positive neurons in the SNc (Liu *et al.*, 2003). The damage to the nigrostriatal system brought about by these molecules could be due to their ability to activate the abundant microglial cells in the SNc (McGeer *et al.*, 2001).

1.10 Risk factors

Environmental factors such as organic insecticides, herbicides, fungicides and exposure to metals as well as neuroinflammation may pose as potential risk factors in PD (Di Monte, 2003; Gao *et al.*, 2003). Also, there was an association in some prevalence studies between severe head injury and PD (Plassman *et al.*, 2000) although other studies failed to establish this correlation between head injury and the later development of PD (Williams, 1991). Although infections caused by microorganisms have been suspected to be risk factors for PD (Ghaemi *et al.*, 2000), epidemiologic studies on the relationship between the infections occurring early in life and PD were inconclusive (Logroscino, 2005). Other suggested risk factors that have not been conclusively proven yet include; personality traits as risk factors for PD, where a premorbid personality dominates (Paulson *et al.*, 1991), and the place of birth as a risk factor in PD (Betemps *et al.*, 1993). Interestingly, the risk of PD is lower in coffee drinkers, and people who smoke also appear to have a decreased risk for the disease (Hernan *et al.*, 2002; Veldman *et al.*, 1998).

1.10.1 Current treatments

PD is a progressive chronic disorder that requires broad-based management which includes patient and family education, the services of support groups, exercise and good nutrition. To this day, there is still no cure for PD, but medications and, in some cases, surgery can provide some relief from the symptoms (Aminoff, 1994; Leung *et al.*, 2005).

The selection of the medication and its dosage is tailored to each individual, where the physician takes into consideration factors such as symptom severity, age, and the presence of other illnesses and the use of other medications. No two people respond

equally to a particular drug or dosage level, so this process involves experimentation, persistence, and patience (Leung *et al.*, 2005).

As the disease is progressive, drug dosages may have to be modified and medication regimens changed, and sometimes there is a need for a combination of drugs.

1.10.2 Medications / Drugs used in PD

1.10.2.1   L-Dihydroxyphenylalanine (L-DOPA)

L-DOPA was the first effective treatment to be used in PD, and is still the mainstay of PD drug therapy. Unfortunately though, 30-50% of patients receiving L-DOPA treatment will suffer from unpleasant and potentially disabling drug induced involuntary movements, this usually happens after 2 to 5 years of using treatment (Poewe *et al.*, 1986; Schrag *et al.*, 2000). After L-DOPA is administered it is distributed in the body and in the brain, it is then metabolized to DA by aromatic L-amino acid decarboxylase enzyme (AADC), which is located in both the brain and the periphery (Shen *et al.*, 2003).

1.10.2.2   DOPA Decarboxylase (DDC) inhibitors

Carbidopa is the most well known inhibitor of DDC, and when given in combination with L-DOPA it decreases the peripheral break down of L-DOPA thus leads to more drug reaching the brain. Carbidopa cannot be given alone as it has no pharmacological action in PD, so its purpose is to increase L-DOPA reaching the brain (Aminoff, 1994). Also, by inhibiting DA formation peripherally, DDC

inhibitors reduce DA related side effects in the periphery. Other DDC inhibitors include benserazide (Aminoff, 1994; Shen *et al.*, 2003).

1.10.2.3   DA agonists

DA agonists mimic the function of DA in the brain and are used primarily as adjuncts to L-DOPA/carbidopa therapy. These DA agonists can be used as monotherapy but are generally less effective in controlling symptoms. Examples of these include: bromocriptine, pergolide, pramipexole and ropinirole (Goetz *et al.*, 2005).

1.10.2.4   Amantadine

Amantadine is an antiviral drug which has DA agonist properties. It increases the release of DA and is often used to treat the early stages of PD alone, with an anticholinergic drug or with L-DOPA. Unfortunately, it generally ceases to be effective within 3 to 4 months (Thomas *et al.*, 2004).

1.10.2.5   MAO-B inhibitors

MAO-B Inhibitors work through inhibiting the oxidation of DA by monoamine oxidase B and thus prolonging its availability. One example is selegiline (Carbex®) which inhibits MAO-B and consequently increases the amount of available DA in the brain. MAO-B inhibitors act synergistically with L-DOPA (Goetz *et al.*, 2005).

1.10.2.6   Anticholinergics

Anticholinergics act by reducing the relative over activity of the neurotransmitter acetylcholine to balance the diminished activity of DA. This class of drugs is most effective in the control of tremor, and they are used as adjuncts to L-DOPA. Examples of these include: benztropine mesylate, biperiden, diphenhydramine and trihyxyphenidyl (Rao et al., 2006).

1.10.2.7   Catechol-O-methyl transferase (COMT) inhibitors

COMT Inhibitors are a class of PD medications that work by inhibiting the COMT enzyme which metabolizes L-DOPA before it reaches the brain. Inhibiting COMT increases the amount of L-DOPA that enters the brain. These drugs are only effective when used with L-DOPA. Examples of these include: entacapone and tolcapone (Deane *et al.*, 2004; Rao *et al.*, 2006).

1.10.3 Surgery

Treating PD surgically was once a common practice. But after the discovery of L-DOPA, surgery was restricted to only a few cases. In the past few decades studies have led to great improvements in surgical techniques, and surgery is again being used in advanced cases of PD where drug therapy is no longer sufficient. Deep brain stimulation is presently the most common surgical procedure used for treatment. Surgery may also be used to implant a drug delivery device, and even to inject stem cells.

1.10.3.1   Deep brain stimulation

A brain implant device is now most commonly used for deep brain stimulation, this implant helps to control many of the PD symptoms. The implant consists of a pacemaker-like unit which is implanted in the chest wall; this transmits electric impulses through a wire to very small electrodes inserted deep within the brain. Deep brain stimulation allows a focal specific electrical stimulation of the subthalamic nucleus in the basal ganglia, this targeted brain center controls many parts of motor function (Kaelin-Lang *et al.*, 2007; Koller *et al.*, 1999). Deep brain stimulation can be effective in improving dyskinesia, bradykinesia, tremor and rigidity and drug treatments can be reduced. Its effectiveness on long term quality of life however has still not been established. Unfortunately, while deep brain stimulation is effective in improving the motor symptoms of PD, it is not helpful at all in patients with severe cognitive, psychiatric and axial symptoms, and any surgery carried out on these patients may even worsen their condition (Kaelin-Lang *et al.*, 2007).

1.10.3.2   Implants

Many strategies have been developed for drug delivery into the CNS, one of these is through the use of implantable polymeric devices for locally controlled drug release. These have evolved from macroscopic implants needing open surgery, into poly(lactide-co-glycolide) microspheres which are safe and biocompatible with brain tissue, can be easily implanted stereotaxically in exact functional areas of the brain without causing damage to the surrounding tissue (Menei *et al.*, 2005), and some of these implants can be used for DA release into the striatum (Winn *et al.*, 1989). Other drug delivery strategies include a localized infusion of a drug using mini-osmotic pumps which allows a greater degree of controlling dosage. Another method for drug delivery is through the transplantation of cell accommodating non-biodegradable devices. The cells inside these devices can synthesize and release deficient

neurotransmitter DA providing a sustained drug delivery (Popovic *et al.*, 2006; Xue *et al.*, 2001).

1.10.3.3 Stem cell therapy

Researchers have been developing stem-cell transplantation for the treatment of many diseases, neurodegenerative disorders such as PD have been candidates for this kind of treatment (Love, 2002). The idea behind cell replacement therapy is to restore the loss in synaptic signaling brought about by neuronal degeneration (Isacson, 2002).

Stem cells were useful as a potential source for cell replacement therapy because they can indefinitely proliferate *in vitro* without differentiating, and also remain capable of this differentiation into most cell types if the proper conditions become present (Bjorklund *et al.*, 2002; Brustle *et al.*, 1999; Kim *et al.*, 2006). Central nervous system (CNS) stem cells have trilineage ability, i.e. they have the ability to generate neurons, oligodendrocytes, and astrocytes (Kalyani *et al.*, 1998), depending on the surrounding host environment. It has been reported that embryonic stem cells can efficiently differentiate into neural precursor cells and then into DA producing neurons with the use of optimal culture conditions and/or some genetic manipulation (Kim *et al.*, 2002). Also, mouse embryonic stem cells have been witnessed to develop into functional DA neurons when transplanted into the damaged host rat striatum, this could lead to the restoration of cerebral function and behaviour in animal models of PD (Bjorklund *et al.*, 2002).

PD patients have also demonstrated improved symptoms and a significant reduction in L-DOPA therapy when they had fetal ventral mesencephalic neurons transplanted into their caudate/putamen and striatum (Lindvall, 1991; Sonntag *et al.*, 2007). The current major problems however when using fetal tissue are the ethical concerns that arise, as well as its impracticality due to the requirement of having tissue from many fetuses because of the low survival of neurons after grafting (Isacson, 2002).

1.10.3.4   Neurotrophic factors

Neurotrophic factors are proteins that activate cell signaling pathways regulating neuronal survival, differentiation, growth and regeneration (Patel *et al.*, 2007).

One very important neuroprotective agent is glial cell line-derived neurotrophic factor (GDNF). This factor induces the injured DA nigrostriatal system to recover, this recovery is reflected in the improved motor function of both rodent and primate animal models of PD (Burke *et al.*, 1998; Gash *et al.*, 1998). Only hours after the administration of GDNF, neuroprotective changes in DA neurons are observed, i.e. after the adult midbrain DA neurons are stimulated by GDNF, they increase in their size (increased fiber density and size of the neurons in SNc) and quantal size (the amount of DA released per synaptic vesicle exocytic event), and both their neurite extent and phenotypic marker expression also increase (Gash *et al.*, 1996; Pothos *et al.*, 1998). These changes could explain the improvements observed in the major PD symptom in non-human primates (Grondin *et al.*, 2002). Unfortunately, GDNF does not cross the blood brain barrier and thus has to be administered intracerebrally. Nevertheless it appears to be effective when administered via intraventricular, intrastriatal and intranigral routes in rodents and non-human primates (Gash *et al.*, 1998). Many studies have been carried out to establish the effectiveness of GDNF, and while some studies showed that GDNF was ineffective in improving motor symptoms in PD accompanied by many side effects upon administration (Nutt *et al.*, 2003), other studies proved GDNF to successfully improve motor symptoms in PD (Gill *et al.*, 2002). The neuroprotective and even neurorestorative features of GDNF observed in preclinical studies indicate the important role trophic factors may play in the treatment of PD (Gash *et al.*, 1998). This led to some controlled clinical studies using GDNF (Fahn *et al.*, 2004).

GDNF was infused into PD patients in some recent studies. One study using bilateral infusion of GDNF into the putamen did not confer the predetermined level of clinical benefits to PD patients (Lang *et al.*, 2006), another more recent study using unilateral intaputamenal infusion concluded that GDNF administration resulted in significant

sustained bilateral benefits in PD patients, but these improvements were lost within 9 months of drug withdrawal (Slevin *et al.*, 2007).

More clinical trials are needed to achieve a decision on the feasibility of employing GDNF infusions as a treatment for PD. Many related compounds to GDNF are currently being examined for their efficacy through the use of alternative delivery methods like implantation of capsules, engineered cells and viral vectors (Savitt *et al.*, 2006).

Unfortunately, to this day there is still no cure or a fully comprehensive long lasting treatment for PD. All the treatments for PD which have been discussed are faced with many limitations which range from inadequate efficacy for long term treatment to high incidence of side effects that could become at times more debilitating than the disease itself. Other more invasive treatments which incorporate surgical procedures have the problem of being financially not viable for the whole population. These procedures also suffer from limited efficacy and with the potential high risks that are involved in this type of surgery.

1.11 Models of PD

To better understand the disease many models that mimic PD in their aetiology, pathogenesis and morphology have been developed. The response of these models to a particular treatment may take it one step closer to clinical use while other experimental treatments can be dismissed. Models that mimic PD can be divided into two main categories, *in situ* and *in vivo*. The most common of these are perhaps the *in vivo* animal models of the disease (Shimohama *et al.*, 2003).

1.11.1 In situ

Many different cell types are used to mimic PD in situ; two very common cells used are the pheochromocytoma cells (PC12) and SHY5Y cells. Both of these DA-neuron-derived cell lines express TH and contain DA transporter (DAT), which are features

of DA neuronal phenotype (Biswas *et al.*, 2005; Deng *et al.*, 2005). Nevertheless these cells have highly variable properties and it is important to verify that every batch possess the DA features. Also, these cells do not share all molecular traits of nigrostriatal DA neurons which may be essential for their susceptibility to PD (Chesselet *et al.*, 2005). Primary mesencephalic cultures from fetal or newborn rats or mice could also be useful, but again these cells are highly heterogeneous and usually contain neurons from both the SNc and ventral tegmental area as well as many other non-DA neurons (Chesselet *et al.*, 2005). Nevertheless these different cell lines are very valuable in studying detailed mechanisms of the degeneration of DA neurons and also for screening molecules with apparent pharmacological potential (Kitamura *et al.*, 1998; Sawada *et al.*, 1996).

To generate models of PD, these different cells are exposed to toxins which show selectivity for DA neurons *in vivo* e.g. 6-OHDA, MPP+ or rotenone (Testa *et al.*, 2005). One example indicating the significance of these *in vitro* models of PD was the identification of protective role growth factors played in PD, especially GDNF and some other related molecules, which have led to clinical trials (Shimohama *et al.*, 2003; Wu *et al.*, 2005a). It is also possible to use cell lines to investigate mutations that lead to PD, either through transfecting the cells with the defective gene, or deriving primary cultures from mice expressing this mutation (Chesselet *et al.*, 2005; Peng *et al.*, 2005). Although these models have contributed considerably to better understanding the disease and to the development of agents for the treatment of PD, they are still limited by the vague relationship of the toxin-induced mechanisms to the pathophysiology of PD (Chesselet *et al.*, 2005)

1.11.2 In vivo

Many animal models have been used to mimic PD; these usually involve the use of either rodents or primates that are given some external manipulation or insult in order

for them to develop PD-like pathology. The external manipulations could be via exposing the animals to a toxin or through genetic engineering; in both cases the animals will develop PD-like effects with different characteristics, such as the time it takes to develop the model, severity and causative mechanisms. Because of the ability of these animal models to reproduce some key features of PD they are crucial for better understanding the aetiology of the disease and are useful for testing emerging new therapies.

Unfortunately these models are not without flaws, as they do not mimic the cause of human PD or the progressive neurodegeneration. Furthermore, as expected, the higher the animals in the developmental tree the closer they are to mimicking the disease in humans, and because of the strong similarity to the human brain in comparison to brains of other species, non-human primate models are considered to be the step before trial of new therapy in humans (Lane *et al.*, 2007).

1.11.2.1 Toxin induced

1.11.2.1.1 Reserpine

Reserpine interferes with the storage of monoamines in synaptic vesicles causing depletion of DA (and other catecholamines) in nerve terminals, this results in sedation, hypokinesia, rigidity and also tremor (van Zwieten *et al.*, 1966). Reserpine is used in animals to produce a model of the motor disruptions that occur in PD because of its effects on spontaneous locomotor activity (Colpaert, 1987) Reserpine is suggested to be a model with some good predictive validity (Skalisz *et al.*, 2002), especially because a number of antiparkinsonian drugs like L-DOPA, amantadine and trihexyphenidyl have led to improvement in the motor deficits caused by reserpine (Menzaghi *et al.*, 1997). Unfortunately there are no morphological changes observed in this model of PD, which may limit its prospects for use as a model for the disease (Carlsson *et al.*, 1957; Hornykiewicz, 1963).

1.11.2.1.2   6-Hydroxydopamine (6-OHDA)

One of the first animal models of PD was generated through the use of 6-OHDA which was the first compound found to have sufficiently specific neurotoxic effects (Sachs *et al.*, 1975; Ungerstedt, 1971). 6-OHDA could produce two models or possible disease scenarios. The first which is more common is via the unilateral injection of 6-OHDA into the medial forebrain bundle which, due to both anterograde and retrograde diffusion, results in substantial destruction of the nigrostriatal pathway in less than a week, and almost complete destruction within two weeks. The other less common scenario is the injection of 6-OHDA directly into the striatum. Here, due to only retrograde degeneration or destruction of the DA neurons in the SNc, the lesion is less severe and takes a longer time to develop (up to 4 weeks). This type of 6-OHDA injection is used when there is a need to mimic the slow progression of the disease (Beal, 2001; Deumens *et al.*, 2002). 6-OHDA is too hydrophilic to cross the blood brain barrier and has to be injected directly into the brain, where it is then taken up by DA neurons via the DAT system (Perese *et al.*, 1989). Other neurons like noradrenergic and seotonirgic neurons could also take up 6-OHDA, but the selectivity to DA neurons is achieved through the co-administration of dismethylimipramine (an inhibitor of the NA and 5-HT transporters) which prevents 6-OHDA uptake by these neurons (Perese *et al.*, 1989; Sachs *et al.*, 1975). 6-OHDA is a neurotoxin which exerts its neurotoxicity mainly through promoting apoptosis, but some evidence indicates that microglial activation may play a role in the toxicity produced by 6-OHDA (Blum *et al.*, 2001; He *et al.*, 2001). Free radical formation or covalent bonding of quinone oxidation products could also be a possible cause of the potent neurodegeneration (Langston, 1988). 6-OHDA can be used in rats, mice, cats and primates as it is an effective DA neurotoxin in all of these animals. The 6-OHDA hemiparkinsonian (unilateral lesion) rat model is especially valuable due to the ability to quantitatively measure the severity of the lesion through behavioural testing using either apomorphine or amphetamine. This has proved to be very useful in the

pharmacological screening of compounds affecting the nigrostriatal DA system (Beal, 2001; Deumens _et al._, 2002). The continuous studies performed using the 6-OHDA animal models have helped in the development of new treatments for PD. This model has also contributed to better understanding the mechanisms hypothesized as representations of the pathological dysfunction sites at the cellular level in PD (Simola _et al._, 2007).

1.11.2.1.3   1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)

MPTP toxicity was first accidentally demonstrated in humans when heroin addicts consumed heroin contaminated with MPTP and consequently rapidly developed clinical symptoms similar to those of PD (Langston _et al._, 1999). MPTP is a neurotoxin which induces parkinsonian features in both primates and rodents through rapid and selective DA neurotoxicity (Langston _et al._, 1983). MPTP itself is not toxic but is very lipophilic and thus readily crosses the blood brain barrier. There it is converted by MAO-B located in glial cells to MPDP which is very unstable and auto-oxidizes to the toxic form MPP+. It appears that DA neurons take up MPP+ through the DA reuptake mechanism where it then resides possibly due to an interaction with neuromelanin. MPP$^{\bullet}$ radical is subsequently formed and actively accumulated by mitochondria, here MPP$^{\bullet}$ leads to ATP depletion through inhibiting complex I in the respiratory chain and thus initiating cell death (Gao _et al._, 2003; Jenner, 1989). It is also possible that there is an involvement of free radical mechanisms through a redox reaction between MPP$^{\bullet}$ and MPP+ which could generate super oxide radicals (Gao _et al._, 2003; Jenner, 1989). Apoptosis has also been suggested to be implicated in the DA neurodegeneration following exposure to MPTP (Novikova _et al._, 2006).

One interesting feature in the MPTP animal model of PD is that following its administration to rodents or monkeys the biochemical and cellular changes are quite similar to those observed in idiopathic PD such as the loss of DA neurons in the SNc and the appearance of α-synuclein-immunoreactive cytoplasmic inclusions (Bohlen _et al._, 2005).

1.11.2.1.4   Lipopolysaccharide (LPS)

LPS is an endotoxin produced by gram-negative bacteria. LPS is a potent microglial cell activator and is an effective initiator of DA neuronal loss in the SNc. This leads to parkinsonian like symptoms in experimental animals treated with LPS (Herrera *et al.*, 2000). LPS is a potent stimulant of neuroinflammation which exerts its actions through binding an LPS membrane receptor complex on microglial cells which lead to the activation and nuclear localization of the transcription factor NFκB and successive activation of pro-inflammatory pathway genes (Liu *et al.*, 2000; Orr *et al.*, 2002).

This inflammatory neurodegeneration seems to be specific to the SNc, as the injection of LPS in other regions such as the hippocampus or cortex does not lead to this degeneration. This is probably due to the high activation and high population of microglia in the SNc. Indeed, activated microglia appear to be up to 8 times more abundant in the SNc than they are in any other region of the brain (Kim *et al.*, 2000)

1.11.2.1.5   Rotenone

Rotenone is a pesticide that is extracted from certain plant roots and when it is systemically and chronically administered to rats, they seem to develop many features of PD such as degeneration of the DA neurons in the SNc and the appearance of cytoplasmic inclusions reminiscent of Lewy bodies, which stain with antibodies for ubiquitin and α-synuclein (Betarbet *et al.*, 2000). It has been established that rotenone is a high-affinity specific inhibitor of mitochondrial complex I (Betarbet *et al.*, 2000). Besides being a mitochondrial complex I inhibitor, rotenone is also suggested to cause neuronal degradation through stimulating the formation of reactive oxygen species (ROS) and causing protein carbonylation. It could also decrease the abundance of the antioxidant glutathione, all of which may lead to apoptosis of DA neurons. It has been suggested that rotenone may increase DA redistribution from the vesicles into the cytosol and this could in turn render DA prone to quinone formation (Watabe *et al.*,

2007). Rats treated with rotenone through chronic systemic exposure from a subcutaneously implanted osmotic pump show symptoms similar to those of human PD, including bradykinesia, postural instability, unsteady gait and some evidence of tremor. These symptoms improved when the DA agonist apomorphine was administered to the rotenone treated rats. This parkinsonian pathology is, however, limited to the Lewis rat strain (Sherer *et al.*, 2003).

1.11.2.1.6 Methamphetamine

Methamphetamine is a psychostimulatory drug that is highly addictive; it has a powerful DA releasing mechanism. When given at very high doses methamphetamine causes neurotoxicity in both rodents and primates (Wagner *et al.*, 1980). It is similar to reserpine in that it does not induce any morphological changes in the SNc and does not lead to loss of nigral cell bodies (Fibiger *et al.*, 1971). Methamphetamine is a DA toxin which enters the neuronal terminals through binding to the DAT or the serotonin transporter SERT, causing the displacement of vesicular and intracellular DA and 5-HT which are subsequently oxidized to ROS via MAO in addition to autoxidation which eventually leads to necrosis (Cubells *et al.*, 1994). Methamphetamine also causes DA redistribution from vesicles to the cytosol like rotenone (Lotharius *et al.*, 2005). There is also a relationship between apoptosis and methamphetamine induced neurotoxicity and this is more recognized on more chronic exposure to the compound (Davidson *et al.*, 2001; Iwasa *et al.*, 1996).

1.11.2.2   Genetically engineered animals

Toxin induced animal models have been important in the understanding of PD as well as in the development of many symptomatic treatments for the disease, yet they all tend to focus on degeneration of the nigrostriatal pathway, and thus are limited in not

having the ability to reproduce the full pathology and progression demonstrated in PD. The recent discovery of specific genes that are involved in the familial form of the disease led to the development of novel genetic mouse models of PD like the α-synuclein over expressor mouse, the Parkin knockout mouse and the DJ-1 knockout mouse. These mice will develop specific pathological traits as a result of the induced mutation (Fleming *et al.*, 2005). Although the most popular genetically engineered animals are mice, other animal species have been used to develop mutations, such as the Drosophila fly where researchers were able to express mutant and normal forms of α-synuclein (Feany *et al.*, 2000).

The table below summarises and compares the most commonly used animal models of PD (table1.2).

Model	PD symptoms	PD pathology	Advantages	Disadvantages
6-OHDA	Motor impairments after bilateral lesion Easily quantifiable turning behavior after unilateral lesion	Reduced DA levels in the striatum Massive loss of dopamine neurons No intracellular aggregates	Works in mice, rats, and monkeys Well characterized Used in dyskinesia models	Does not pass the blood–brain- barrier (needs intracerebral injection, which increases variability) Fast, massive neurodegeneration Poor construct validity
MPTP	Motor impairments	Reduced DA levels in the striatum Massive loss of dopamine neurons With chronic administration, formation of aggregates with little LB resemblance	Lipophilic Systemic administration Works mainly in mice Well characterized Good construct validity	Highly toxic to humans (dangerous to administer) Reduced reliability
Reserpine	Motor impairments	Reduced catecholamines levels in the striatum Loss of dopamine neurons in the SN No aggregate formation	Systemic administration	Transient non-specific depletion of catecholamines. Hypothermia
LPS	Motor impairments after bilateral lesion Easily quantifiable turning behavior after unilateral lesion	Reduced DA levels in the striatum Massive loss of dopamine neurons. Some changes in α-synuclein observed.	Distinct neuro-inflammatory damage through activating microglia in SNc.	Needs intracerebral injection, which increases variability, damage limited to inflammatory causes.
Methamphetamine	No clear parkinsonian symptoms	At very high doses leads to loss of TH in the striatum, possible loss of DA cells in the SNc	Screening of antioxidant therapies for their DA cell protective effects	Acute, limited histopathological changes

Rotenone	Motor impairments	Reduced DA levels in the striatum Massive loss of dopamine neurons Inclusions reminiscent to Lewy bodies	Systemic administration Works only In rats with variable susceptability	Toxic for the whole organism Low construct validity
Dj-1 KO, Pink1 KO, Parkin KO	Little motor impairment	Only slight DA pathology	Good construct validity	Slight DA pathology. Expensive and time consuming.
a-Synuclein wild-type and A53T, A30P overexpression	Little motor impairment	Little DA pathology Intracellular aggregates with little Lewy body resemblance	Good construct validity	Slight DA pathology. Expensive and time consuming

Table 1.2 Examples of some of the most commonly used animal models of PD.
Adapted from (Betarbet *et al.*, 2002; Lane *et al.*, 2007; Terzioglu *et al.*, 2008)

1.12 Current experimental compounds for the treatment of PD

As there is still no cure for PD, the medicines available are symptomatic and due to the progressive nature of the disease, drug dosages need to be increased and different drugs may need to given in combination until eventually they will not have any effect as the nigrostriatal pathway will eventually completely degenerate. For this reason researchers are looking for treatments that would prevent the progression of the disease and would protect the remaining neurons from degradation. To achieve this, many approaches have been used, most of which were based on the uncovered mechanisms implicated in PD to attempt to stop or modify the state of the disease. Many neuroprotective agents have been investigated and are still being assessed for their disease modifying actions in PD. These alleged neuroprotective agents include pro-mitochondrial molecules like Ginko biloba, while others are supposed anti-excitotoxic agents such as NMDA antagonists. Also anti oxidants were thought to have protective action, including radical scavengers (vitamin C, E) and GSH enhancers. Many antiapoptotics were also investigated for their disease modifying abilities; examples include cyclosporine and caspase inhibitors. Finally, and due to the recent implication of neuroinflammation in the aetiology of PD (McGeer *et al.*, 2001), anti-inflammatory agents have been investigated for their protective actions against the further degeneration of neurons; compounds investigated have included

steroids, COX 2 inhibitors, minocycline, and, more recently, urocortin (UCN) (Schapira, 2005).

1.13 Urocortin (UCN)

1.13.1 Background

UCN is a 40 amino acid peptide that was originally discovered in the midbrain of the rat. It is a member of the corticotropin-releasing factor (CRF) neuropeptide family. The amino acid sequence of human urocortin is 43% identical to that of CRF (Takahashi *et al.*, 2004), while cloned rat UCN is 45% identical to CRF (Vaughan *et al.*, 1995).

In 1996, Donaldson et al. mapped the UCN gene to chromosome 2 by Southern blot analysis of human/rodent somatic cell hybrids (Donaldson *et al.*, 1996). In 2002, Delplanque et al. used radiation hybrid analysis to map the UCN gene to chromosome 2p23-p21 (Delplanque *et al.*, 2002).

Urocortins are distributed in a distinct pattern, both in the brain and the gastrointestinal tract. Human UCN is expressed in both the CNS and the periphery, it is found in the placenta, fetal membranes, pituitary, brain, gastrointestinal tract, ovaries, synovial tissue, and lymphocytes (Latchman, 2002).

Generally there is minimum overlapping in the distribution of UCN and CRF in the brain, this suggests different functional roles of the two peptides (Skelton *et al.*, 2000).

Although researchers had agreed that UCN expression is most prominent in the Edinger-Westphal nucleus and the latteral superior olive (Skelton *et al.*, 2000), more recently it has been suggested that UCN is produced by midbrain sympathetic neurons adjacent to the Edinger-Westphal nucleus (Pan *et al.*, 2008). Here, UCN production is believed to be involved in mediating stress through the sympathetic

nervous system (De Fanti *et al.*, 2002). Additionally, while UCN has been observed to have low basal levels , it appears to undergo rapid induction (Pan *et al.*, 2008).

It was observed that UCN production in the GI tract correlates with severity of inflammation in patients suffering from ulcerative colitis (Saruta *et al.*, 2004). Also, UCN levels were higher in the gastric mucosa of people with active H.pylori gastritis, indicating a relationship with inflammation (Chatzaki *et al.*, 2003). Moreover, in addition to its expression in the thymus and spleen, human UCN is also produced by lymphocytes, again indicating a role in inflammatory immune responses (Pan *et al.*, 2008). UCN central administration has been shown to interfere with immune system reactions, leading to decreased proliferative response of splenic lymphocytes to mitogen (Okamoto *et al.*, 1998).

In the SNc and ventral tegmental nucleus, UCN immunoreactivity has been located exclusively to neurons which co-express tyrosine hydroxylase, suggesting that within these regions, UCN is co-localized with DA (Yamamoto *et al.*, 1998). Interestingly, the female hormone estrogen exerts a direct and differential transcriptional regulation of the UCN gene (Haeger *et al.*, 2006), suggesting the possible involvement of UCN in the reduced incidence of PD in women.

1.13.2 Corticotropin-Releasing Factor (CRF)

CRF is a 41-amino acid peptide which is secreted in response to stress by the paraventricular nucleus of the hypothalamus. After its release into the primary capillary plexus of the hypothalamo-hypophyseal portal system, CRF is carried by the portal system to the anterior lobe of the pituitary, where it stimulates the secretion of corticotropin and other biologically-active substances, such as β-endorphin (Hauger et al., 2006). CRF binds to the corticotropin releasing factor receptors (CRF-R). These G protein coupled receptors are classified as CRF-R1 and CRF-R2 (Hauger et al., 2006).

CRF plays a key role in mediating stress in the central nervous system. CRF also participates in inducing various changes such as stimulation of the pituitary-adrenal axis, autonomic nervous system, immune system, behaviour, and mood (Takahashi, 2001).

It has been shown that brain CRF plays a role in neuropsychiatric, neurodegenerative, metabolic, and feeding disorders (Lovenberg *et al.*, 1995b; Nemeroff *et al.*, 1988).

The excessive activation of the hypothalamic-pituitary–adrenal axis and consequent hypersecretion of glucocorticoids may underlie a number of conditions, including depression, certain anxiety disorders and anorexia nervosa (Arborelius et al., 1999). Also, it has been observed that decreased CRF signaling may play a role in motor disorders related to the basal ganglia (Heuser et al., 1991), as well as in olivopontocerebellar atrophy and spinocerebellar degeneration (De Souza, 1995). It has also been noted that there is a trend for CRF to decrease in the striatum as a consequence of aging (De Souza, 1995). This shortage in brain CRF may lead to dementia associated with neurodegenerative disorders such as Alzheimer'sdisease (De Souza, 1990). Whitehouse et al. also observed changes in CRF concentrations in PD cases (Whitehouse et al., 1987), a phenomenon that was also confirmed when deficits in brain CRF were apparent in neurodegenerative disorders, including PD (De Souza, 1995).

Apart from its obvious physiological roles in mediating stress responses, CRF also possesses neuroprotective properties. Many studies have suggested that CRF is able to elicit CRF-R1 mediated neuroprotection (Bayatti *et al.*, 2003; Facci *et al.*, 2003; Lezoualc'h *et al.*, 2000; Pedersen *et al.*, 2002). After binding to the CRF-R1 receptor, CRF can activate a series of intracellular signalling pathways which leads to the production of protective factors, including induction of the expression of neurotrophic factors. CRF can also trigger a CRF-R1 mediated-inhibition of other intracellular pathways that may be potentially toxic and lead to apoptosis (Bayatti *et al.*, 2005).

1.13.3 The UCN family

The urocortins are a family of three related peptides belonging to the CRF family; urocortin (UCN) is known as urocortin I to distinguish it from the two further isoforms; urocortin II (UCNII) and urocortin III (UCNIII), which are identified in humans. UCN binds with high affinity to both CRF type 1 (CRF-R1) and type 2 (CRF-R2) receptors but with a much higher affinity to CRF-R2, infact it has been posulated that UCN is the endogenous ligand for CRF-R2 receptors (Parkes *et al.*, 2001). UCN 2 and UCN 3 however are are selective CRF-R2 ligands (Hashimoto *et al.*, 2004). The CRF-R2 receptor is further divided into 2 isotypes (α and β) in rodents and 3 isotypes (α, β, and γ) in humans (Takahashi, 2001). Whilst UCN is non selective and binds both CRF-R1 and CRF-R2, what determines which receptor is involved in mediating its effects could be the relative abundance of the receptor type at the site of action (Pedersen *et al.*, 2002).

1.13.4 Function

Urocortin has many functions in the body, which to date have mainly been described in the cardiovascular system and the nervous system. In the cardiovascular system UCN appears to increase cardiac contractility, decrease blood pressure and also confers cardiac protection. UCN is also functionally active in the nervous system where it elicits many effects such as reducing appetite, increasing activity and raising anxiety levels. Figure 1.13.1 illustrates these effects (Latchman, 2002).
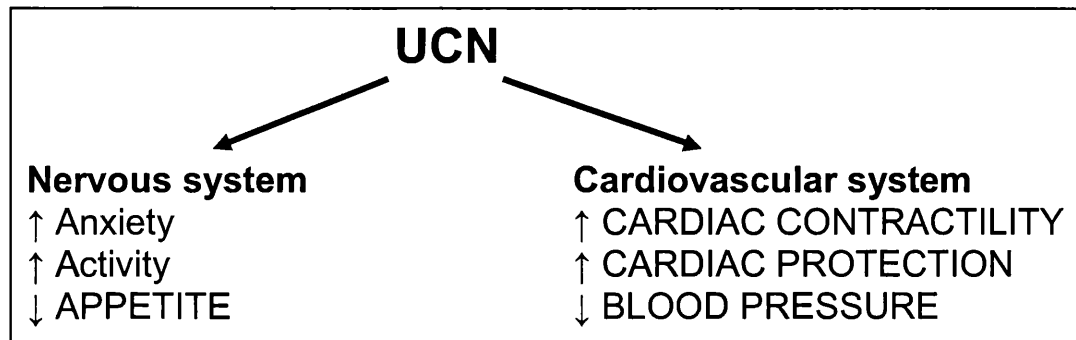
Figure 1.13.1 Diagram illustrating the role of UCN in the nervous and cardiovascular systems.
Words in capitals represent the effects where urocortin is more potent than CRF. Adapted from (Latchman, 2002).

As UCN is a CRF agonist, it would also lead to similar effects to those observed following stimulation of the hypothalamic-pituitary-adrenal (HPA) axis, which plays an important role in controlling reactions to stress. Stimulating the HPA axis also regulates many physiological processes including digestion, the immune system, mood and emotions, sexuality and energy storage and expenditure (Engelmann *et al.*, 2004). Because of its similarity to CRF, UCN is capable of producing behavioral and physiological effects that are similar to CRF. However, although these effects are qualitatively similar, they seem to be quantitatively different, whereby UCN appears to be more potent in suppressing food and water intake and less potent in inducing anxiogenic behavior than CRF (Skelton *et al.*, 2000). Even though UCN is more potent than CRF in releasing ACTH from the pituitary, CRF is the predominant physiological mediator of the activity of the HPA axis (Turnbull *et al.*, 1999).

Also, while CRF is expressed widely in neurons in the brain, UCN seems to have a more restricted expression pattern (Morin *et al.*, 1999). This may account for some of the differences between the effects which are mediated by these peptides.

1.13.5 CRF receptors

As mentioned before, CRF receptors (CRF-R) are mainly classified as CRF-R1 and CRF-R2 (Hauger *et al.*, 2006). The CRF-R1 receptor is a seven transmembrane spanning Gs-protein coupled receptor which is localized primarily to cortical and cerebellar regions in the brain. The CRF-R2 receptor, which is 70% identical to CRF-R1 is also coupled to Gs-protein. This CRF-R2 exists as three splice variants, CRF-R2α is mainly found in the brain, where it is localized to subcortical regions including the lateral septum, paraventricular and ventromedial nuclei of the hypothalamus and the olfactory bulb (Lovenberg *et al.*, 1995a). The CRF-R2β however, is more abundant in the non-neuronal parts of the brain and the periphery, where it is mainly localized to heart, skeletal muscle, and in the brain to cerebral arterioles and choroid plexus (Lovenberg *et al.*, 1995a; McCarthy *et al.*, 1999). The CRF2γ receptor was also later identified in the amygdale, hippocampus and septum of the human brain (Kostich *et al.*, 1998; McCarthy *et al.*, 1999).

Because overlap of the CRF-R1 and the CRF-R2 isoforms is limited it is most likely that these possess distinct functional roles (Skelton *et al.*, 2000). For example, activation of the CRF-R1 in dispersed rat and human adrenal chromaffin cells induces catecholamine secretion, while activation of the CRF-R2 suppresses catecholamine secretion (Dermitzaki *et al.*, 2007).

CRF-R1 and CRF-R2 receptors are mainly postsynaptic (Rominger *et al.*, 1998).

In the brain, the anxiogenic effect of UCN is mediated through the CRF-R1 receptor only (Swiergiel *et al.*, 1999), while activation of the CRF-R2 receptor mediates an anxiolytic effect that apposes anxiogenic functions of CRF-R1 receptors. Because of this, CRF-R2 activation is thought to mediate stress-coping responses (Kishimoto *et al.*, 2000; Liebsch *et al.*, 1999).

1.13.5.1   CRF-R signalling

CRF-R1 and CRF-R2 are highly homologous, and both these receptor subtypes couple to the same Gα protein and consequently signal through similar second messengers (Hauger *et al.*, 2006). These seven-transmembrane domain receptors are linked to AC through Gs protein activation; the activation of AC leads to the production of cAMP which causes PKA activation, this mechanism is responsible for most CRF-R mediated events including corticotrope secretion of ACTH (De Souza, 1995).

Stimulation of CRF-R also activates the phospholipase C-protein kinase C (PLC-PKC) pathway which is possibly achieved through coupling to Gqα. The activation of this pathway eventually leads to nuclear factor-kappaB (NF-κB) activation which ultimately leads to upregulation of gene expression (Hauger *et al.*, 2006). Moreover, it has been observed that CRF-R1 association to Gq is through activated PKA. Once activated, PKA inhibits association of CRF-R1 to Gq leading to inhibition of the PLC-PKC pathway (Nemoto *et al.*, 2005). CRF-R signalling through the PLC-PKC pathway is, until now, still not completely understood (Hauger *et al.*, 2006).

CRF-R signalling is also mediated by the extracellular signal-regulated kinase (ERK)-MAP kinase cascade. This signalling pathway is activated through activation of either the PKA or the PKC pathway. In CRF-R1 receptors, activation of the PKA pathway mediates MAP kinase signalling. However, CRF-R2 receptors appear to activate this pathway through PKC activation (Rossant *et al.*, 1999). Activation of the (ERK)-MAP kinase pathway will eventually activate transcription factors which control gene expression, with an overall outcome of regulation of synaptic plasticity, cell motility, chemotaxis, and apoptosis (Lefkowitz *et al.*, 2005; Sweatt, 2004). Stimulation of the CRF-R could also lead to Gi activation which stimulates ERK1/2 activation through the Gβ/γ-Ras-Raf pathway. This activation leads to TH phosphorylation and subsequently catecholamine synthesis in PC12 cells (Nemoto *et al.*, 2005).

The neuroprotective effects of CRF and UCN have been associated with activation of PKA, which subsequently leads to the activation of transcription factor CREB and

eventually induces BDNF expression (Bayatti *et al.*, 2005). Figure 1.13.2 illustrates a simplified diagram of CRF-R signalling pathways.
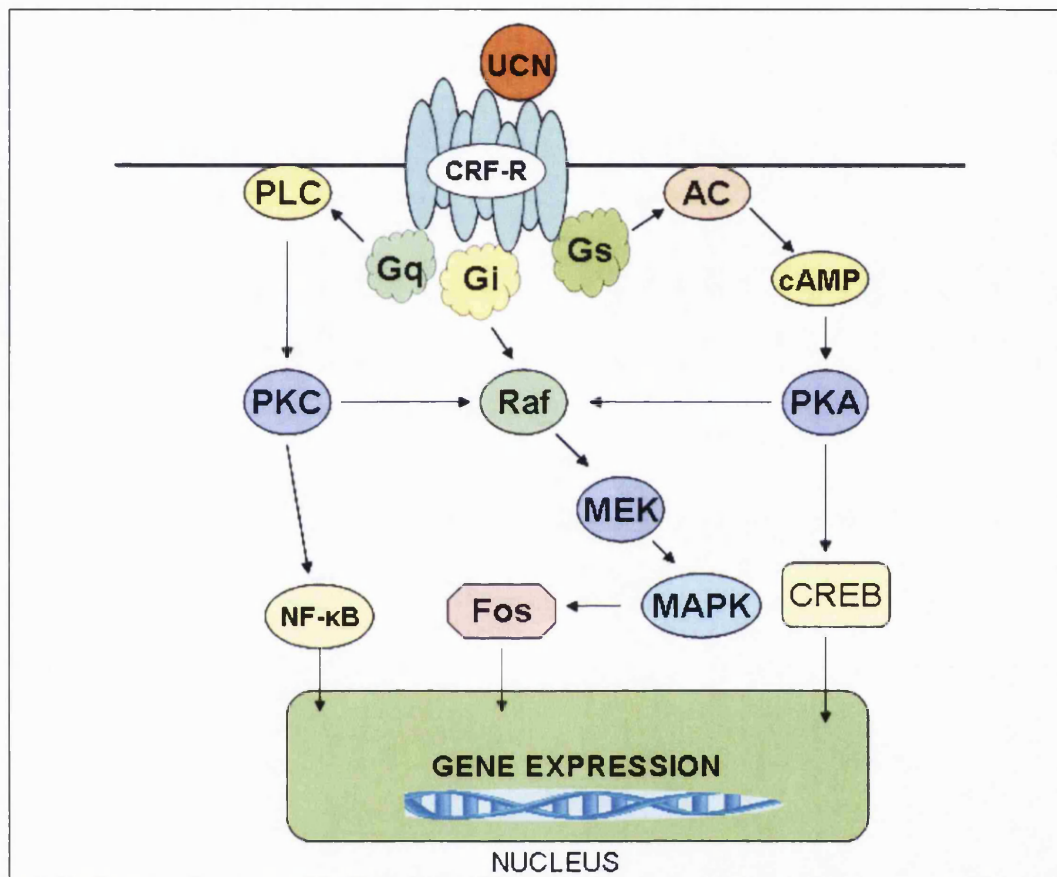
Figure 1.13.2 Schematic representation of simplified CRF-R cellular signalling after UCN stimulation.
Adapted from (Bayatti *et al.*, 2005; Hauger *et al.*, 2006; Nemoto *et al.*, 2005; Punn *et al.*, 2006).

1.13.6 Uses as novel therapy

After UCN was cloned and became available to researchers, it was investigated for its roles in the CNS and cardiovascular system. UCN was also examined for its anti-inflammatory effects to determine any therapeutic advantage this molecule may hold.

1.13.6.1 Urocortin and Heart disease

Both UCN mRNA and UCN are expressed in the heart of the rat, where cardiomyocytes express UCN. These expression levels are increased by heat shock and ischemia (Sohail *et al*, 2004). It has also been recently reported that the human myocardium is immunohistochemically positive for UCN, with this immunohistochemical staining appearing to be more intense in the failing heart (Nishikimi *et al.*, 2000). Also, UCN has been proven to have potent coronary vasodilatory and cardiac inotropic effects in conscious sheep (Parkes *et al.*, 2001). These findings have shown that UCN has important pathophysiological roles in the heart.

In rats, UCN was found to produce CRF-R2-mediated effects of decreasing mean arterial pressure and causing tachycardia (Hashimoto *et al.*, 2004).

In sheep, UCN was also observed to possess a protective compensatory role in heart failure where it contributes to suppression of vascular tone and renin-angiotensin-aldosterone/endothelin activation (Charles *et al.*, 2006; Rademaker *et al.*, 2005).

Also, autophagy, which is a known feature of cardiomyopathies and chronic ischaemia, is reduced by UCN in cardiac myocytes after ischemia/reperfusion injury, which is thought to be through UCN's activation of the PI3 kinase/Akt pathway (Valentim *et al.*, 2006).

Brar et al. (2000) demonstrated that UCN has protective effects in rat cardiac myocytes following ischemic injury. Here, UCN administration prevented cell death to primary cardiac myocyte cultures. This was observed when UCN was given before or even after simulated hypoxia/ischemia. UCN also reduced damage in isolated rat hearts *ex vivo* subjected to regional ischemia/reperfusion. This protective effect was observed when UCN was administered either before ischemia, or at the time of reperfusion after ischemia, which proposes the usefulness of this peptide even after ischemia occurs (Brar *et al.*, 2000).

Scarabelli et al. (2002) also observed that UCN still produced cytoprotective and functional benefits even when it was given only at reperfusion; this was presented as

improvement in ventricular function associated with reduction in mycocardial damage and a decrease in endothelial cell and cardiomyocyte apoptosis.

The findings of Brar et al. (2000) and Scarabelli et al. (2002), where UCN was able to still elicit cardioprotective/preservative effects even after ischemia and only at reperfusion, were also demonstrated when Townsted et al. found that acute UCN treatment decreased necrosis and improved hemodynamic recovery during reperfusion after 30 min of global ischemia in Langendorff perfused rat hearts. These reaserchers also observed that UCN reduced cell death following simulated ischemia and re-oxygenation in adult and neonatal rat isolated cardiac myocytes after both acute and chronic UCN treatment. It was suggested that UCN's ability to reduce myocyte vulnerability to reperfusion injury is through a PKC-mediated reduction of oxidative stress (Townsend *et al.*, 2007).

The result of the latter studies, where UCN was observed to produce protective/restorative effects after injury occurs, present it as a potential candidate for use in disorders like PD where treatment always takes place after neuronal damage has occured.

1.13.6.2   Urocortin and PD

1.13.6.2.1   In situ

As well as having protective effects on cardiac myocytes, UCN was also observed to produce similar effects on other cell types after injury. Pedersen et al. (2002) observed that UCN protected against oxidative and excitotoxic cell death in cultured hippocampal neurons. This finding was significant as the hippocampus, which is important in the regulation of stress responses, houses neurons that are vulnerable during disease conditions including Alzheimer's disease and anxiety disorders (Pedersen *et al.*, 2002). The UCN mediated neuroprotective effects observed here seemed to involve cAMP-dependent PKA, PKC and MAPK (Pedersen *et al.*, 2002).

These neuroprotective effects were also supported when UCN was found to be neuroprotective as well as having the ability to suppress apoptosis in cerebellar granule neurons. UCN was also able to stimulate cAMP synthesis in these cultured neurons (Facci *et al.*, 2003). These researchers also found UCN to rescue both hippocampal and cortical neurons from β-amyloid peptide toxicity (Facci *et al.*, 2003), a finding that may be of relevance to Alzheimer's disease and possibly PD.

Researchers have also observed that both CRF and UCN are potent regulators of dendritic development in rat Purkinje neurons, where they induced proliferation and dendritic growth. These observed effects were sugested to be mediated by the protein kinase A and mitogen-activating protein kinase pathways (Swinny *et al.*, 2004).

Two research groups in 2005 carried out studies that investigated the direct effect of UCN and UCN II on catecholamine release or catecholamine synthesis in PC12 rat pheochromocytoma cells. Their results showed that UCN significantly induced an increase in cAMP levels and TH enzyme activity. Furthermore, UCN also significantly increased TH mRNA. These results indicate that UCN and UCN II stimulate TH enzyme activity and synthesis, suggesting that they both contribute to catecholamine biosynthesis in PC12 cells, possibly via the cAMP/protein kinase A and the PKA/ERK1/2 pathway (Nanmoku *et al.*, 2005; Nemoto *et al.*, 2005).

Disruption in the CRF system was shown to be closely linked with the progression of Alzheimer's disease, indicating the potential neuroprotective effects of CRF and related peptides like UCN (Bayatti *et al.*, 2005).

The potential neuroprotective role that UCN may play in neurodegenerative disorders such as Alzheimer's disease and PD still needs further investigation. One aspect worth examining is the effect of UCN on models of neurodegenerative disease, especially *in vivo*.

1.13.6.2.2   *Aims of the project*

In light of the above, the work in this project was carried out to evaluate possible effects of UCN in animal models of PD, where the aim was to establish whether or

not this peptide possesses neuroprotective properties. Mechanisms through which this peptide exerts its effects were also studied to establish individual targets. Two distinct rodent models of PD, the 6-OHDA model and the LPS model, were used to determine whether UCN confers neuroprotection or not. These two models were used to avoid the possibility that UCN might be uniquely effective against a specific component of one model. The study outcomes were evaluated through multiple *in vivo* and *ex vivo* assessments. These assessments included: behavioural apomorphine challenge, *in vivo* measurement of extracellular DA, measurement of tissue DA, determining TH activity in the nigrostriatal system, testing for TH protein content, and TH immunostaining in the SNc to estimate the density of TH positive staining. By performing these different and complementary assessments it was possible to ascertain the effects of UCN in these animal models of the disease with confidence.

Chapter 2

Materials and Methods

2   Materials and Methods

2.1   Chemicals and regents

All reagents were purchased from Sigma-Aldrich (Sigma-Aldrich, UK) unless otherwise stated.

2.2   Animals

Male Wistar rats (240-260 g) were purchased from Harlan, U.K. and housed in an animal care facility. The animal unit was maintained at constant temperature, humidity and fixed dark: light cycles. The animals were provided free access to tap water and rodent chow feed. All procedures were in accordance with the institutional and Home Office animal care guidelines.

2.2.1   Stereotaxic surgery

Stereotaxic surgery was performed to administer both insults used; 6-OHDA and LPS, all UCNs and α-helical-CRF. Animals were anesthetized using isoflurane 4% v/v in O_2 for induction of anesthesia (Abbot Laboratories Ltd., Kent) and then secured on a stereotaxic frame (David Kopf, US), a maintenance isoflurane concentration of 1.5% v/v in O_2 was then used throughout the duration of the surgery. Stereotaxic surgery was performed by first exposing the surface of the skull using a sterile blade. This was done by laterally reflecting two skin flaps and removing excess membranous material and blood until the scull was fully visible, the bregma was then located and marked, and using stereotaxic coordinates from the atlas of Paxinos and Watson (1982), the SNc, medial forebrain bundle (MFB) and striatum were located.

For all intracerebral (i.c) injections and before actual injections could be made, the skull was penetrated using a dental drill, after these openings in the skull were drilled the injections were carried out using a Hamilton 10 μl syringe. *All injections through out this work were performed unilaterally,* on the left side of the brain, to achieve a unilateral animal model of PD. In this manner it was possible to compare the nigrostriatal pathway of the treated side to the untreated side, in the same animal, in addition to comparisons to other groups.

2.2.1.1 Injection of 6-OHDA

In the case of 6-OHDA, and prior to its intracerebral injection; animals were treated intraperitoneally (i.p) with pargyline (50 mg/kg) and desmethylimipramine (25 mg/kg), this was done to maximize the selectivity of 6-OHDA for the DA neurons. After these injections the rats were fixed in the stereotaxic frame and then injected with 6-OHDA (8μg/4μl) of saline containing 0.2% ascorbic acid in saline or vehicle into the left medial forebrain bundle MFB (from bregma in mm; A -4.3, L 1.4 and V 8.2; Paxinos and Watson, 1982). The injections were performed at a rate of 1 μl per minute, and after the total injection volume was infused, a waiting period of approximately 5 minutes was allowed to permit the local diffusion of the injected solution. The syringe was then slowly raised to prevent any back flow from occurring. The scalp was finally either sutured or bonded using Michele clips, and the rats were then returned to their cages and observed until they regained consciousness (generally within 5 minutes).

2.2.1.2 Injection of LPS

After the rats were fixed in the stereotaxic frame, they were injected with LPS (from E.coli, stertype 0111:B4) $2\mu g/2\mu l$ or normal saline directly into the left SNc (from bregma in mm; A -5.2, L 2.2 and V 8.3; Paxinos and Watson, 1982). These injections were performed at a rate of 1 μl per minute, and after the total injection volume was infused; a waiting period of about 5 minutes was allowed to permit the diffusion of the injected solution. The syringe was then slowly raised to prevent any back flow that may happen. The scalp was finally either sutured or bonded using Michele clips, and the rats were returned to their cages and observed until they regained consciousness.

2.2.1.3 Injection of UCN, UCNII and UCN III

Rats co-treated with UCN after injection of either insult (6-OHDA or LPS) received UCN injections of 2pmol in $2\mu l$ of saline directly into the left SNc (from bregma in mm; A -5.2, L 2.2 and V 8.3; Paxinos and Watson, 1982). These injections were made either directly after the injection of the insult (6-OHDA or LPS) or in other groups seven days after the injection of either insult. The doses of UCN used were decided according to doses of UCN used in PC12 cells where it seemed to confer a preservative or neuroprotective role (Nanmoku *et al.*, 2005; Pedersen *et al.*, 2002).

2.2.1.3.1 UCN administration at time 0

In this group of rats, the injection of the UCNs was carried on the first treatment day, and only a few minutes after the injection of either insult. 2pmol/$2\mu l$ of UCN, UCNII or UCNIII in saline were injected directly into the SNc, this was done 5 minutes after insult treatment. These rats were assessed after 14 days (figure 2.2.1)
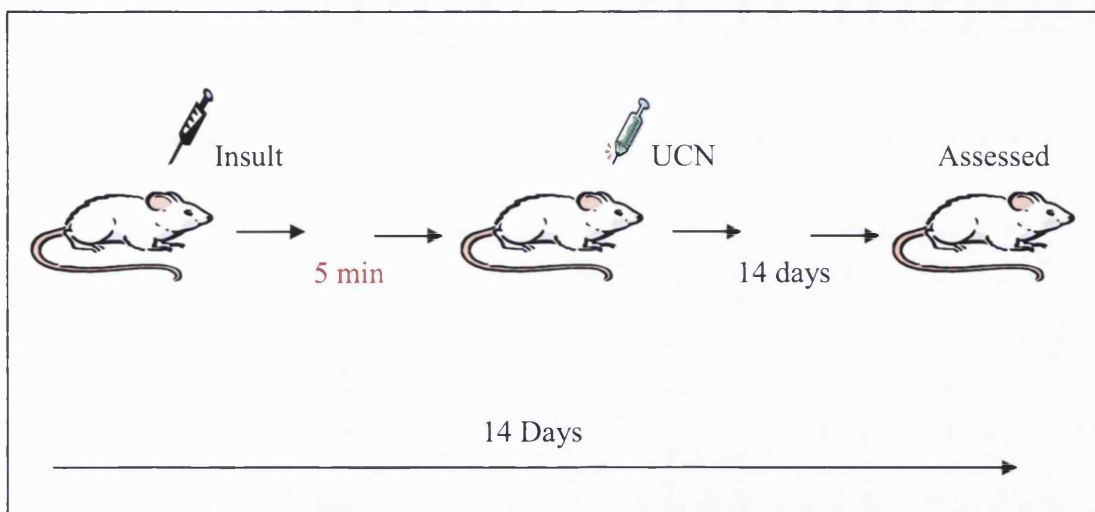
Figure 2.2.1 A schematic diagram showing the time line of UCN injection at time zero.
Insult (LPS/6-OHDA) injection was followed with UCN and assessed after 14 days. All injections were unilateral.

2.2.1.3.2 UCN administration after seven days

In this group, the injection of UCN was made 7 days after initial treatment with insult. 2pmol of UCN in 2µl saline were injected directly into the SNc. Rats were left for a further 7 days and assessed on day 14 (figure 2.2.2).
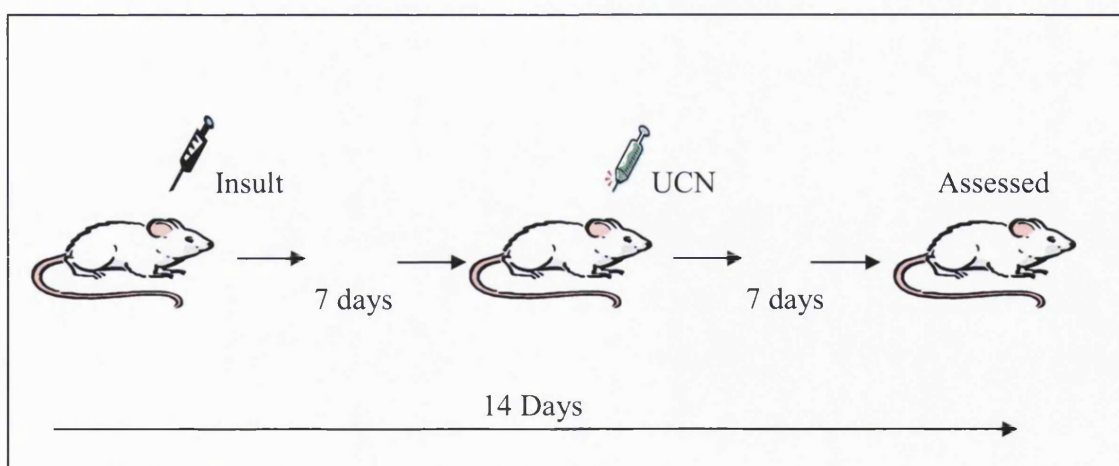
Figure 2.2.2 A schematic diagram showing the time line of UCN injection after 7 days.
Seven days after insult (LPS/6-OHDA) injection, animals were ttreated with UCN and then left for a further seven days before assessment on day 14. All injections were unilateral.

2.2.1.4 Injection of antagonists

Two antagonists were used in this work, α-helical CRF and NBI-27914. α-helical CRF is a non selective CRF receptor antagonist which does not cross the blood brain barrier. α-helical CRF is similar in structure to CRF peptides and would be expected to have the similar half life and diffusion properties as any of the UCNs and remain in the injected region for the same duration as the agonist (UCN) would. The second antagonist used was NBI-27914, a selective CRF-R1 antagonist which is very lipophilic and readily crosses the blood brain barrier, and due to its high lipophilicity and the highly lipophilic nature of the CNS, NBI-27914 remains for a long period in the brain once it is present there (Chen, 2006; McCarthy *et al.*, 1999).

2.2.1.4.1 Alpha-helical CRF

After being treated with 6-OHDA, and prior to UCN injection, rats were subject to α-helical CRF injection 1nmol in 2μl of saline directly into the SNc (Baram *et al.*, 1997; Baram *et al.*, 1996). After this non-selective antagonist was allowed to diffuse for 5 min, UCN was injected, also directly into the SNc. Rats were assessed after 14 days (figure 2.2.3).
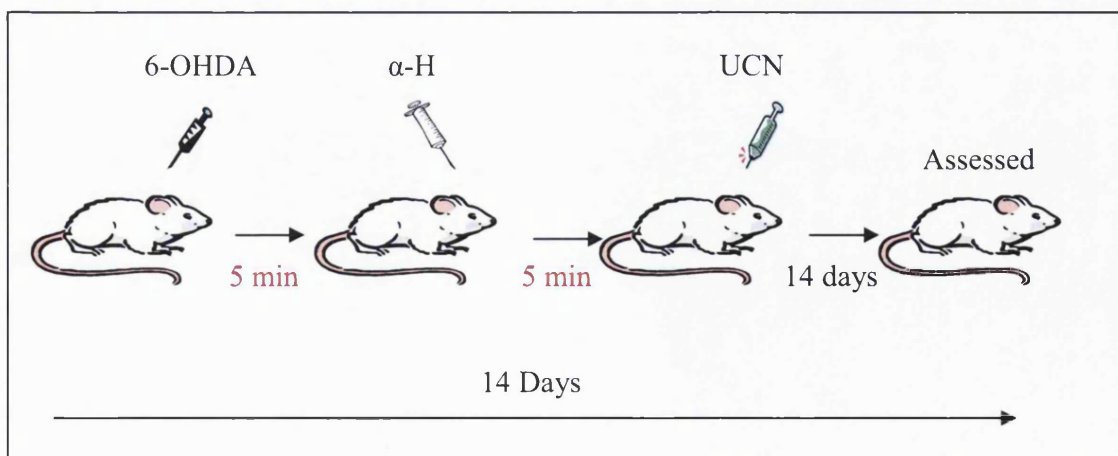
Figure 2.2.3. A schematic diagram showing the time line of α-helical CRF and UCN injections after lesioning with 6-OHDA,
6-OHDA injection was followed with α-helical CRF treatment before administering UCN; animals were assessed after 14 days from initial injection. All injections were unilateral.

2.2.1.4.2 NBI-27914

NBI-27914 is a small highly lipophilic molecule, it possesses high plasma protein and tissue binding, which leads to long elimination half life and accumulation in tissues like the brain (Chen, 2006). These properties enable NBI-27914 to cross the blood brain barrier (BBB) and remain there. NBI-27914 was injected i.p at a dose of 10mg/kg (Baram *et al.*, 1997), this injection was carried out 30 minutes before UCN injection (Baram *et al.*, 1997) and one week after treatment with 6-OHDA. These animals were assessed after seven days from NBI/UCN treatment and 14 days after initial 6-OHDA injection as shown in diagram below (figure 2.2.4).
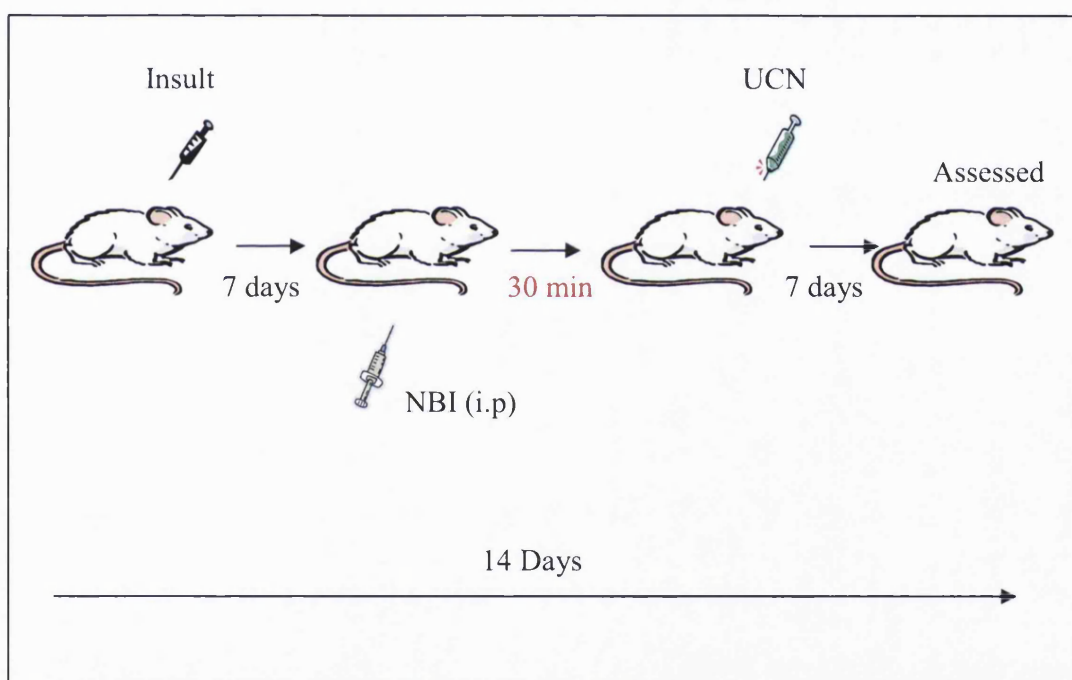
Figure 2.2.4 A schematic diagram showing the time line of NBI-27914 and UCN injections after lesioning with 6-OHDA,
After seven days from the initial 6-OHDA injection, rats were treated with NBI-27914 followed 30 minutes later with the administration of UCN; animals were assessed after 14 days from initial injection. All injections were unilateral and NBI-27914 was injected systemically.

2.2.2 Post mortem handling

As there were different assessments used, it was necessary to have different handling conditions for brain tissue depending on the nature of the assessment intended.

2.2.2.1 Flash freezing

Flash freezing was carried out on brain tissue intended for measuring tissue DA in striata and SNc, TH enzyme assays and some immunohistochemistry. Here rats were lightly anesthetized with 5% isoflurane then quickly placed under guillotine and decapitated. Brains were then very quickly removed, flash frozen on dry ice and stored at -80 until use.

2.2.2.2 Perfuse fixing

Perfuse fixing of rats was carried out when the only assessment intended was immunohistochemistry. In this procedure animals were administered a lethal dose of pentobarbital (0.6ml of 100 mg/ml solution; Euthatal, Merial, UK), and once animals were under deep anaesthesia, the heart was surgically exposed and a butterfly cannula (Butterfly-21, Hospira Venisystems, Ireland) was inserted into the left ventricle while the right atrium was incised to bleed the animal. At first animals were perfused transcardially with 100 ml of heparin-PBS solution 10 units/mL (Multiparin, Wockhardt, UK) to prevent coagulation of blood, followed by another 100 ml of 4% paraformaldehyde (PFA) solution in 0.1M PBS to fix the tissue, after 30 minutes rats were decapitated and the brains carefully removed and post fixed again in 4% PFA in 0.1% PBS for 24 hours at 4° C . Brains were then cryo-protected in a 30% sucrose solution for 24 hours at 4°C then flash frozen on dry ice and stored at -80°C until use.

2.3 Tissue handling (Brains)

Frozen brains were removed from -80°C storage and left at -20°C overnight, they were then placed on a ceramic tile over ice for a few minutes to defrost. Brains were then cut using a surgical blade into 2mm thick cross sections, these sections were made according to coordinates from the atlas of Paxinos and Watson (1982) for the striatum and confirmed by the marks left by needle injections or dialysis probes. From this cross section both right and left striata were punched out using a stainless steel punch. The dissected striata were then placed into a 1 ml microcentrifuge tube containing 1 ml ice cold PBS which was then immediately homogenized using a pestle and mortar 2 ml homogenizer (Kartell, Cambridge, UK) and frozen at -80 until further use.

As for the SNc, a cross section with a thickness of about 1mm was made according to coordinates from the atlas of Paxinos and Watson (1982) for the SNc. From this brain cross section SNc was punched out using a stainless steel punch, the dissected SNc was then placed into a 1 ml microcentrifuge tube containing 1 ml ice cold PBS, which was immediately homogenized and then frozen at -80 until further use.

2.4 HPLC

2.4.1 System components

The HPLC components start with a reservoir containing mobile phase which was pumped into the system by a piston pump (P580, Dionex, Germany). Flow then continued to pass through a Triathlon refrigerated autosampler (4°C; Spark-Holland, Netherlands), this led to a C18 reverse phase column maintained at 40°C (ODS 3 µM, 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 µM, 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 tissue homogenates. Another C18 reverse phase column also maintained at 40°C (ODS 3 μM, 4.6 mm I.D. x 150 mm; Rainin Dynamax Instruments Co. INC., U.S.A.) was used for L-DOPA measurement from the TH enzyme assays. Columns then led to an Antec-Intro electrochemical detector (Antec Leyden BV, Holland) fitted with a VTO3 flow cell (V_{cell} + 625 mV filtered to 5 abu with range set on 0.5 nA/volt for a full scale deflection). Data capture was achieved and analysed by Dell Corporation PC system 310 (Dell Corporation, U.S.A.) equipped with Chromperfect for Windows software (Justice Innovations chromatography data systems, CA, U.S.A.). Chromatograms were printed out using an Epson LX-300 printer as illustrated in the diagram (figure 2.4.1).

The separation technique used was based on that of Hutson et al. (1989) with some modifications. All seperations 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 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.
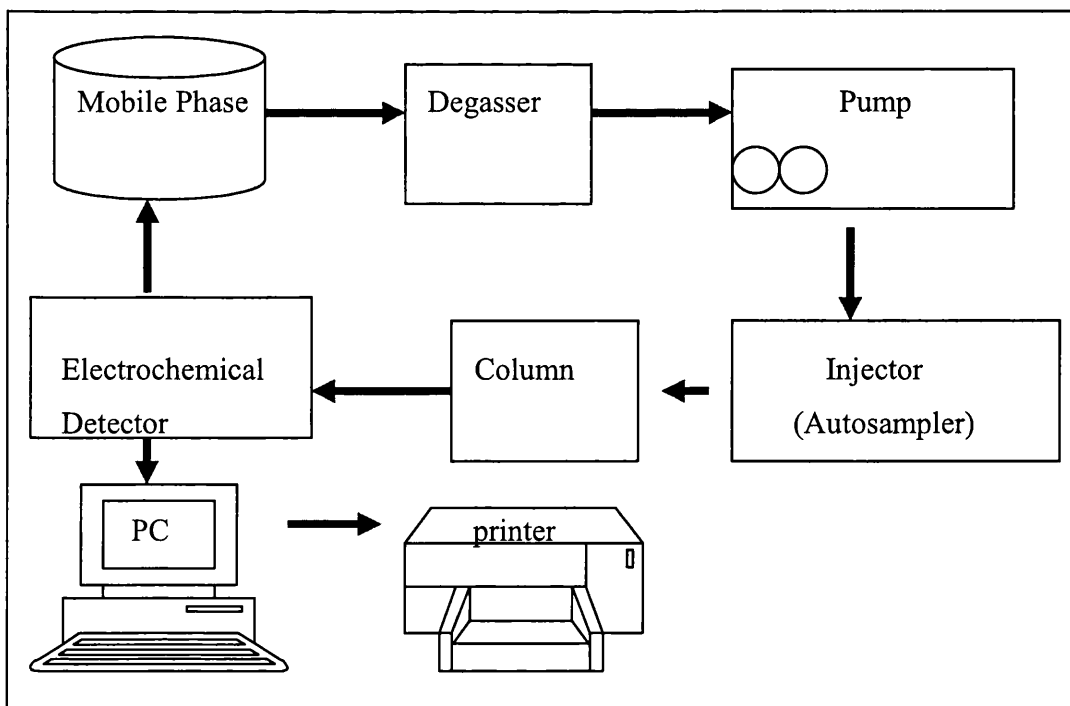
Figure 2.4.1 Schematic diagram of an HPLC-ED system.

2.4.2 Chromatograms

The catecholamines were first identified through comparing their retention times with authentic monoamine standards. They were then quantified through measuring peak area values generated from the analysis software and then comparing these values to a calibration curve.

Catecholamine peaks were presented as chromatograms and were displayed on a PC and then printed out. Figure 2.4.2 shows an example of a typical chromatogram of catecholamines using HPLC-ED.
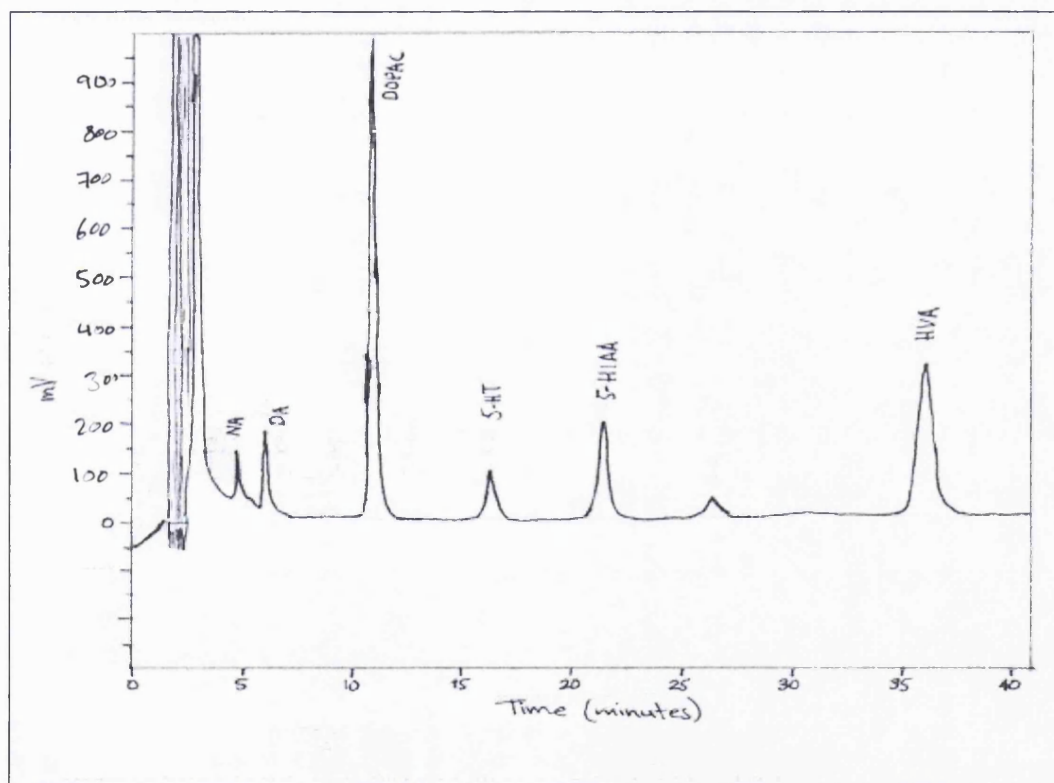
Figure 2.4.2 An example of a typical chromatogram of catecholamines using HPLC-ED.
The X axis indicates the retention times of analytes in minutes, and the Y axis gives an indication of amount or quantity shown as electrical in terms of current expressed in milliVolts. Chromatograms were generated using Chromperfect® software. NA: noradrenaline, DA: Dopamine, DOPAC: 3,4-dihydroxyphenylacetic acid , 5-HT: 5-hudroxytryptamine, 5-HIAA: 5-hydroxyindoleacetic acid , HVA: homovanillic acid.

2.4.3 Internal standard

An internal standard is added as a known quantity to the sample and has to resolve adequately from the analytes of interest to achieve accurate measurements. DA was used as the internal standard for measuring the amount of L-DOPA produced after the TH enzyme assay was performed; the calibration curve used here was peak area ratio vs. concentration as shown below in the diagram (figure 2.4.4). An example of a sample used to generate the calibration curve is shown in figure 2.4.3.

Figure 2.4.3 An example of one of the concentrations used to construct the calibration curve.
L-DOPA peak shown had a retention time of about 4 minutes and was generated from 30 pmol of L-DOPA; the amount of the internal standard (DA) was constant at 10 pmol.
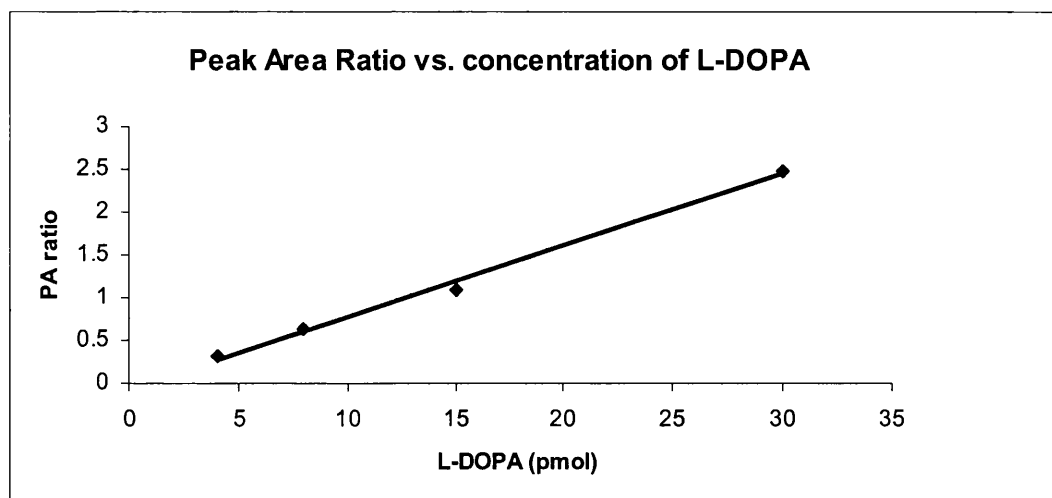
Figure 2.4.4 Calibration curve of L-DOPA amount vs. Peak Area Ratio (PAR).
This calibration curve was generated using internal standard method through fixing
the internal standard amount (DA) and changing the amount of L-DOPA.

2.4.4 External standard

The external standard was used for for quantifying DA from microdialysis samples
and from SNc and striatal homogenates, where a suitable internal standard that could
be separated from the components of the mixture could not be selected. The external
standard used was DA and different concentrations were run to achieve a calibration
curve as shown in figure 2.4.6, this was run as a separate chromatogram under exactly
the same conditions as samples, an example of one of the concentrations used for the
calibration curve is provided in figure 2.4.5.
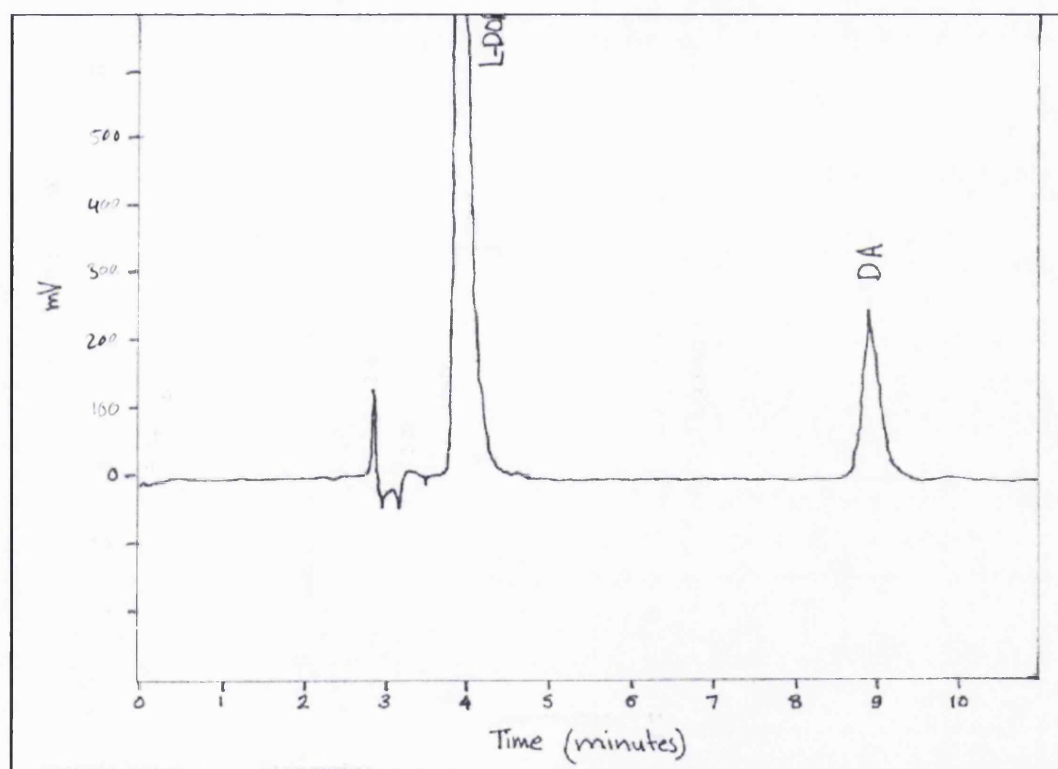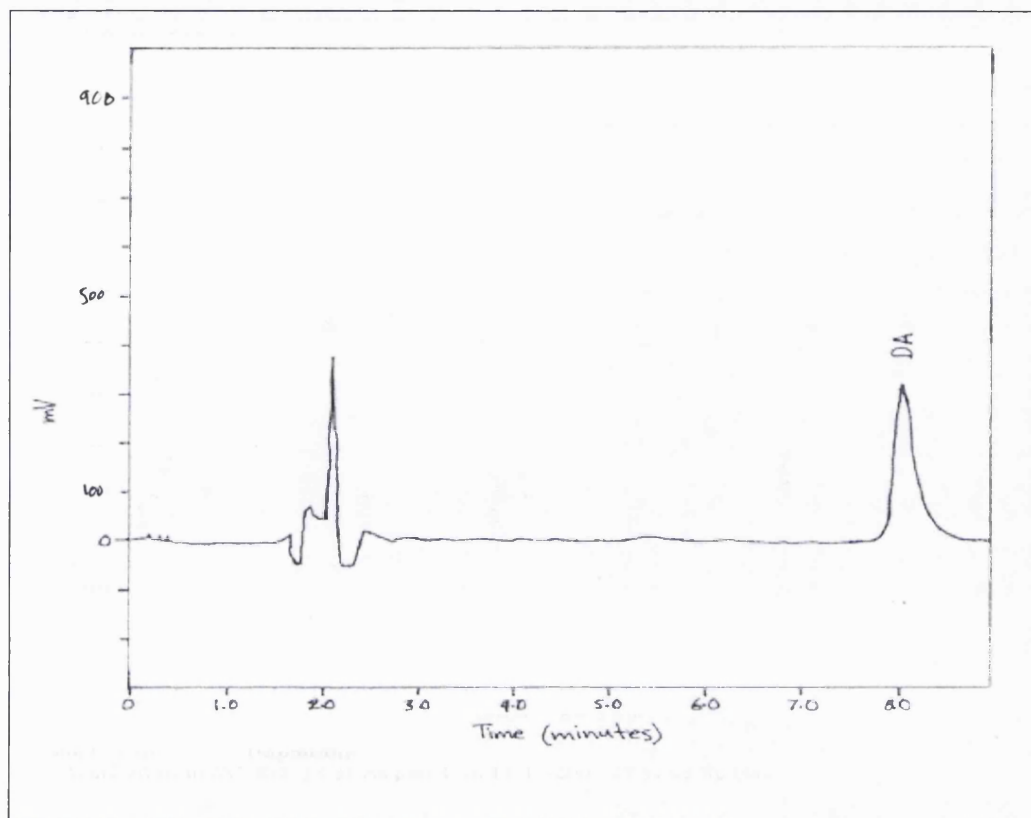
Figure 2.4.5 An example of one of the concentrations used to construct the calibration curve.
Peak shown had a retention time of approximately 8 minutes and was generated from 10 pmol of DA.
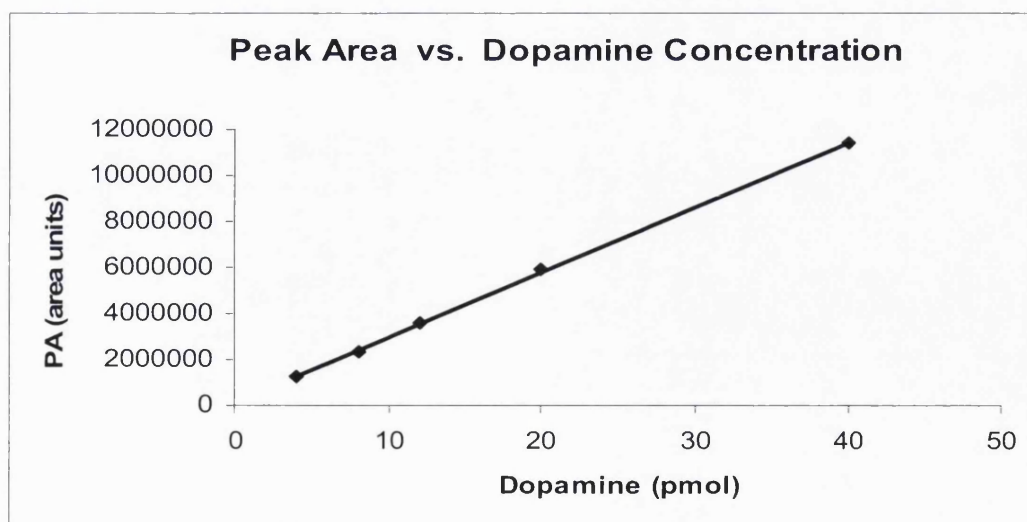
Figure 2.4.6 Calibration curve of DA amount vs. Peak Area.
This calibration curve was generated using different concentrations of DA.

2.5 Assessment

In order to evaluate the effects that UCN had on the animal models of PD employed, different assessment methods were used. These assessment included *in vivo* and *in vitro* evaluations which, when taken together, would give a better more comprehensive reflection of the disease state than would each one individually.

2.5.1 Apomorphine challenge

The first line of assessment is the behavioural apomorphine challenge. Apomorphine is a non-selective DA agonist which penetrates into the CNS upon subcutaneous (s.c) administration and is used as a measure of neurotoxic lesion severity. After its administration, apomorphine activates DA receptors, this actvation is much more pronounced on the lesioned side due to receptor upregulation and therefore contralateral (i.e. away from the lesioned side) circling behaviour can be observed. Fourteen days after initial surgery, the animals were injected with apomorphine (0.5mg/kg s.c.), after 10-15 minutes the rats were placed in a circular test arena, where they were left for two minutes to acclimatise to their surroundings. The number of consecutive turns they performed was then counted over a period of two minutes. The number of turns is indicative of the severity of the neurotoxic lesion (Pinter *et al.*, 1999).

2.5.2 *In vivo* microdialysis

Microdialysis was used to measure striatal extracellular levels of DA *in vivo*; this was performed bilaterally in order to compare these DA levels in treated and untreated sides of the same rat as well as comparing with DA levels of sham treated rats.

Animals were anaesthetized with isoflurane (4% induction and 1.5% maintenance) and then secured on a stereotaxic frame. The surface of the skull was exposed as mentioned previously and two concentric microdialysis probes, constructed as described by Whitton et al. (1992), were stereotaxically implanted and inserted into the striata on both sides of the brain (in mm from bregma A 0.2, L/R 3.0, V 8.0). The probes were then fixed in to the skull with dental acrylic (DuraLay, Reliance, Illinois, U.S.A.). The animals were then placed in individual microdialysis cages and allowed to recover for 24 hours before the dialysis was started. After recovery, rats were perfused with artificial cerebrospinal fluid (aCSF; 2.5mM KCl, 125mM NaCl, 1.18mM $MgCl_2,.6H_2O$, 1.26mM $CaCl_2$, pH:7.2) at a rate of 1µl/min, collecting samples every 30 minutes for 4 hours, samples were immediately frozen at -80°C after collection until analysed. The first four consecutive samples were collected and used to establish basal extracellular levels of DA. After collecting the fourth sample, the aCSF was switched to a higher (100mM) K^+ CSF and Na^+ was reduced to account for a change in molarity (100mM KCl, 27.5mM NaCl, 1.18mM $MgCl_2,.6H_2O$, 1.26mM $CaCl_2$, pH: 7.2), This was done for only 30 minutes after which the conditions were returned as before. The high potassium aCSF induces the release of DA from storage vesicles in nerve terminals and is a measure of the maximum nerve cell secretion capability. After collecting the last sample the animals were sacrificed using a guillotine and the brains were removed and frozen at -80°C until further use (Biggs *et al.*, 1992).

2.5.2.1 Analysis of microdialysis samples

Frozen microdialysis samples were removed from the -80°C freezer and placed in the refrigerated auto sampler at 4°C to defrost, analysis was then carried out using HPLC-ED. Samples were then quantified using external standard method. A calibration curve between peak areas vs. concentrations for DA was constructed and used to translate peak areas into concentrations (figure 2.4.6). An example of typical microdialysis chromatograms showing basal (figure 2.5.1) and potassium induced

catecholamine   levels   (figure   2.5.2)   is   shown   in   the   figures.
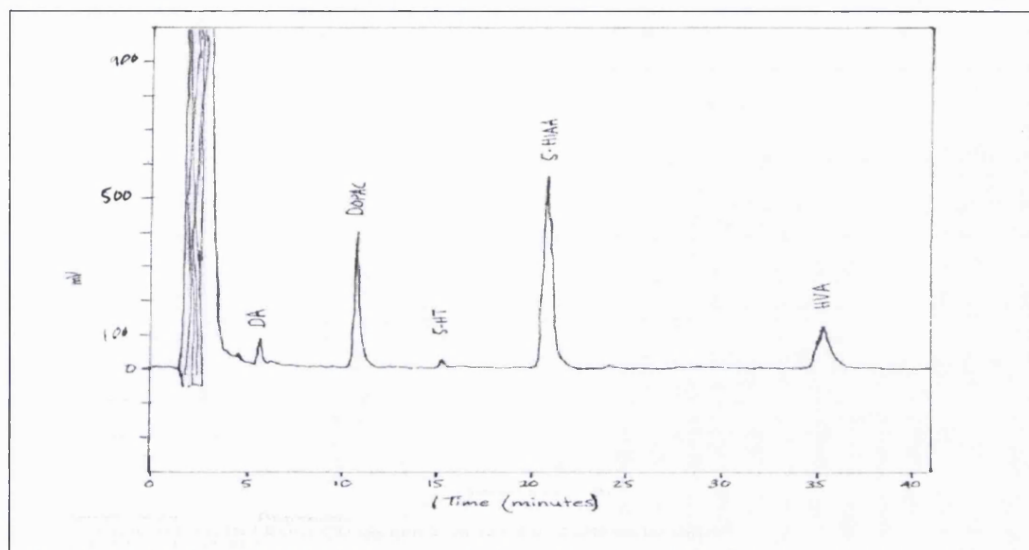
Figure 2.5.1 An example of a typical chromatogram generated from a dialysis sample illustrating basal levels of catecholamines.
NA: noradrenaline, DA: Dopamine, DOPAC: 3,4-dihydroxyphenylacetic acid , 5-HT: 5-hudroxytryptamine, 5-HIAA: 5-hydroxyindoleacetic acid , HVA: homovanillic acid.
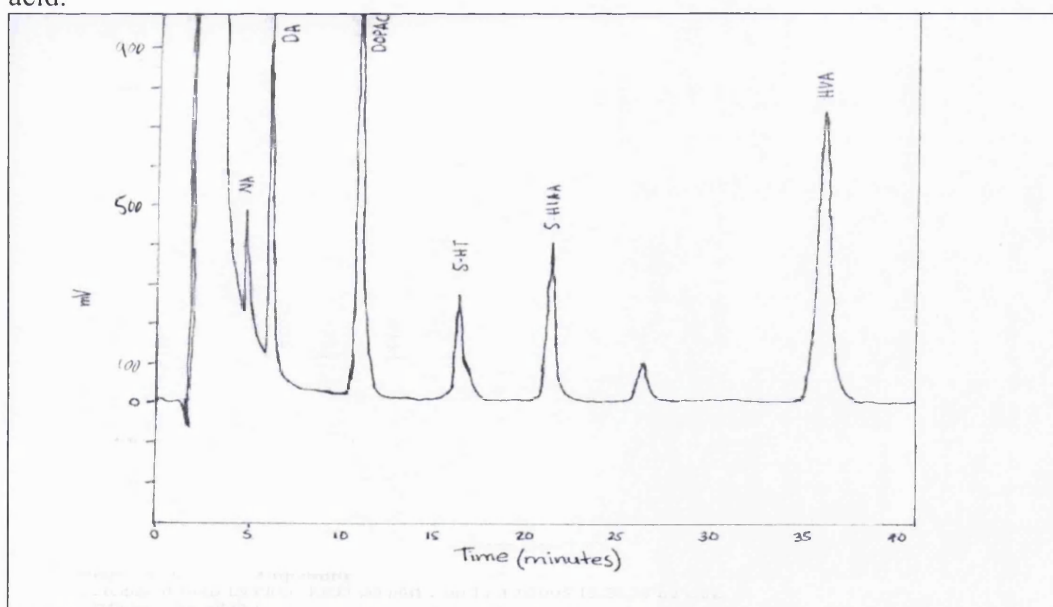
Figure 2.5.2 An example of a typical chromatogram generated from a dialysis sample illustrating high potassium induced levels of catecholamines
NA: noradrenaline, DA: Dopamine, DOPAC: 3,4-dihydroxyphenylacetic acid , 5-HT: 5-hudroxytryptamine, 5-HIAA: 5-hydroxyindoleacetic acid , HVA: homovanillic acid.

2.5.3 Tissue DA

To evaluate the neurochemistry of the lesion, tissue DA levels were estimated in the nigrostriatal pathway. After homogenising the brain tissue, 40 μL of supernatant from either striatal or SNc homogenates (prepared as described before) of the treated animals were mixed with 10 μL of 0.2M perchloric acid to precipitate proteins, these homogenates were then centrifuged at 10,000 x g (Biofuge, Heraeus Instruments, Germany) for 10 minutes, supernatants were placed in sample vials and whole tissue DA levels were estimated using HPLC-ED (Biggs *et al.*, 1992). An example of a typical tissue DA chromatogram is illustrated in figure 2.5.3, peak areas were converted to concentrations using external standard method (figure 2.4.6).
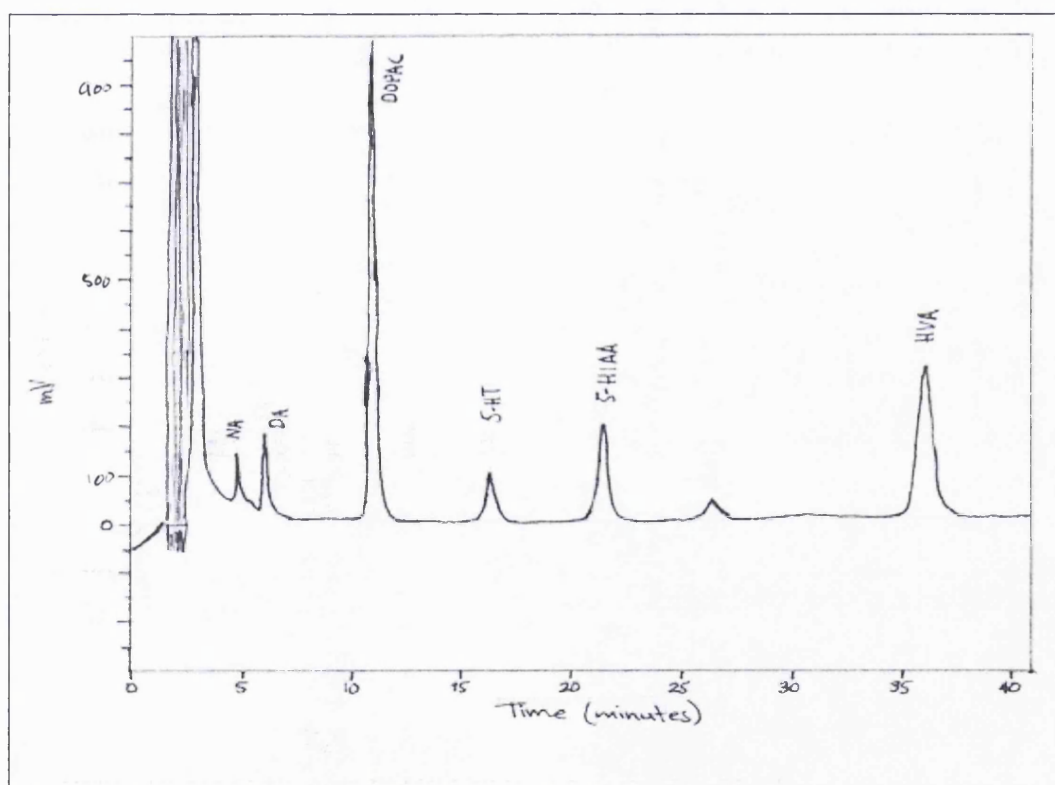
Figure 2.5.3 An example of a typical chromatogram generated from striatal homogenate illustrating tissue levels of catecholamines
NA: nore adrenaline, DA: Dopamine, DOPAC: 3,4-dihydroxyphenylacetic acid, 5-HT: 5-hudroxytryptamine, 5-HIAA: , HVA: homovanillic acid.

2.5.3.1 Protein estimation

Total protein content was determined using BCA reagent (Pierce Biotechnology, Inc. U.S.A.) according to the manufacturer's instructions.

2.5.4 Tyrosine Hydroxylase Assay

The TH enzyme assay was performed as one measure of the functional integrity of the nigrostriatal system, as L-DOPA synthetic capacity was measured. Brain homogenates from SNc and straiatum were incubated in duplicates with 200 μM L-tyrosine in a 100 μl total volume of the reaction mixture in 100 mM sodium acetate-acetic acid buffer (pH 6.0). The reaction mixture also contained 2 mM ferrous ammonium sulphate, 1 mM 6MPH$_4$ as co-factor, 10 μg of catalase, 1 mM benzirezide (an inhibitor of AADC). The 6MPH$_4$ solution was made as 10 mM in 1 M mercaptoethanol. The incubation mixture, except for tyrosine and the pteridin cofactor, was preincubated at 37°C for 5 min, and the reaction was started by adding the L-tyrosine and the pteridin cofactor. After incubation at 37°C for 10 min, the reaction was terminated by addition of 100 μl of 0.1 M perchloric acid, containing 0.4 mM sodium metabisulphite and 0.1 mM disodium EDTA.

The sample was then vortexed and left to stand on ice for 10 min and then centrifuged at 1000 x g for 10 min. The supernatant was diluted to 1 in 1000 in mobile phase to minimise the background, then analyzed using HPLC-ED to measure the amount of L-DOPA. As blank, a similar reaction mixture containing D-tyrosine instead of the L-isomer and 100 μM 3-iodo-L-tyrosine was used (Naoi et. al., 1988).

An example of a typical chromatogram measuring L-DOPA with DA as an internal standard is illustrated in figure 2.5.6. The identity of L-DOPA was determined after running a standard (figure 2.5.5). PA values were converted into concentrations using the calibration curve through internal standard method (figure 2.4.4).

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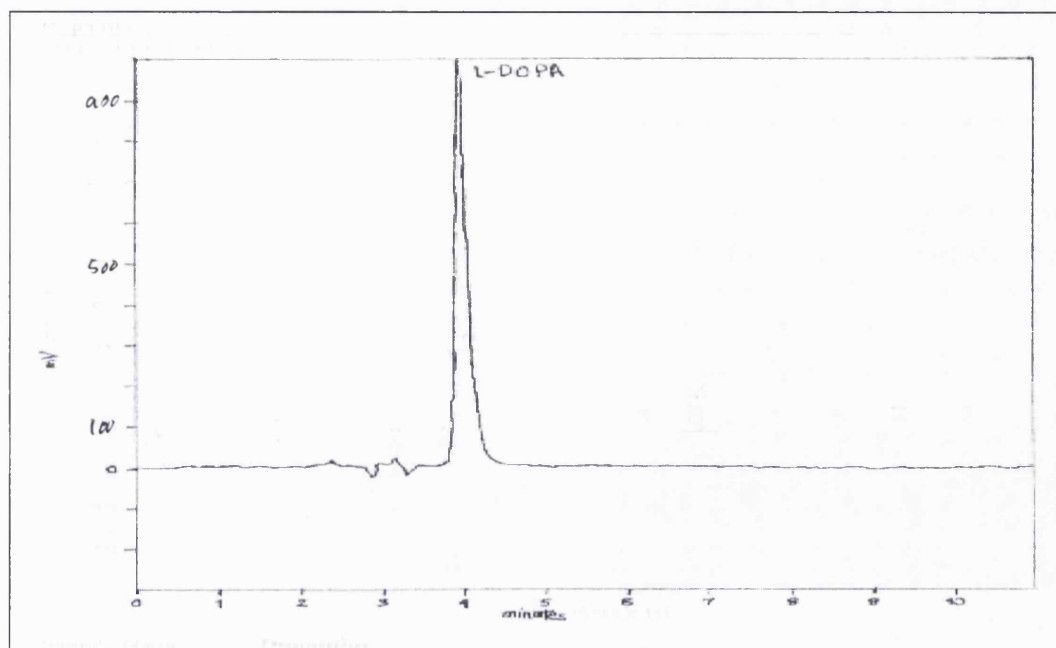
Figure 2.5.4 Chromatogram of an L-DOPA standard used before enzyme assay to determine retention time.
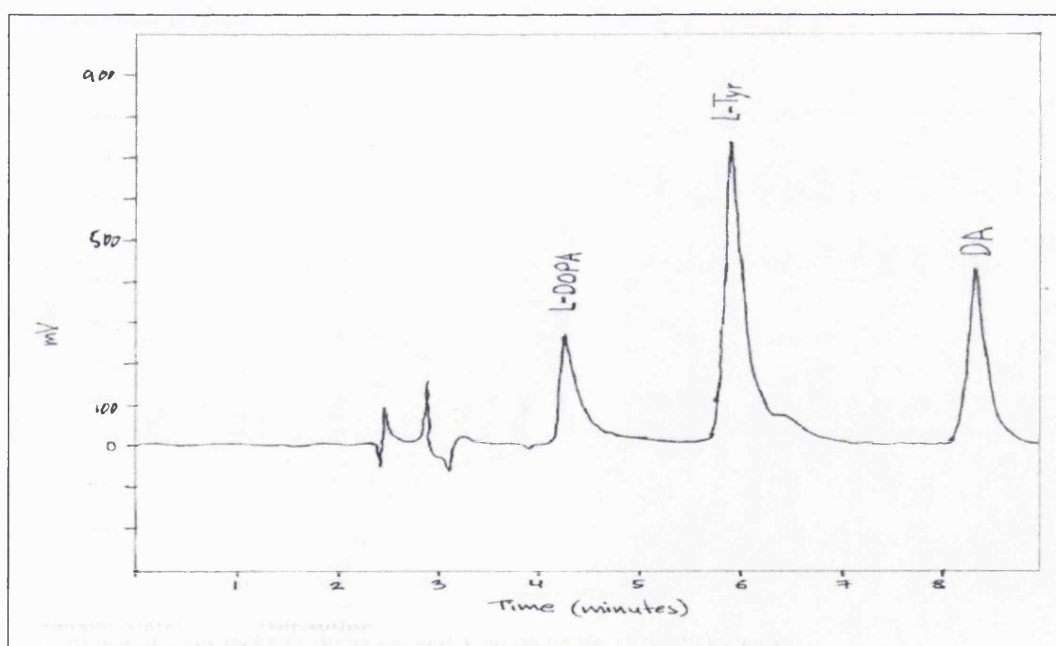
Figure 2.5.5 Chromatogram generated from the product of the TH enzyme assay.

2.5.5 Western Blot

To establish whether the loss of functionally active TH was associated with loss of protein a western blot was carried out. Brain striatal homogenate suspension was prepared in phosphate buffer (10.9 g Na2HPO4; 3.2 g NaH2PO4 in 1000 ml d.H2O; pH 7.4) containing protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin). Following centrifugation, supernatants were stored at -20 °C until analysis.

The supernatants were mixed with the same volume of SDS-PAGE sample buffer containing 100mM dithiothreitol (DTT) and the mixtures were heated to 95 °C for 4 minutes in order to denature proteins. Following this, samples and a molecular weight marker (Dual colour, Bio-rad, U.K.) were centrifuged at 13, 000 x g for 2 minutes, after which 25 µl of each sample and 15 µl of protein standard were separated according to molecular weight (Thermo EC570-90) in 12% tris-glycine gels (Invitrogen, U.K.), submerged in running buffer (3 g Trizma-base; Sigma-Aldrich Ltd., U.K; 14.4 g glycine; AnalaR, U.K; 1 g SDS in 1000 ml of d.H2O) for 45 minutes at 200 V.

The gels were then transferred to nitrocellulose membrane (Whattman, Kent, U.K.) for 1.5 h with a constant voltage of 15 V. Membranes were blocked for 30 minutes at room temperature with 5% powdered skimmed milk in PBS-Tween and then probed overnight at room temperature using primary antibodies for total TH (rabbit polyclonal, 1:500 dilution, PhosphoSolutions, Aurora, CO, U.S.A.). After probing with primary antibodies and washing with PBS-Tween buffer (3×5 min), membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Cell Signaling, MA, U.S.A.). Proteins were visualized using a chemiluminescence ECL kit (Amersham). Bands were visualized using an imaging system for chemiluminescence (GeneGnome, Vacutec, JHB). Results were expressed by comparison to the normalized control, and sham operated animals.

2.5.6 Immunohistochemistry

Slide-mounted 12 μm cryostat sections (sections were cut using a -20° CM3050 cryostat, Leica, Germany) from flash-frozen rat brain were removed from the freezer and allowed to equilibrate to room temperature for 30 minutes prior to post fixation in 4% paraformaldehyde, containing 1% gluteraldehyde for 5 minutes at 0°C. Following rinsing in 0.1M PBS for 5 minutes, sections were dehydrated through graded alcohols and endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in methanol for 10 minutes. The sections were then rehydrated and non-specific immunoreactivity was blocked with 10% swine serum (Dako, Denmark) in PBS for 10 minutes. Sections were then incubated in primary antibody (rabbit anti-rat tyrosine hydroxylase antibody, Calbiochem, UK) at 1 / 700 in PBS for 16 hours at 4°C. After rinsing, the sections were incubated sequentially in biotinylated swine anti-rabbit antibody 1 / 250 in PBS (Dako, Denmark) for 30 minutes at room temperature and ABC complex (Vector Laboratories, UK) following the manufacturer's instructions. Immunoreactivity was visualized through incubation in 0.5 mg/ml 3-diaminobenzidine (DAB) containing 0.0003% H_2O_2 for 2 minutes at room temperature. The sections were counterstained using Harris haematoxylin, dehydrated, cleared and mounted for microscopic examination (Pablo et al., 2006; Stephenson et al. 2006).

2.6 Data analysis

The data obtained from the apomorphine challenge, tissue DA assay and TH enzyme assay were all subjected to one way ANOVA, microdialysis data however was subject to a two way ANOVA. Data were then subject to a *post hoc* Bonferroni's test to establish significant differences between the groups and data were presented as mean ± s. e. mean. GraphPad Prizm, version 4, software was used for all of the

statistical analysis. Post hoc analysis was only carried out when ANOVA F values indicated significant difference between variances in a 95% confidence interval.

Chapter 3

UCN reverses toxin induced PD-like deficits

3   UCN reverses toxin induced PD-like deficits.

3.1   Introduction

PD is largely the result of a selective degeneration of nigrostriatal neurons. Before the disease presents clinically, death of nigrostriatal neurons occurs in the SNc asymptomatically, probably as a result of concurrent apoptotic, excitotoxic, free-radical mediated and neuroinflammatory events (Gandhi et al., 2005; Vaux et al., 1999; Whitton, 2007). Progression of the disease continues for the remainder of the patient's lifetime causing a decrease in the quality of life. Unfortunately current drug therapies are mostly symptomatic and diminish in efficacy, often rapidly with many side effects that frequently become, in time, almost as debilitating as the disease itself. Although alternative treatment strategies such as neurosurgery or the use of stem cells hold potential long term promise, these approaches have significant problems, the most obvious being 'when' or even 'if' neurosurgery will become available on the scale required to address the problem of PD. Prevention of nigro striatal neuronal destruction once established, or preferably reversal of the lesion, thus represents a crucial future therapeutic goal.

The CRF like peptide UCN has recently been proposed as a cytoprotectant. Evidence for this exists in a diverse range of mammalian tissues (Brar et al., 2000; Facci et al., 2003; Intekhad-Alam N.Y. et al., 2004; Pedersen et al., 2002; Scarabelli et al., 2002), including cultured hippocampal neurons (Pedersen et al., 2002) and cerebellar granule cells (Facci et al., 2003). Furthermore, UCN can restore function to cardiac myocytes after hypoxic damage has occurred (Brar *et al.*, 2000). This makes it an ideal candidate to study in models of PD

3.1.1 PD models used

Although a specific cause of PD remains to be revealed, evidence indicates interplay between apoptotic, excitotoxic and free radical mediated events (Gandhi *et al.*, 2005; Vaux *et al.*, 1999). However, understanding of the pathogenic background of this disease has been confounded by the fact that epidemiological studies have provided little evidence for a single causative factor. This in turn makes it difficult to achieve one model that will comprehensively and completely mimic the disease. Nevertheless, 6-OHDA-induced neurotoxic damage presents a useful and well characterised model of nigral DA neurodegeneration and is commonly used as a paradigm of PD. 6-OHDA leads to the destruction of DA neurons through both ROS and quinones (Cohen, 1984) and many studies have shown that the neurotoxicity caused by 6-OHDA brings about molecular changes similar to those encountered in PD (Blum et al., 2001). Researchers have long considered that 6-OHDA provides a very good model of PD; indeed it was suggested that 6-OHDA might exist as an endogenous neurotoxin which can naturally occur in both rat (Axelrod *et al.*, 1958a; Axelrod *et al.*, 1958b) and human brain (Curtius *et al.*, 1974), and that its excessive production due to an imbalance or trigger could be a causative factor for PD. 6-OHDA also provides a good model due to its relatively predictable outcome, which is almost complete destruction of the nigrostriatal pathway within 2 weeks of injection.

Recently, however, it has been found that in both PD patients and animal models of this disease, neuroinflammation appears to be a common factor; comprising phagocyte activation, increased synthesis and release of proinflammatory cytokines and complement activation. These findings indicate the usefulness of more recently characterised neuroinflammatory paradigms of PD. LPS is a bacterial endotoxin able to evoke a degeneration of nigral DA neurons whilst sparing other cell types such as ACh and GAD containing neurons (Herrera *et al.*, 2000). The manner in which LPS produces the lesion makes it even more comparable to PD, in terms of severity and selectivity, especially when compared to the harsher 6-OHDA paradigm (Castano *et al.*, 2002; De Pablos *et al.*, 2005). However, this distinction makes it an excellent candidate to use alongside the 6-OHDA model to generate a more comprehensive

overview of PD, as one model may mimic the nature of the disease in ways that the other fails to.

Moreover, when employing two distinct animal models of PD, any neuronal effects that the CRF-like peptide UCN offers are less likely to be toxin or model specific.

Experiments were performed to investigate whether UCN confers neuroprotection in rats treated either with 6-OHDA or LPS. The effects of UCN were established through the use of different assessments which included _in vivo_ apomorphine challenge, _in vivo_ microdialysis, _in vitro_ TH activity assay and estimation of tissue DA level. Finally, the surviving TH positive neurons in the SNc were assessed using immunohistochemistry.

3.2 Experiment design

Rats were divided into 4 treatment groups; the first group of rats (sham surgical group) were given 4 µl i.c. injections of vehicle (0.2% ascorbic acid) into the medial forebrain bundle, as described in chapter 2. The second group received 8 µg of 6-OHDA delivered in 4 µl of 0.2% ascorbic acid, followed by an i.c. injection of 2 µl saline directly into the SNc. The third group of rats received 8 µg of 6-OHDA delivered in 4 µl of 0.2% ascorbic acid and then were treated with a further i.c. injection of 2 µg UCN, delivered in 2 µl saline, directly into the SNc. The fourth group of rats received 4 µl i.c injections of 0.2% ascorbic acid into the medial forebrain bundle followed by 2 µg UCN, delivered in 2 µl saline, directly into the SNc in order to assess the the effects of UCN in the absence of a nigrostriatal lesion. In the case of more than one i.c injection in the same rat, there was a 5 minute waiting period between these i.c injections. Another 4 groups of rats were treated as above but with LPS as the insult, whereby 2µg LPS in 2 µl PBS was injected directly into the SNc in lieu of 6-OHDA. All rats were assessed 14 days after initial injections. All results in bar graphs are shown as mean ± SEM.

A schematic time bar is provided to clarify times of injections and procedures carried out in each experimental group (diagram 1 for 6-OHDA and diagram 2 for LPS). In total 64 rats were used in this section.

Diagram 1 Schematic time bar indicating times of injections and procedures carried out in each experimental group.
Where A is SHAM group, B is 6-OHDA only group, C is 6-OHDA + UCN group and D is UCN only group. Number of rats used in this expirement: n = 33
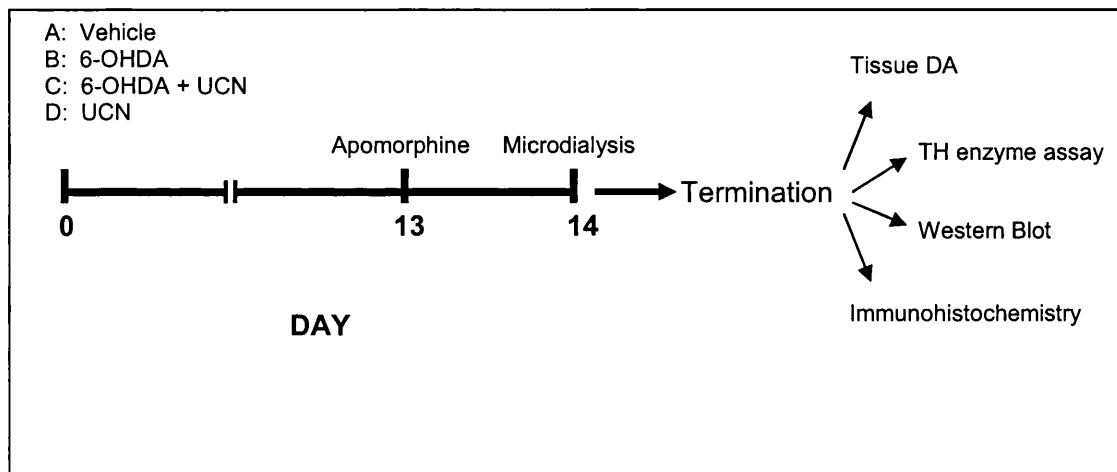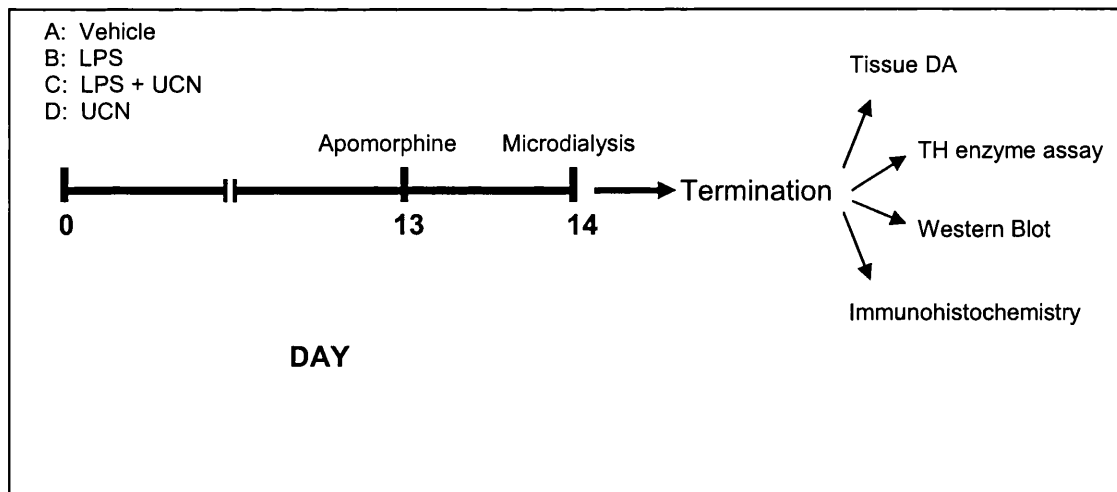

Diagram 2 Schematic time bar indicating times of injections and procedures carried out in each experimental group.
Where A is SHAM group, B is LPS only group, C is LPS + UCN group and D is UCN only group. Number of rats used in this expirement: n = 31

3.3 The effect of UCN in 6-OHDA lesioned rats

3.3.1 Apomorphine challenge

Rats that were treated with 6-OHDA and vehicle displayed characteristics of unilateral nigrostriatal degeneration after being subjected to the apomorphine challenge where they demonstrated intense, tight contraversive circling, around 25 turns in 120 second, which was significantly different than in sham treated rats where no turning was observed (Figure 3.1.1). In contrast, rats treated with 6-OHDA and co-treated with UCN showed reduced sensitivity to the apomorphine challenge; this was indicated by the significant reduction of about 80% in the number of turns from the 6-OHDA + vehicle treated animals, leaving a residual number of turns that was not significantly different to that seen in sham treated animals. Rats treated with UCN alone, with vehicle in place of 6-OHDA, did not exhibit any turns and were comparable to sham treated animals (Figure 3.2.1).
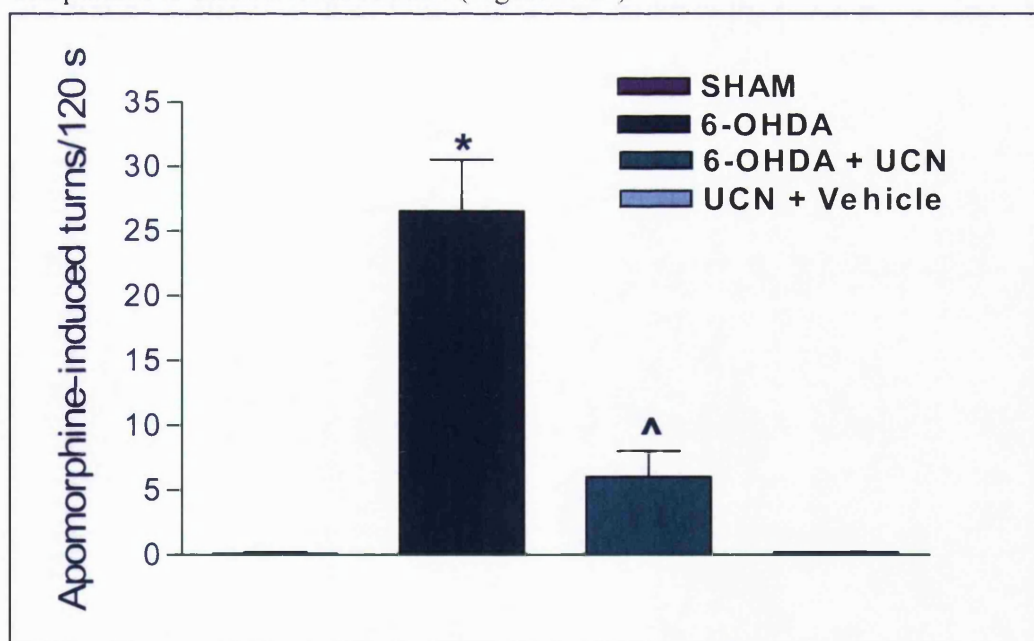
Figure 3.3.1 Effects of UCN on 6-OHDA-induced contraversive turns in response to apomorphine.
Apomorphine injection (0.5 mg/kg) was delivered through s.c injection. Turns were recorded in a 1.2 metre circular arena for all animals as mean counts / 120 s.
* Significantly different to sham and UCN treated groups (p<0.001), ^ significantly different to 6-OHDA + vehicle (p<0.001). 6-OHDA + UCN not significantly different to sham (p>0.05; n = 6-7 rats in each treatment group)

3.3.2 *In vivo* microdialysis

In vivo microdialysis of the striatum was performed to evaluate the functional integrity of the nigrostriatal pathway, through the measurement of extracellular DA levels; measurements were performed for both basal and potassium induced levels (Figure 3.3.2). Extracellular DA levels in rats treated with 6-OHDA and vehicle were significantly reduced when compared to sham operated animals, both basally and in response to high potassium concentration. However, levels measured in animals treated with 6-OHDA and UCN were comparable to sham equivalents, with no significant difference observed. All contra-lateral sides displayed extracellular DA levels very similar to those of sham treated animals both at basal and potassium induced levels (Figure 3.3.2).
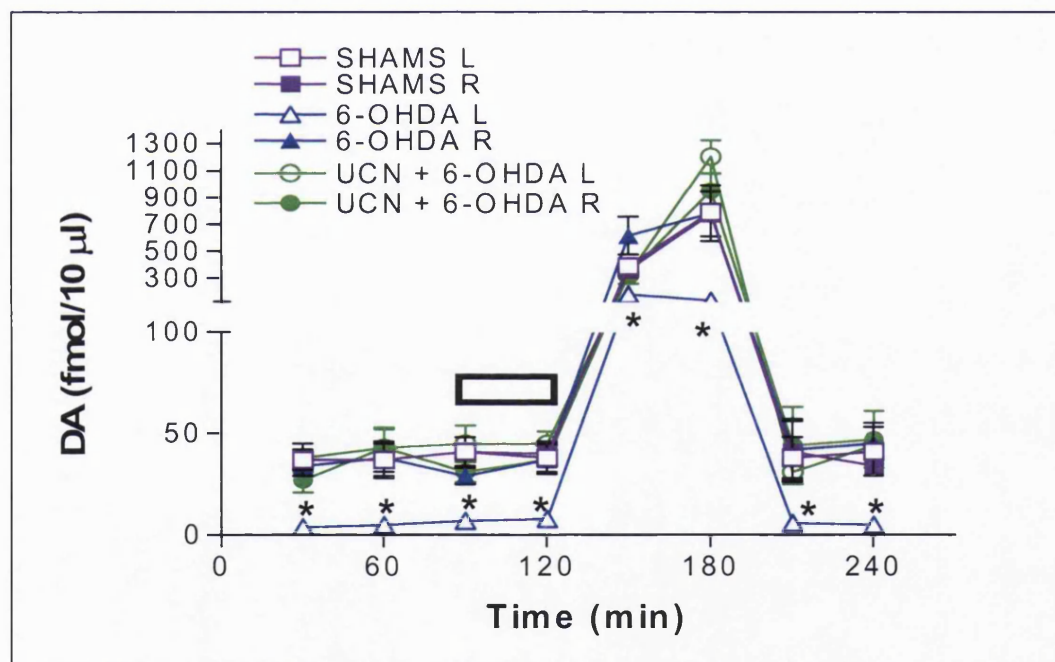
Figure 3.3.2 The effect of UCN on loss of extracellular striatal DA levels as a result of 6-OHDA unilateral treatment where data was obtained from *in vivo* microdialysis.
Microdialysis was performed over a period of 4 hours both at basal levels, and potassium induced levels where animals were infused with high potassium (ACSF) indicated by black rectangle. * Significantly different to sham ($p < 0.05$) at basal levels and ($p < 0.001$) at potassium induced levels. 6-OHDA + UCN not significantly different to sham. Bilateral micro dialysis was performed with L indicating left or treated side and R indicating right or untreated side. Break in Y axis to facilitate data expression. n= 4-6 rats in each treatment group.

3.3.3 Measurement of tissue DA

To estimate the intracellular functionality of the TH enzyme, tissue DA levels were assessed in the both nigra and striata of rats. In this case, animals treated with 6-OHDA and vehicle showed dramatic depletion in both nigral and striatal DA levels which was significantly lower than in sham operated animals (Figure 3.3.3 a and b). By contrast, rats co-treated with UCN in addition to 6-OHDA showed almost complete preservation of tissue DA levels to control (sham group) levels. Animals that received UCN and vehicle treatment did not produce any significant change in DA tissue levels in comparison to sham treated animals.
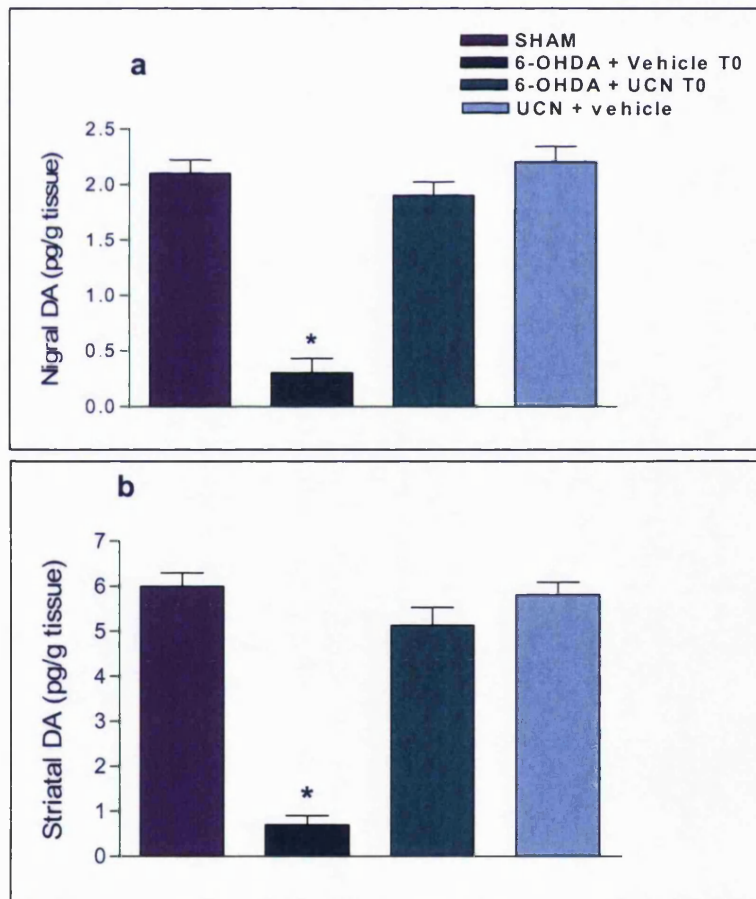
Figure 3.3.3 Effects of UCN on 6-OHDA-induced tissue DA loss.
 DA levels were measured in either SNc (a) or striata (b) through HPLC-ED
 * Significantly different to sham and UCN treated groups (p<0.001). 6-OHDA + UCN not significantly different to sham (p>0.05). n=4-6 in each treatment group.

3.3.4 TH enzyme assay

The functional integrity of the nigrostriatal DA system was also evaluated through the L-DOPA synthesising capacity of the TH enzyme. This was carried out using an *in vitro* enzyme assay, using nigral and striatal homogenates as the source of the TH, with L-tyrosine as the substrate and L-DOPA as the measured end product. TH activity was significantly reduced in nigra and striata obtained from 6-OHDA + vehicle treated rats when compared to sham treated controls. This loss of TH activity due to 6-OHDA treatment was prevented by co-treatment with UCN whereby levels were not significantly different to sham controls in both nigra and striata. UCN alone did not significantly influence TH activity (Figure 3.3.4 a and b).
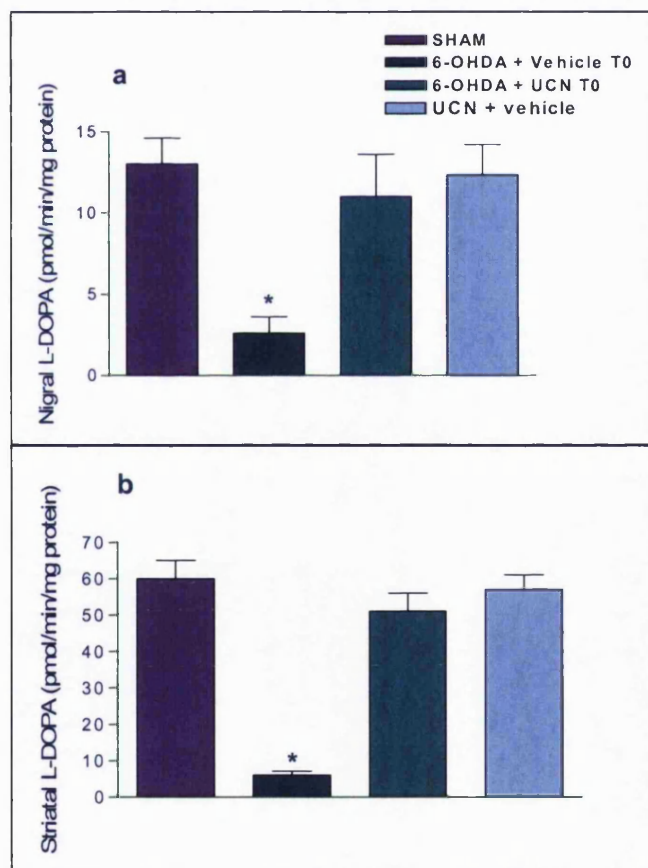
Figure 3.3.4 The effect of UCN on 6-OHDA-induced TH activity loss.
TH activity was measured as moles L-DOPA produced per min per mg protein after a 10 min enzyme assay using homogenates from SNc (a) and from striatum (b). L-DOPA was quantified using HPLC-ED after the termination of the reaction and DA was used as the internal standard. * Significantly different to sham and UCN treated groups ($p<0.01$) in a and ($p<0.001$) in b. 6-OHDA + UCN not significantly different to sham. n=4-6 rats in each treatment group.

3.3.5 Western blotting

The approximate amount of TH protein expressed was measured using Western blotting; this was carried out to assess whether the loss or retention of TH activity was due to changes in protein content. It is apparent from the results obtained (Figure 3.3.5) that TH protein content was greatly reduced in striata from 6-OHDA + vehicle treated animals and that this protein loss was greatly reduced when animals were co-treated with UCN.
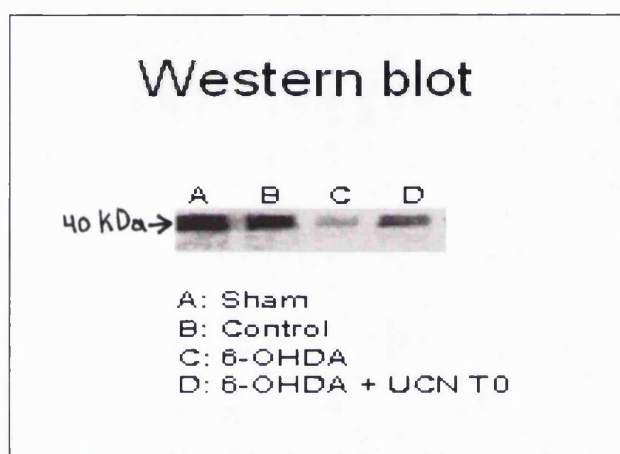
Figure 3.3.5 Effects of UCN on 6-OHDA induced TH protein loss
Representative western blot analysis of TH protein content in striatal homogenates. 6-OHDA + UCN T0 indicate rats that were administered 6-OHDA and UCN concomitantly.

3.3.6 Immunohistochemistry

Nigral sections were subject to TH staining in order to determine whether the changes in DA activity were due to the loss of DA neurons or alterations in nigral TH. It can clearly be seen from the results that there was a substantial difference between the TH positive neurons in the 6-OHDA lesioned SNc than in the non lesioned SNc, as illustrated in Figures 3.3.6 d and c respectively, and also in comparison to sham treated animals (Figure 3.3.6 b). The effects of UCN can clearly be seen, showing preservation of the normal (comparable to sham or untreated SNc) TH positive neurons in the SNc of lesioned animals (Figure 3.3.6 f) in comparison to animals that were 6-OHDA-lesioned in the absence of UCN treatment (Figure 3.3.6 d).

a: SHAM untreated side   b: SHAM Treated side

c: 6-OHDA Untreated   d: 6-OHDA Treated

e: 6-OHDA + UCN untreated   f: 6-OHDA +UCN treated
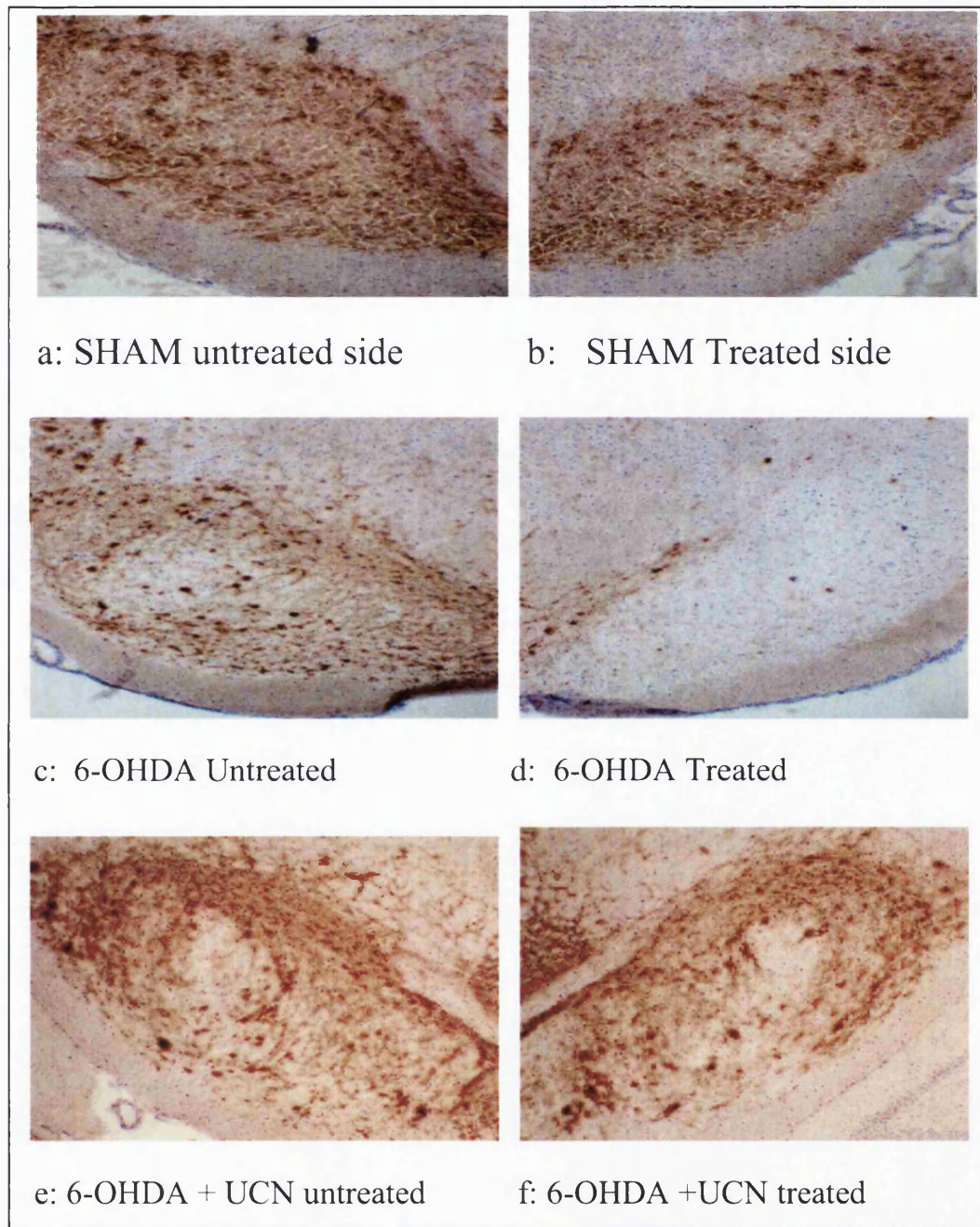
Figure 3.3.6 Representative photomicrographic images of rat brain cross sections at the SNc showing UCN effect on TH neuron survival after 6-OHDA unilateral lesion was induced.

For each section a comparison can be made between treated and untreated SNc in an individual rat (image shown is one example from a group of 4), sides labelled treated were treated with either vehicle only (b), 6-OHDA+ vehicle (d) or 6-OHDA + UCN (f), sides labelled untreated are shown for comparison. DAB stain (orange brown colour) is indicative of TH positive neurons.

3.4   The effect of UCN on LPS lesioned rats

3.4.1 Apomorphine challenge

Rats that were treated with LPS and vehicle displayed characteristics of unilateral nigrostriatal degeneration, although not to the same extent as in 6-OHDA treated animals. When LPS-treated animals were subjected to apomorphine challenge they demonstrated intense, tight contraversive circling of around 13 turns per 120 s; whilst significant, apomorphine induced circling in LPS-lesioned animals was less than that observed in the 6-OHDA model (circa 13 turns per 120s LPS; circa 25 turns per 120s 6-OHDA). No turning was observed in sham treated animals (Figure 3.4.1). However, as seen in 6-OHDA treated animals, rats treated with LPS and co-treated with UCN showed reduced sensitivity to apomorphine challenge; this was indicated by a significant reduction of more than 50% in the number of turns compared to the LPS + vehicle treated animals, leaving a residual number of turns that did not differ significantly from sham controls. Rats treated with UCN and vehicle did not exhibit any turns in response to apomorphine (Figure 3.4.1).
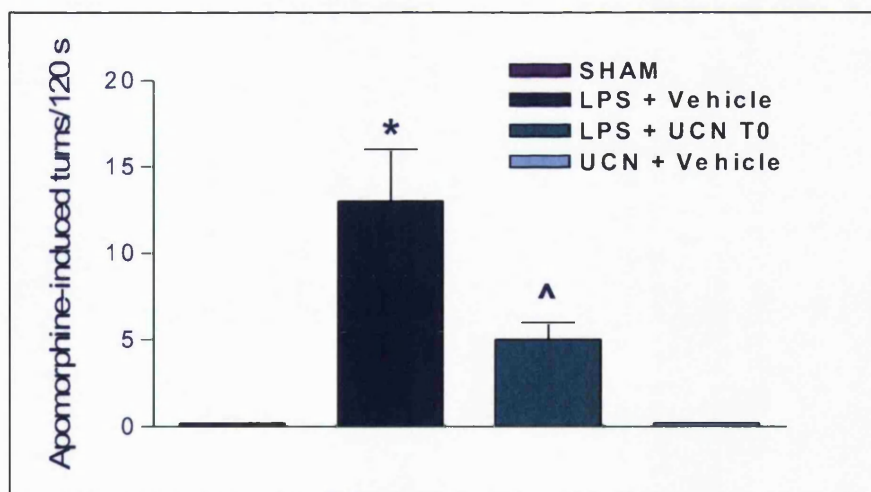
Figure 3.4.1 Effects of UCN on LPS-induced contraversive turns in response to apomorphine.
Apomorphine (0.5 mg/kg) was delivered through s.c injection. Turns were recorded in a 1.2 metre circular arena from all animals as mean counts / 120 s. * Significantly different to sham and UCN treated groups (p<0.001), ^ significantly different to LPS + vehicle (p<0.05). 6-OHDA + UCN not significantly different to sham (p>0.05). n=5-6 rats per treatment group.

3.4.2 *In vivo* microdialysis

Microdialysis was performed bilaterally in the striata of freely moving animals to evaluate the functional integrity of the nigrostriatal pathway through the measurement of extracellular DA levels. These measurements were done both at basal levels and potassium induced levels (Figure 3.4.2). Extracellular DA levels in rats treated with LPS + vehicle were significantly lower than the levels in sham-operated animals both at basal and at potassium induced levels. DA levels estimated in animals treated with LPS and co-treated with UCN were comparable with sham equivalents and there was no observed significant difference at both basal and potassium induced levels. All right hand sides were untreated and displayed extracellular DA levels parallel to those of shams both at basal and potassium induced levels (Figure 3.4.2).
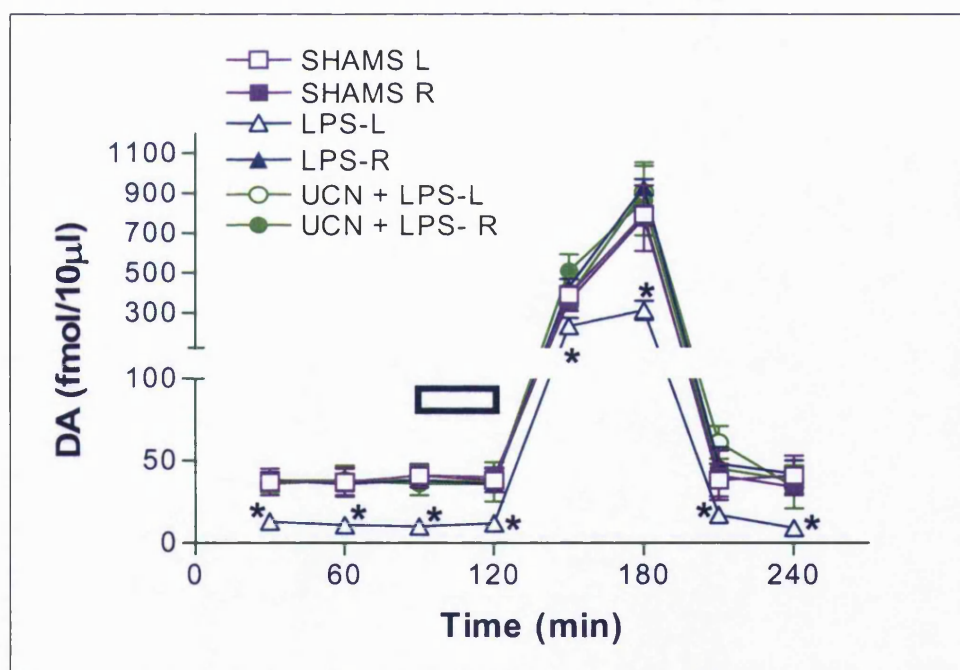
Figure 3.4.2 The effect of UCN on LPS induced loss of extracellular striatal DA levels obtained from *in vivo* microdialysis.
Microdialysis was performed over a period of 4 hours both at basal levels, and potassium induced levels where animals were infused with high potassium (ACSF) indicated by black rectangle. * Significantly different to sham and UCN treated groups ($p < 0.01$). LPS + UCN not significantly different to sham. Bilateral micro dialysis was performed with L indicating left or treated side and R indicating right or untreated side. Break in Y to facilitate data expression. n=3-5 rats in each treatment group.

3.4.3 Measurement of tissue DA

Tissue DA levels were assessed in both the nigra and striata of rats. Animals treated with LPS and vehicle showed dramatic depletion of both nigral and striatal DA levels which were significantly lower than sham in operated animals (Figure 3.4.3 a and b). In contrast, rats that were co-treated with UCN in addition to LPS showed an almost complete restoration of tissue DA levels to those measured in sham treated controls. Animals that recieved UCN and vehicle did not show any significant change in DA tissue levels in comparison to sham treated rats.
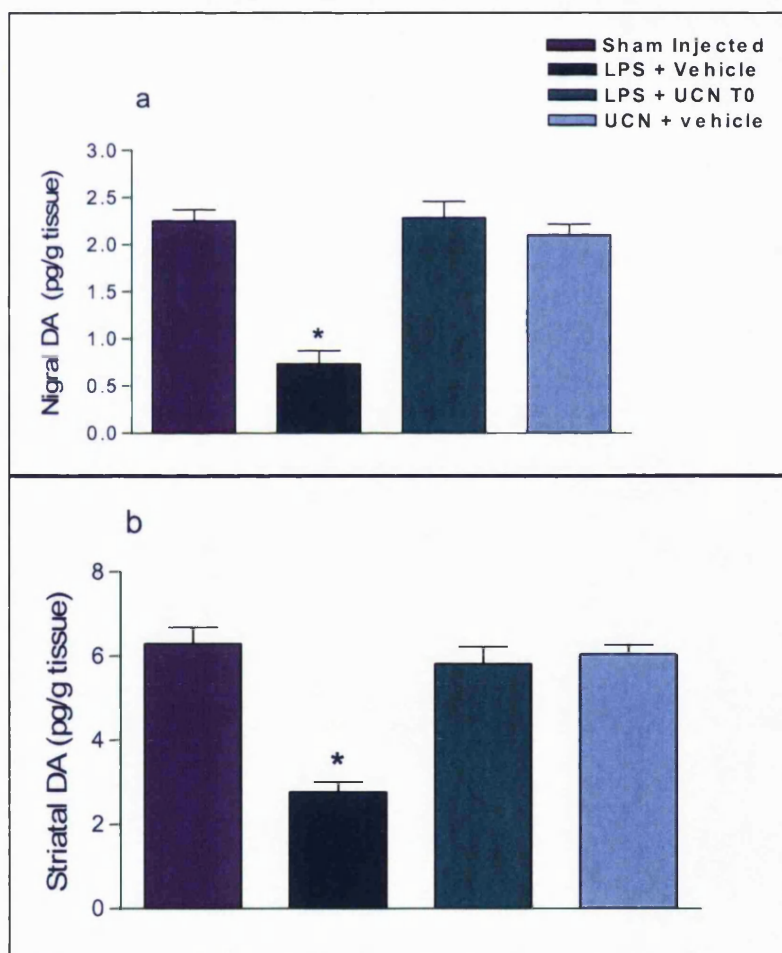
Figure 3.4.3 Effects of UCN on LPS-induced tissue DA loss.
DA levels measured in either SNc (a) or striata (b) through HPLC-ED.
* Significantly different to sham and UCN treated groups (p<0.001). LPS + UCN not significantly different to sham (p>0.05). n=4-6 rats in each treatment group.

3.4.4 TH assay

The functional integrity of the nigrostriatal DA system after LPS treatment was also evaluated through the L-DOPA synthesising capacity of the TH enzyme. This was again performed using an *in vitro* enzyme assay, using nigral and striatal homogenates as the source of the TH with L-tyrosine as the substrate and L-DOPA as the measured end product. TH activity was significantly reduced in nigra and striata obtained from LPS + vehicle treated rats when compared to sham controls. This loss of TH activity was prevented by co-treatment with UCN, whereby levels were not significantly different to those measured in sham controls in both nigra and striata. UCN alone did not have any significant effect on TH activity (Figure 3.4.4 a and b).
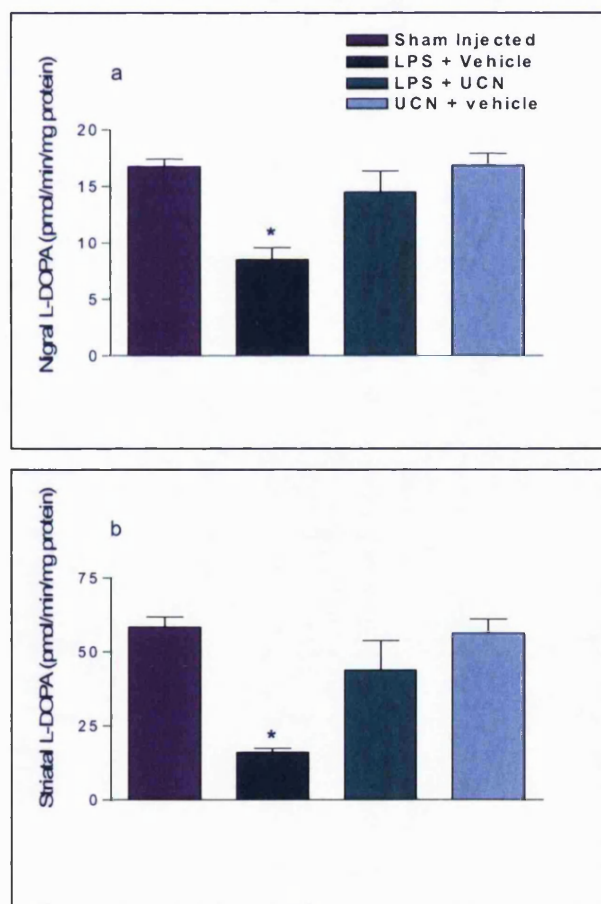
Figure 3.4.4 The effect of UCN on LPS-induced TH activity loss.
TH activity was measured as moles L-DOPA produced per min per mg protein after a 10 min enzyme assay using homogenates from SNc (a) and from striatum (b). L-DOPA was quantified using HPLC-ED after the termination of the reaction and DA was used as the internal standard. * Significantly different to sham and UCN treated groups (p<0.001). 6-OHDA + UCN not significantly different to sham. n=4-6 rats in each treatment group.

3.4.5 Western blotting

The amount of TH protein expressed was measured using western blotting; this was done to assess whether the loss or retention of TH activity was due to changes in protein content. It is obvious from the results obtained (Figure 3.4.5) that TH protein content was greatly reduced in striata from LPS + vehicle treated animalsand that this protein loss was reduced when animals were co-treated with UCN.
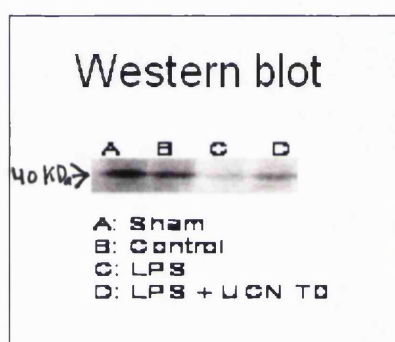
Figure 3.4.5 Effects of UCN on 6-OHDA LPS induced TH protein loss.
Representative western blot analysis of TH protein content in striatal homogenates. LPS + UCN T0 indicate rats that were administered LPS and UCN concomitantly.

3.4.6 Immunohistochemistry

Nigral sections were subject to TH staining to determine whether the changes in DA activity were due to the modification in the number of DA neurons or alterations in the amount of TH in individual cells. It is clear from the images that there was a substantial difference between TH positive neurons in the LPS lesioned SNc than in the non lesioned SNc, as illustrated in figures 3.4.6 d and c respectively, and also in comparison to sham treated animals (figure 3.4.6 b). The effects of UCN can clearly be seen, with preservation of the normal (comparable to sham or untreated SNc) TH positive staining in the SNc of lesioned animals (figure 3.4.6 f) in comparison to animals that were LPS-lesioned (figure 3.4.6 d).

a: Sham untreated side   b: Sham treated side

c: LPS Untreated side   d: LPS Treated side

e: LPS+UCN T0 untreated side   f: LPS+UCN T0 treated side
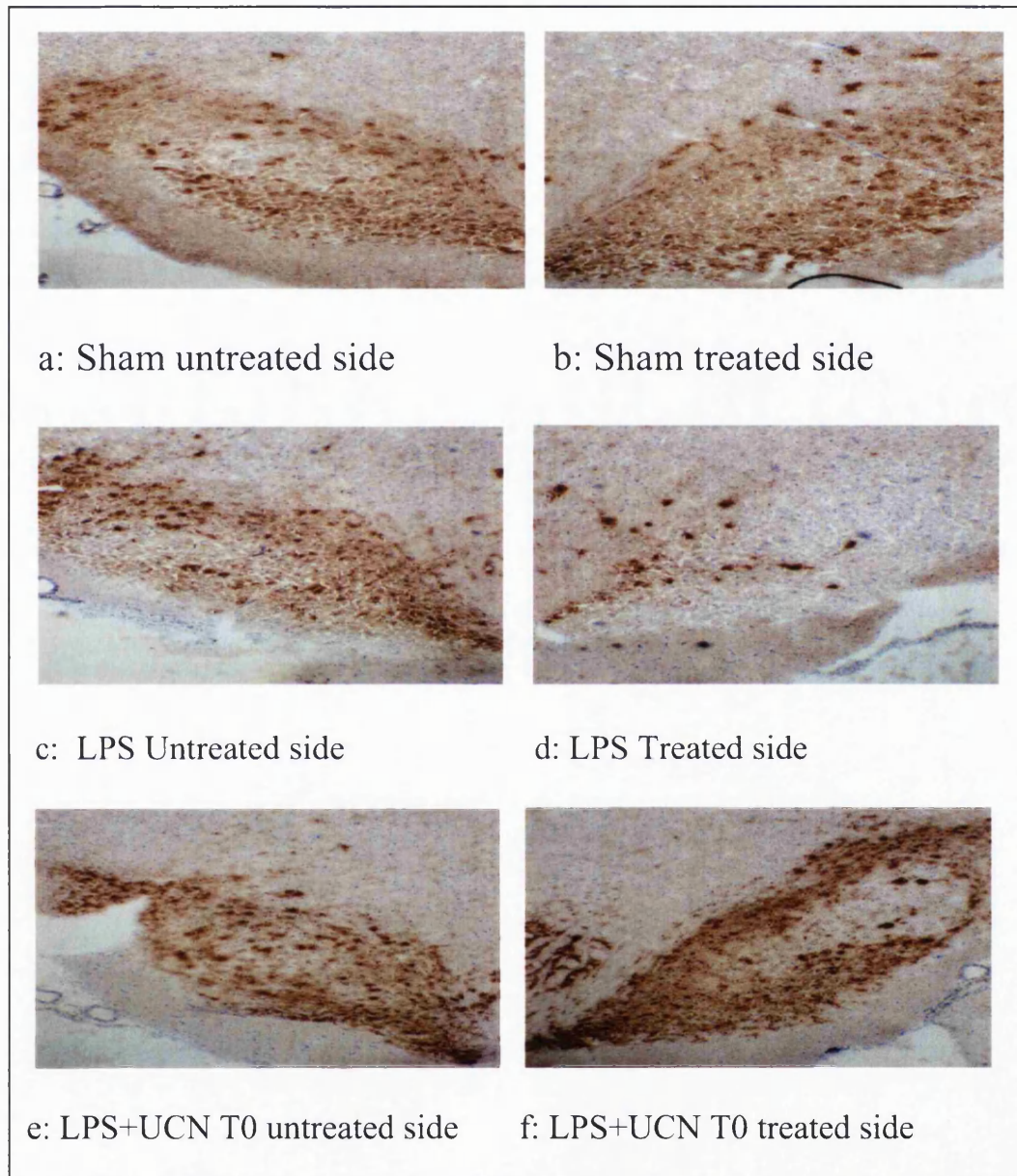
Figure 3.4.6 Representative photomicrographic images of rat brain cross sections showing both the right and the left SNc

In each section a comparison can be made between treated and untreated SNc in an individual rat (image shown is one example from a group of 4), sides labelled treated were treated with either vehicle only (b), LPS + vehicle (d) or LPS + UCN (f), sides labelled untreated are shown for better comparison. The orange brown colour (DAB stain) is indicative of TH positive neurons.

3.5 Discussion

The current findings demonstrate that UCN greatly attenuates the development of PD-like pathology in a commonly employed 6-OHDA rodent model of PD and a more recently proposed paradigm of the disease, the LPS rat model. The validity of the latter model is being increasingly appreciated, especially when the role of neuroinflammation as a probable causative factor in the aetiology of PD is gaining substantial support from studies in patients and animal-models (Block *et al.*, 2007; Whitton, 2007; Wu *et al.*, 2005b).

When animals were subjected to the two neurotoxic insults and co-treated with UCN it was observed that this peptide attenuated a behavioural indicator of PD-like damage, namely apomorphine induced circling, which is regarded as a quantitative index of NS lesion severity (Abercrombie *et al.*, 1990), an attenuation thus being predictive of potential anti-parkinsonian activity. The neurochemistry of the toxin induced lesions was evaluated through estimation of whole tissue DA levels in the ipsilateral striata of animals, where UCN prevented any dramatic depletion in DA levels by either 6-OHDA or LPS treatment. This phenomenon was also observed when estimating the functional integrity of nigral neurons ipsilateral to injection sites, where L-DOPA synthetic capacity of both nigral and striatal homogenates was measured and a clear protective effect of UCN against the significant reductions in ex vivo TH activity caused by either insult was observed.

From the Western blot results it was established that TH activitiy in homogenates correlated closely with the visual intensity of immunostaining for the enzyme as Western blots revealed that UCN clearly prevented the loss of TH immunoreactivity in homogenates caused by 6-OHDA or LPS treatments.

Having established that intracerebral administration of UCN prevents the loss of nigrostriatal function post-toxin in both rodent models, it was crucial to ascertain whether this could be explained by preservation of discrete nigral cells or, alternatively, by an upregulation of TH protein in surviving cells. In order to test these possibilities, immunohistochemistry was carried out through staining cryo-preserved brain sections for TH immunoreactivity and estimation of nigral cell numbers in the SNc. UCN almost completely protected against loss of cells and

dendrites in response to 6-OHDA administration, with a similar pattern of TH immunoreactivity seen in LPS lesioned rats. This discloses a clear neuroprotective effect of UCN, indicated by the surviving neurons, suggesting the ability of UCN to protect against neuronal degeneration caused by 6-OHDA and LPS.

A major question which required consideration was whether the behavioural, neurochemical and histological indicators of NS recovery could be supported by data indicating the release of striatal DA. In order to determine this we used *in vivo* microdialysis in freely moving rats. When UCN was administered 5 minutes after treatment with 6-OHDA or LPS, extracellular basal and evoked release of DA was significantly higher than in rats that were lesioned without UCN treatment. This clearly indicates that UCN mediated a functional preservation of DA transmission in the striata of these animals; most likely, this released DA would engage striatal DA receptors to underlie the observed preservation of motor behaviour. Also one very interesting observation was the proportionally significant amounts of potassium-induced extracellular DA released in the striatum in 6-OHDA treated animals, where there was almost complete loss of the TH positive neurons in SNc. While in these animals basal extracellular DA levels (as obtained from microdialysis) were barely detectable, when subject to an impulse of high potassium aCSF, although DA levels were significantly lower than normal, these DA levels were proportionally considerably elevated. This phenomenon was also met during the microdialysis procedure, where these 6-OHDA treated animals exhibited significant contra lateral turning after the high potassium impulse, which indicates that the proportionally high levels of potassium-induced extracellular DA observed from microdialysis was still functional. This observation could be due to the release of DA stored in vesicles in dying neurons, whether this was the case or not, this observation could explain why PD symptoms start to arise only when significant damage has occurred.

These data reveal that UCN can act as an effective neuroprotective agent in two distinct rodent models of PD at a dose that was found to be effective in a variety of previous studies in other systems (Brar *et al.*, 2000; Brar *et al.*, 2002; Choi *et al.*,

2006; Facci *et al.*, 2003). Moreover, I have assessed multiple parameters of NS pathway activity and nigral neuronal integrity. This approach has important strengths over 'single parameter' studies, given that human PD pathology is complex and multi-component (Blum *et al.*, 2001; Gandhi *et al.*, 2005; Tatton *et al.*, 1998).

Chapter 4

UCN confers neuroprotection after the lesion develops

4 UCN confers neuroprotection after the lesion develops?

4.1 Introduction

Perhaps the most unfortunate aspect of PD is that it develops asymptomatically for years prior to a definitive diagnosis, based on specific motor signs. The result of this is that once symptoms become manifest some 60-70 % of nigrostriatal DA neurons have already been lost.

The well-characterised features of PD are largely the result of a selective degeneration of nigral neurons. Before the disease presents clinically, death of these neurons occurs in the SNc 'silently' as a result of multiple and convergent neurodegenerative processes (Gandhi _et al._, 2005). Prevention of further nigrostriatal pathway destruction once established and, ultimately, reversal of the severity of the lesion therefore represents a highly desirable therapeutic goal. However, as indicated by Meissner et al. (2004), rodent and primate studies utilizing 'anti-parkinsonian' agents have involved administration of these agents prior to or concurrent with the experimental lesion. A brief consideration of the progressive events that occur in PD genesis suggests such an approach to have little relevance to the clinical situation where, in the substantial majority of cases, the neuropathological lesion has become well established once symptoms appear. In an attempt to address this important issue, this work has tested the possibility that manipulation of the central UCN/CRF receptor axis may offer a novel therapeutic target in human PD. This possibility is supported by studies suggesting that the UCN peptide may function as a cytoprotectant in a diverse range of mammalian tissues and cerebellar granule cells (Brar _et al._, 2000; Pedersen _et al._, 2002; Scarabelli _et al._, 2002). This observation was also confirmed from my findings where UCN produced dramatic effects when it was administered at the time of lesioning in two distinct models of PD, assessed by quantification of the effects of intracerebral UCN injection on critical behavioural, neurochemical and histological indices routinely employed to assess the extent of

nigrostriatal pathway destruction in models of PD. In order to establish whether UCN would have any potential future therapeutic use, it was important to examine the effects of UCN when given after the lesion had developed significantly, which represents a scenario analogous to diagnosed cases of PD.

Again, a dual model approach was employed to reduce the possibility that UCN might be uniquely active against a single neurotoxic agent and to better mimic complex nature of PD pathophysiology, which is likely to be multi-factorial (Blum *et al.*, 2001; Gandhi *et al.*, 2005; Tatton *et al.*, 1998)

4.2 Experiment design

Rats were divided into 5 groups. The first group was sham treated where rats recieved 4 µl i.c injections of vehicle (0.2% ascorbic acid) into the medial forebrain bundle. The second group received 8 µg of 6-OHDA delivered in 4 µl of 0.2% ascorbic acid into the medial forebrain bundle, followed by i.c injection of 2 µl UCN directly into the SNc. The third group received 8 µg of 6-OHDA delivered in 4 µl of 0.2% ascorbic acid into the medial forebrain bundle, and then seven days later rats were subject to another i.c injection of 2 µl saline directly into the SNc. The fourth group of rats received 8 µg of 6-OHDA delivered in 4 µl of 0.2% ascorbic acid into the medial forebrain bundle and then seven days later they were treated with another i.c injection of 2 µg UCN delivered in 2 µl saline directly into the SNc. Finally the fifth group of rats received 8 µg of 6-OHDA delivered in 4 µl of 0.2% ascorbic acid into the medial forebrain bundle and after seven days rats were sacrificed.

All rats, apart from the fifth group, were assessed after 14 days from initial injection and seven days after second injection; assessments included Apomorphine challenge, *in vivo* Microdialysis, measurement of tissue DA, measurement of TH activity and Immunohistochemistry as described in chapter 2. Another 24 male Wistar rats were used as previously described, but this time using LPS instead of 6-OHDA. LPS was injected directly into the SNc, and the vehicle used here was saline. All rats were assessed 14 days after initial injections except for a group that received 6-OHDA only and was assessed after 7days. All results in bar graphs are shown as mean ± SEM.

A schematic time bar is provided to clarify times of injections and procedures carried out in each experimental group (diagram 3 for 6-OHDA and diagram 4 for LPS). In total 52 rats were used in this section.

Diagram 3 Schematic time bar indicating times of injections and procedures carried out in each experimental group.
Where A is SHAM group, B is 6-OHDA + UCN at day 7 group, C is 6-OHDA only group, D is 6-OHDA + UCN where UCN was given 5 minutes after 6-OHDA and E is 6-OHDA only but animals here were terminated at day 7.
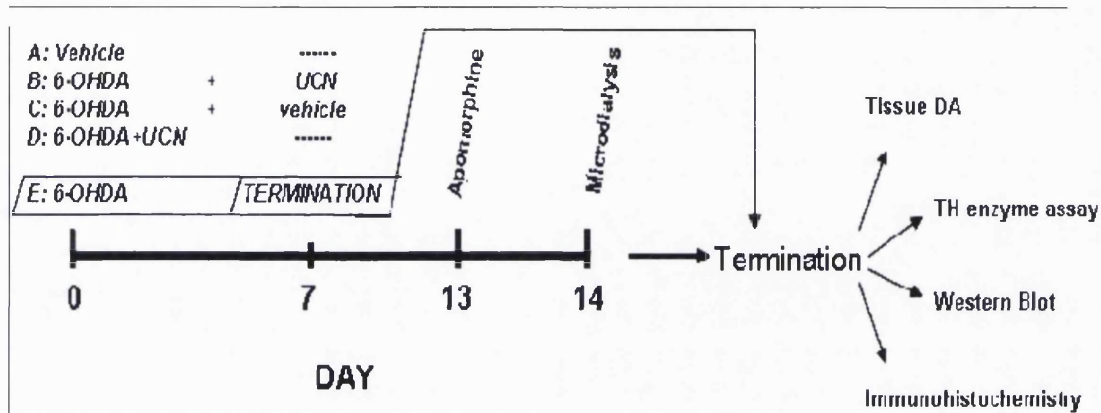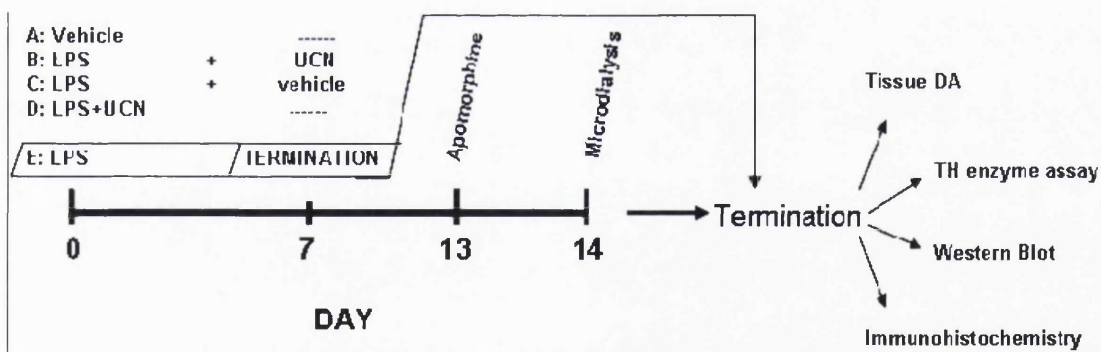Number of rats used in this expirement: n = 25.

Diagram 4 Schematic time bar indicating times of injections and procedures carried out in each experimental group.
Where A is SHAM group, B is LPS + UCN at day 7 group, C is 6-LPS only group, D is LPS + UCN where UCN was given 5 minutes after LPS and E is LPS only but animals here were terminated at day 7. Number of rats used in this expirement: n = 27.

4.3 Results

4.3.1 Apomorphine challenge

Rats that were treated with either 6-OHDA or LPS and vehicle displayed characteristics of unilateral nigrostriatal degeneration, this was clear after the animals were subjected to the apomorphine challenge where they demonstrated intense, tight contraversive circling (6-OHDA Figure 4.3.1 a and Figure 4.3.1 b). At the doses of toxins used the number of turns was consistently lower in LPS than 6-OHDA treated rats (Figure 4.3.1 a and b) but in both cases, circling was significantly greater than in sham controls where no circling was observed. As before, the rats that were treated with the insults (6-OHDA or LPS) and co-treated with UCN at the same time showed much reduced sensitivity to the apomorphine challenge, and more interestingly when the rats were lesioned and treated with UCN seven days *after the injection of toxin*, reduced sensitivity to the apomorphine challenge was also displayed (6-OHDA figure 4.3.1 a and LPS figure 4.3.1 b). Rats treated with 6-OHDA + vehicle and sacrificed seven days later turned significantly more than sham controls, but were not significantly different to UCN treated animals. No turns were recorded for sham-operated rats.
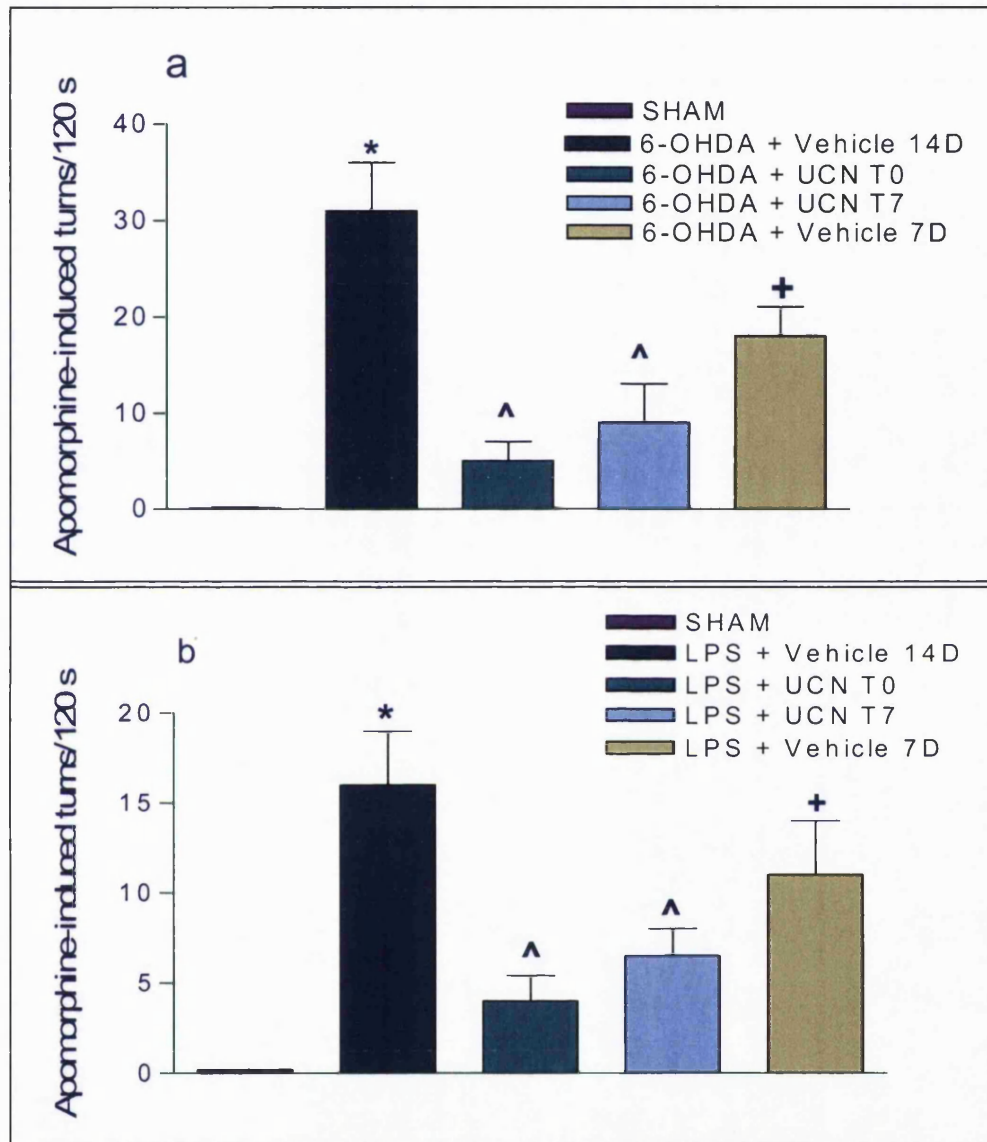
Figure 4.3.1 Effects of UCN on either 6-OHDA-induced (A) or LPS-induced (B) contraversive turns in response to apomorphine.

Apomorphine (0.5 mg/kg) was administered through s.c injection. Turns were recorded in a 1.2 metre circular arena from all animals as mean counts / 120 s.

* Significantly different to sham and UCN treated groups (p<0.001), ^ significantly different than 6-OHDA + vehicle (6-OHDA + UCN T0 (p<0.001); 6-OHDA + UCN T7 (p<0.01)). + Significantly different to sham (p<0.01 in a, and p<0.05 in b). 6-OHDA + UCN T0 or UCN T7 not significantly different than sham (p>0.05). UCN T0 indicates rats that were given toxins and UCN concomitantly. UCN T7 indicates rats given UCN 7 days after injection of toxins. 7D indicates that rats were sacrificed 7 days after inititial injections and 14D indicates sacrifice was after 14 days. n=5-6 rats for each treatment group.

4.3.2 *In vivo* microdialysis

Results from *in vivo* microdialysis were used to evaluate the functional integrity of the nigrostriatal pathway through measuring striatal extracellular DA at both basal and potassium induced levels (Figure 4.3.2). Striatal extracellular DA levels in rats treated with either 6-OHDA + vehicle (a) or LPS + vehicle (b) were significantly lower than the levels in sham-operated animals both at basal and at potassium induced levels. By contrast, when rats were lesioned and treated with UCN seven days *after the injection of toxin*, normal levels of extracellular DA were restored in both basal and potassium evoked samples (6-OHDA Figure 4.3.2 a and LPS Figure 4.3.2 b). DA levels measured in animals treated with either LPS or 6-OHDA followed by UCN seven days later were comparable with those seen in sham equivalents with respect to both basal and potassium elevated levels. All contralateral sides were untreated and displayed extracellular DA levels similar to those of sham controls (Figure 4.3.2)
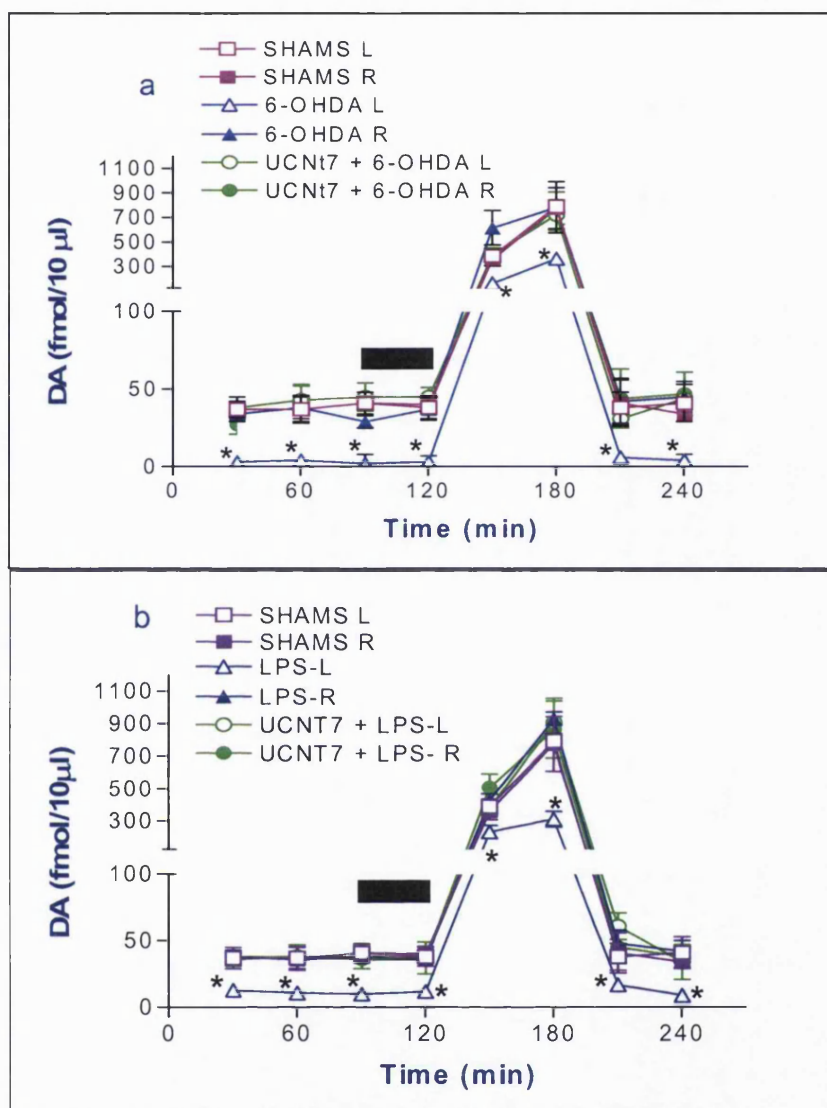
Figure 4.3.2 The effect of UCN on extracellular striatal DA levels 7 days after 6-OHDA (a) or LPS (b) treatement. DA levels were obtained from *in vivo* microdialysis.

Microdialysis was performed over a period of 4 hours both at basal levels, and potassium induced levels where animals were infused with high potassium (ACSF) for 30 min indicated by black rectangle (dialysis samples were collected every 30 minutes). * Significantly different to sham and UCN treated groups (p<0.01). LPS + UCN T7 and 6-OHDA + UCN T7 not significantly different to sham. Bilateral micro dialysis was performed with L indicating left or treated side and R indicating right or untreated side. Break in Y axis to facilitate data expression. n= 4-5 rats in each treatment group.

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4.3.3 Measurement of tissue DA

Tissue DA levels were assessed in the both nigra and striata of rats. Animals treated with 6-OHDA and vehicle exhibited dramatic depletion of both nigral and striatal DA levels, which was significantly lower than in sham operated animals (figure 4.3.3 a and b). Animals treated with LPS and vehicle also showed depletion in both nigral and striatal DA levels, not as severe as with 6-OHDA, but still significantly lower than sham operated animals (figure 4.3.4 a and b). Rats that were co-treated with UCN in addition to the insults showed almost complete preservation of tissue DA levels, which did not differ significantly from sham controls. Interestingly, in animals that recieved UCN seven days after treatment with toxins, when it can be clearly seen that DA levels have declined significantly compared to controls (6-OHDA + vehicle 7D in figure 4.3.3 and LPS + vehicle 7D in figure 4.3.4), a dramatic restoration in tissue DA levels was observed when compared to DA levels in rats treated with the toxins and vehicle.

Figure 4.3.3 Effects of UCN on 6-OHDA-induced tissue DA loss in SNc (a) and striatum (b)
DA levels were measured in SNc or striata through HPLC-ED.
* Significantly different to sham and UCN treated groups ($p < 0.001$). 6-OHDA treatment with UCN either at the same time (T0) or seven days after (T7) was not significantly different to sham. 7D indicates that rats were sacrificed 7 days after inititial injections and 14D after 14 days. n=4-6 rats in each treatment group.
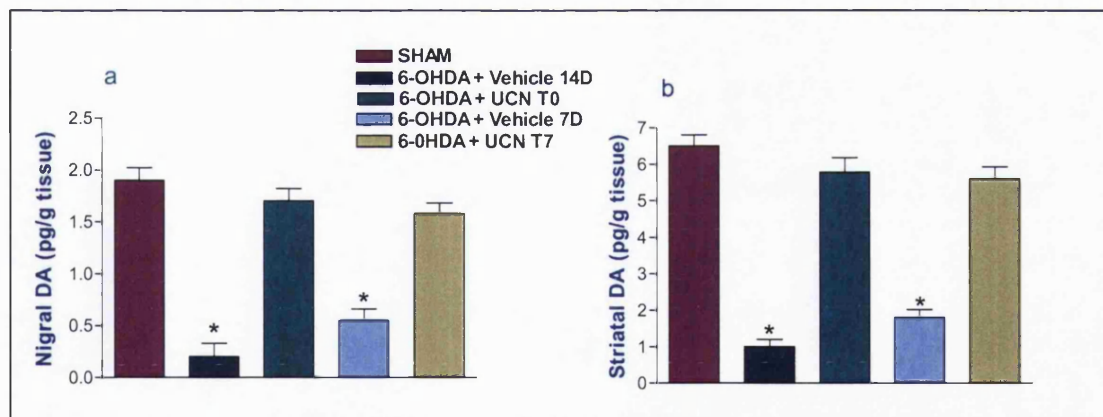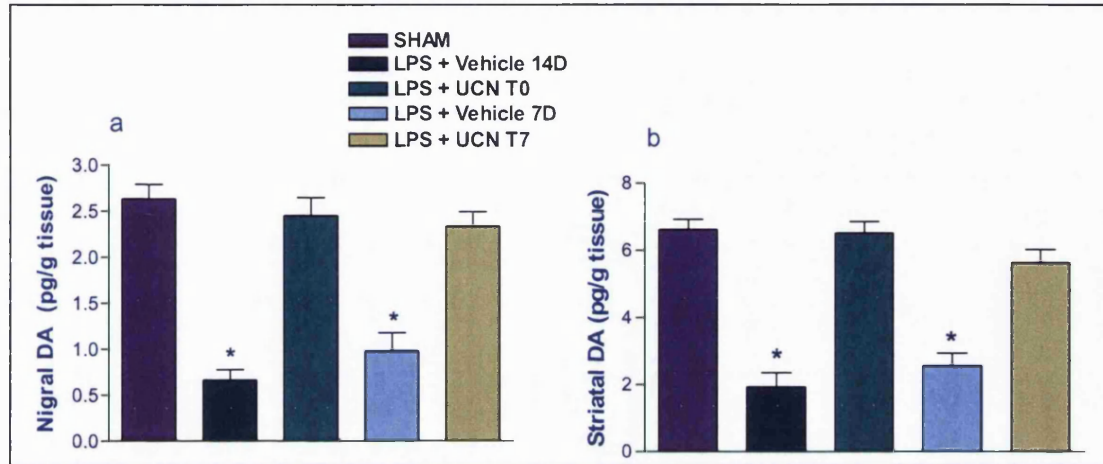
Figure 4.3.4 Effects of UCN on LPS-induced tissue DA loss in SNc (a) striatum (b)
DA levels measured in SNc or striata through HPLC-ED. *Significantly different to sham and UCN treated groups (p<0.001). LPS treated with either UCN T0 or UCN T7 not significantly different to sham. 7D indicates that rats were sacrificed 7 days after inititial injections and 14D after 14 days.n=4-5 rats in each treatment group.

4.3.4 TH assay

When the integrity of the DA system was studied on the basis of L-DOPA synthetic capacity, TH activity was greatly reduced in both the nigra and striata of 6-OHDA + vehicle and LPS + vehicle treated rats to less than 20% in comparison to sham treatment (nominally 100%; Figures 4.3.5 and 4.3.6). Co-administration of UCN with 6-OHDA or LPS however conserved the TH activity when UCN was administered at the same time as the toxins. When UCN was administered 7days after the toxins, when TH activity had significantly declined in both models, UCN appeared to reverse the loss of TH activity to levels not significantly lower than control (Figures 4.3.6 and 4.3.7).
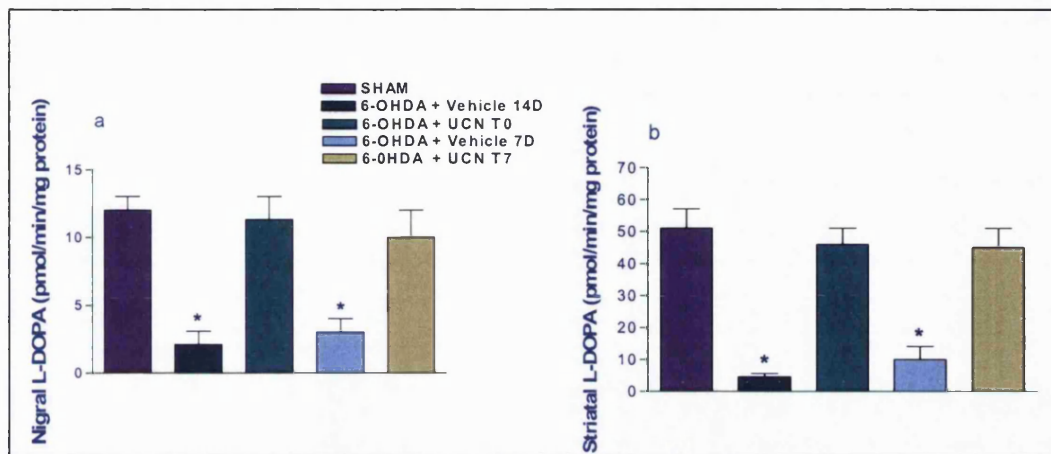
Figure 4.3.5 The effect of UCN on 6-OHDA-induced TH activity loss 7 days after lesioning.

TH activity was measured as moles L-DOPA produced per min per gm protein after a 10 min enzyme assay using homogenates from SNc (a) and from Striatum (b). L-DOPA was quantified using HPLC-ED after reaction termination and DA was used as the internal standard. * Significantly different to sham and UCN treated groups ($p < 0.001$) for 6-OHDA + vehicle 14Dand ($p < 0.01$) for 6-OHDA + vehicle 7D. 6-OHDA + UCN T0 or T7 not significantly different to sham. 6-OHDA + UCN T 7 indicate rats given UCN 7 days after 6-OHDA injection, and 6-OHDA + vehicle 14D and 6-OHDA + vehicle 7D indicate day of termination after injection of insult at 14 and 7 days respectively. n=4-6 rats in each treatment group.
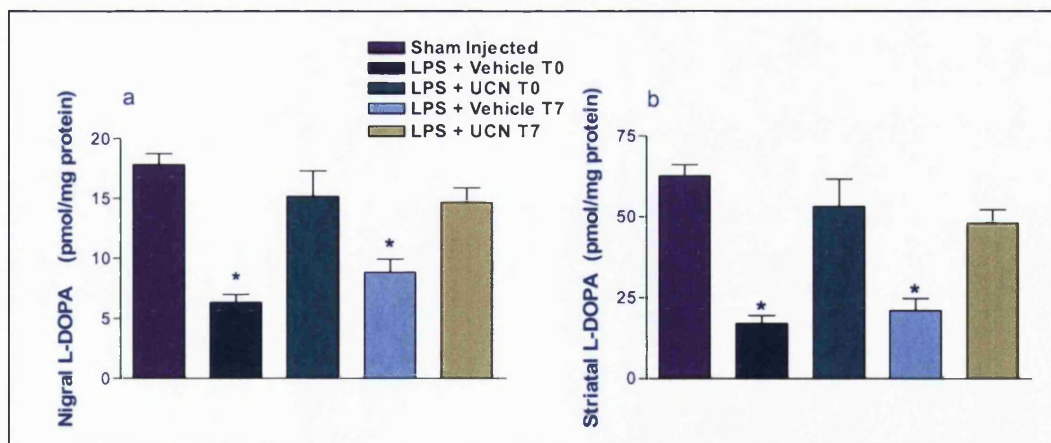
Figure 4.3.6 The effect of UCN on LPS-induced TH activity loss 7 days after lesioning

TH activity was measured as described in Figure 4.3.5. * Significantly different to sham and UCN treated groups ($p < 0.001$) for LPS + vehicle 14Dand ($p < 0.01$) for LPS + vehicle 7D. 6-OHDA + UCN T0 or T7 not significantly different to sham. LPS + UCN T 7 indicate rats given UCN 7 days after 6-OHDA injection, and LPS + vehicle 14D and LPS + vehicle 7D indicate day of termination after injection of insult at 14 and 7 days respectively. n=4-5 rats in each treatment group.

4.3.5 Western blotting

Relative TH protein expression in the different groups was measured using western blotting to assess whether the loss of activity of the TH enzyme was due to loss of protein. It is clear from the results obtained (Figure 4.3.7) that TH protein content was greatly reduced in the striata of 6-OHDA or LPS / vehicle treated rats in comparison to sham treatment. Co-administration of UCN with 6-OHDA or LPS, or injection of UCN seven days post-toxin both resulted in less reduction in TH protein (Figure 4.3.8).
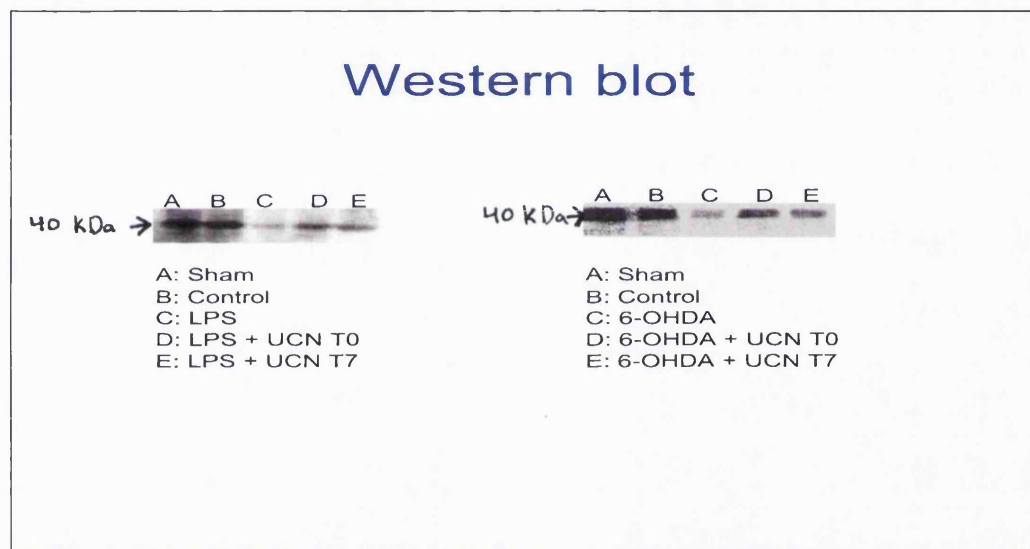
Figure 4.3.7 Effects of UCN on 6-OHDA and LPS-induced TH protein loss
Representative western blot analysis of TH protein content in striatal homogenates (in both 6-OHDA and LPS-lesioned rats). 6-OHDA + UCN T0 indicate rats that were administered 6-OHDA and UCN concomitantly. 6-OHDA + UCN T7 indicate rats administered UCN 7 days after 6-OHDA injection. LPS + UCN T0 indicate rats that were administered LPS and UCN concomitantly. LPS + UCN T7 indicate rats recieving UCN 7 days after LPS injection.

4.3.6 Immunohistochemistry

Nigral sections were subjected to TH staining to determine whether the changes in DA activity were due to modification in the number of DA neurons or alterations in the amount of TH in individual cells. It was clear from the images that there was a substantial loss in TH positive neurons after 7 days of insult injection, as illustrated in

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image D in comparison to sham controls (Figure 4.3.8 for 6-OHDA and 4.3.9 for LPS), and when UCN was administered at this time point, i.e. after this significant cell loss, it can be observed that there was restoration of TH positive cells (image F; Figure 4.3.8 for 6-OHDA and 4.3.9 for LPS).

a: Sham untreated side   b: Sham treated side

c: 6-OHDA Untreated side (7Days)   d: 6-OHDA Treated side (7days)

e: 6-OHDA+UCN T7 Untreated   f: 6-OHDA+UCNT7 Treated

Figure 4.3.8 Representative photomicrographic images of rat brain SNc cross sections showing the effect of UCN on TH neuron loss after seven days from unilateral 6-OHDA lesioning

In each section a comparison can be made between treated and untreated SNc in an individual rat (image shown is one example from a group of 4), sides labelled treated were treated with either vehicle only (a), 6-OHDA + vehicle (d) or 6-OHDA+ UCN T7 (f), sides labelled untreated are shown for better comparison. a: untreated SNc of rat that was treated with vehicle only on the other SNc b, c: untreated SNc of rat that was treated with 6-OHDA + vehicle on the other SNc D and terminated after 7 days. E: untreated SNc of rat that was treated with 6-OHDA + UCN T7 on the other SNc f. The orange brown colour (DAB stain) is indicative of TH positive neurons.

a: Sham Untreated side   b: Sham Treated side

c: LPS Untreated side (7 days)   d: LPS Treated side (7days)

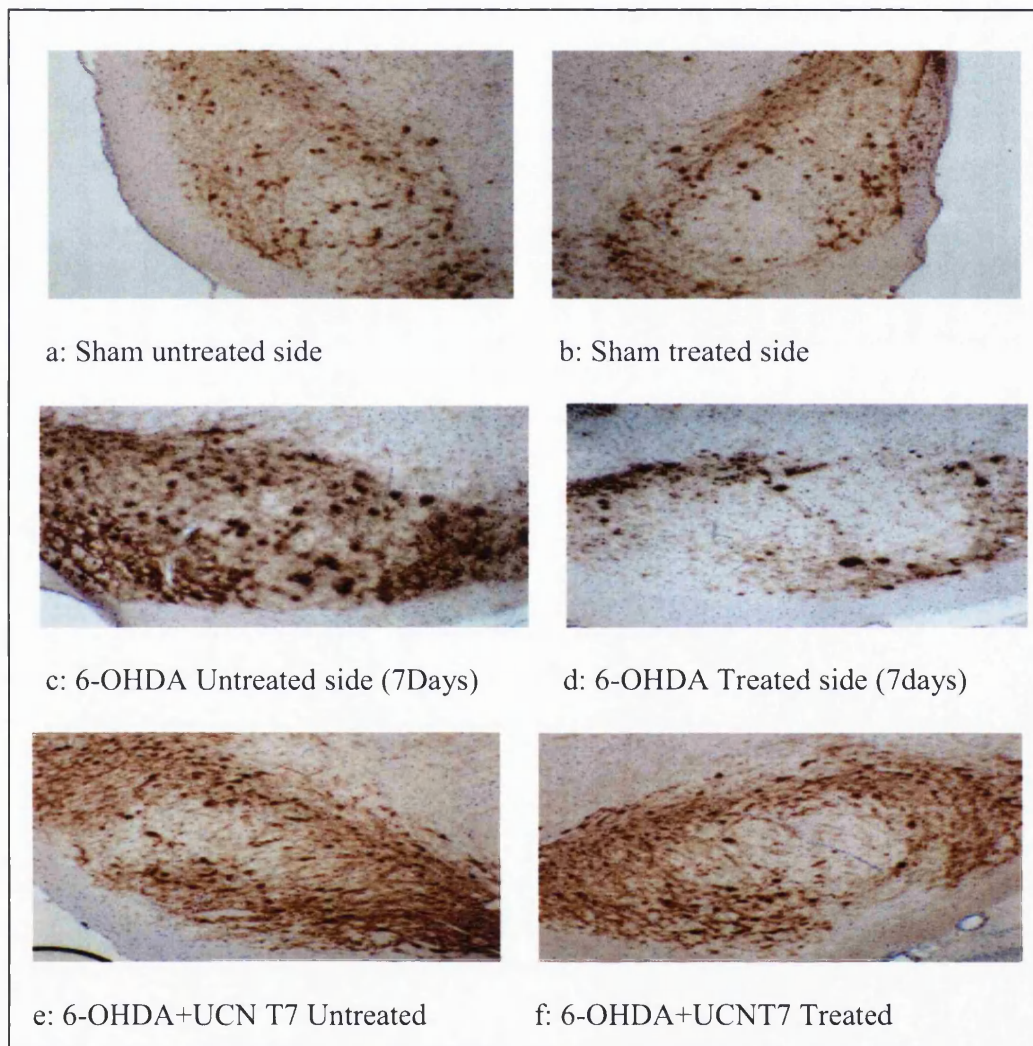e: LPS+UCN T7 Untreated   f: LPS+UCN T7 Treated

Figure 4.3.9 Representative photomicrographic images of rat brain SNc cross sections showing the effect of UCN on TH neuron cell loss seven days after LPS lesioning

In each section a comparison can be made between treated and untreated SNc in an individual rat (image shown is one example from a group of 4), sides labelled treated were treated with either vehicle only (b), LPS + vehicle (d) or LPS + UCN T7 (f), sides labelled untreated are shown for better comparison. a: untreated SNc of rat that was treated with vehicle only on the other SNc b, c: untreated SNc of rat that was treated with LPS + vehicle on the other SNc d and terminated after 7 days. e: untreated SNc of rat that was treated with LPS + UCN T7 on the other SNc f. The orange brown colour (DAB stain) is indicative of TH positive neurons.
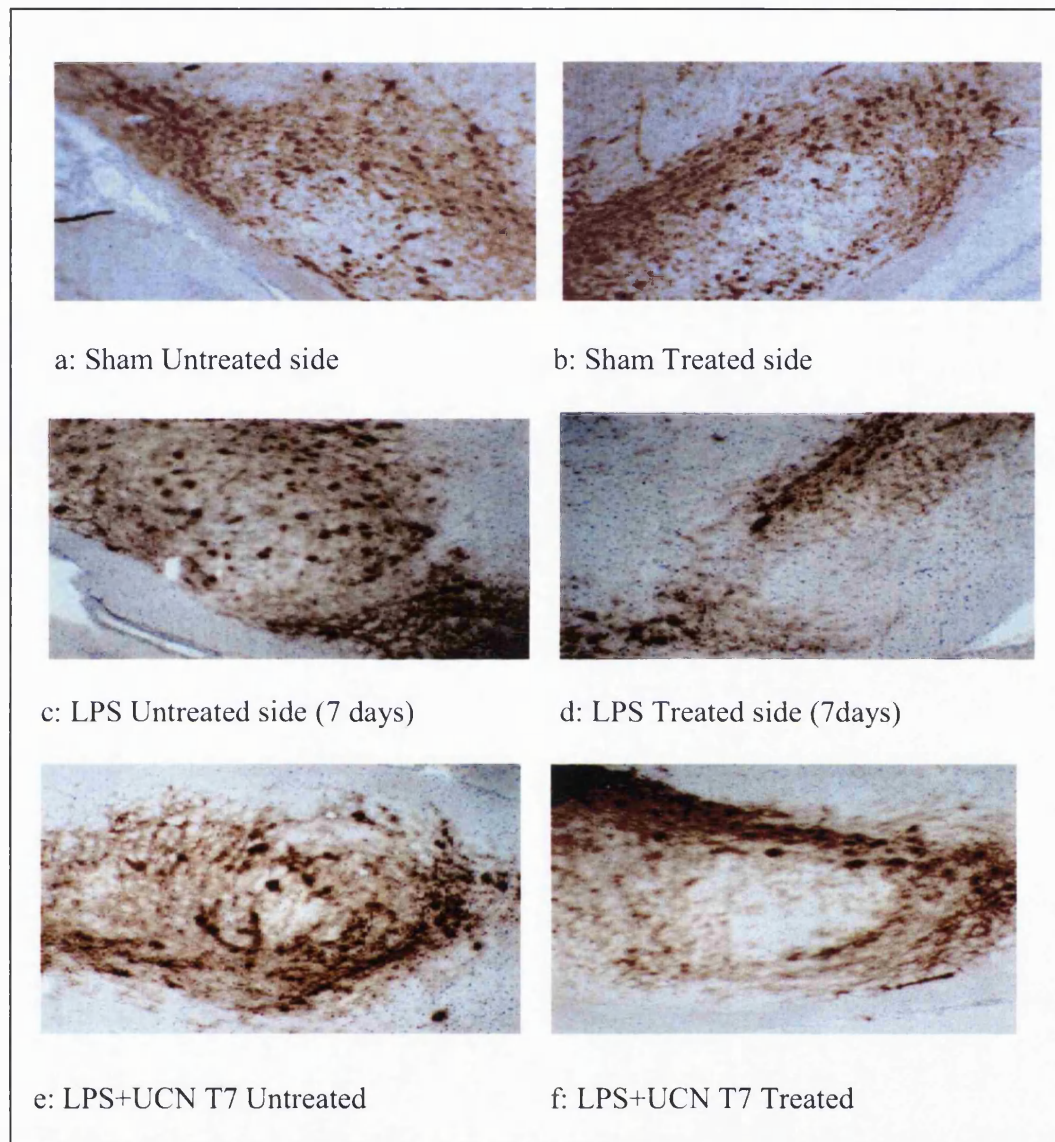
4.4 Discussion

It was previously observed that UCN, when given at the same time as 6-OHDA or LPS induced nigrostriatal lesions, was able to effect a dramatic reduction in key indicators of nigrostriatal neurodegeneration (chapter 3). Using these two models of PD the current data show that UCN is able to effect a significant reduction in apomorphine-induced circling behaviour, a restoration in striatal and nigral DA levels, TH activity and a retention of TH positive cells within the nigra. Crucially, in both paradigms, UCN is 'neuroprotective' both when given at the same time as the neurotoxic insult (chapter 3) and also when given seven days afterwards (where I have determined that the lesion is clearly established but still developing). Previous studies have revealed UCN to have cytoprotectant properties, for example Brar et al. (2002) found UCN to be protective against ischaemia/reperfusion injury in cardiac myocytes; an effect which was apparent after the cytotoxic insult had been delivered (Brar *et al.*, 2000; Brar *et al.*, 2002; Scarabelli *et al.*, 2002). UCN also confers protection in neuronal cell cultures, if given up to 8 h after the neurotoxic insult (Choi *et al.*, 2006; Facci *et al.*, 2003; Pedersen *et al.*, 2002)..

Of particular significance is the finding that UCN is able to arrest and possibly reverse nigrostriatal lesions, once the neurodegenerative process has commenced. This principle, if reproduced in the human brain, would have obvious clinical significance since patients generally present with symptoms only once nigral neuronal loss reaches 70-80% of the total nigral complement and striatal DA levels have fallen significantly (Abercrombie *et al.*, 1990). The ability to restore the indices of PD-like damage in DA nigrostriatal neurons seven days after administration of the neurotoxic insult is particularly significant, as it was determined that at this time point in the two models used the lesion had become established but was evidently still unstable since degeneration continued to proceed for at least a further seven days.

The key point from the present study is the ability of UCN to effect a preservation or restoration of nigrostriatal function once a lesion is established. Recent findings have shown that other drugs, particularly anti-inflammatory medicines, possess 'anti-parkinsonian' activity (Block *et al.*, 2007; Whitton, 2007). However, these drugs are

almost invariably given at the same time or prior to the neurotoxic insult (Hunter *et al.*, 2007). Since PD presents once the underlying lesion is well advanced, and very early diagnosis is quite difficult, the best these strategies can hope for would appear to be a deceleration in the rate of neuronal loss. Although of clear value this probable stabilization of hostile conditions (e.g. elevated proinflamatory cytokines, ROS) within the nigra is not a 'cure'. Realistically, the patients who are likely to benefit from these new findings are individuals taking drugs such as aspirin for cardiovascular disease in which the CNS is maintained in a tonic 'anti-inflammatory' state which may lessen the incidence or time of onset of PD. This does not, however, constitute a prophylactic treatment strategy for the population as a whole.

It is still unclear which molecular mechanisms are responsible for the effect of UCN. Because this peptide itself is unlikely to be of therapeutic use due to its physicochemical properties (MW: 4700), further understanding of the mechanism of action by which UCN elicits its neuroprotective effects would be of great value, and would hopefully uncover a target site that would be viable for future therapeutic applications.

Chapter 5

Receptor targets for UCN in rat models of PD

5 Receptor targets for UCN in rat models of PD

5.1 Introduction

Most of the work that has been carried out to investigate the UCN peptide family as agents that ameliorate inflammatory responses has concluded that this group of peptides produce their effects through binding at CRF receptors and not through other mechanisms (Tao *et al.*, 2005). These CRF receptors are G protein-coupled receptors which bind CRF and, as mentioned previously, CRF receptors have two main forms, type I and 2 or CRF-R1 and CRF-R2 (Hauger *et al.*, 2003). CRF-R mediated anti-inflammatory effects were indicated when UCN, which activates both CRF-R1 and CRF-R2 receptor types, and UCN III, which is highly selective for CRF2, brought about a reduction of the inflammatory response in the intestine and the lungs (Gonzalez-Rey *et al.*, 2006; Moffatt et al., 2006; Reyes *et al.*, 2001). UCN I, II and III have also been found to protect against ischaemia-reperfusion injury to cardiac myocytes, an effect seen when UCN was given either prior to ischemia or at the time of reperfusion after ischemia. This is believed to be due to binding of these peptides to the CRF-R2β, which is expressed in the heart (Brar *et al.*, 2000). Since both receptors are present in the nigrostriatal pathway of the rat, with greater abundance of the CRF-R1 and only few CRF-R2 in the fundus of striatum (Potter *et al.*, 1994; Van Pett *et al.*, 2000), it was assumed that the effects UCN produced to reverse key parkinsonian features were achieved through binding to at least one of these receptors. The theory that UCN was conferring its effects through the CRF receptor family was tested through studying the effects of a non-selective CRF receptor antagonist, α-helical CRF, on UCN elicited effects.

While UCN itself is relatively non-selective for its target receptors (CRF-R1 and CRF-R2), UCN II and UCN III are far more selective for the CRF-R2 receptor (Reyes *et al.*, 2001). Therefore, establishing whether these two peptides possess any of the neuroprotective properties of UCN would further indicate the specific receptor UCN binds to elicit these effects.

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Finally, the selective CRF-R1 antagonist, NBI-27914 was employed to determine the CRF-R1-dependency of the effects UCN had produced. The co-treatment of this antagonist with UCN seven days after injection of 6-OHDA, after the lesion significantly progresses, would also indicate if the CRF-R1 receptors survive the toxin lesioning at that time point.

5.2 Experiment design

It is reasonable to assume that there is no interaction between UCN and the toxins, 6-OHDA and LPS, as similar results were obtained with both of these animal models (chapter 3 and 4). Therefore I chose to use a single model of the disease in experiments investigating the target receptors through which UCN elicits its effects. The model chosen here was the 6-OHDA model; this was because this model was the harsher and the more established of the two.

In some experiments, UCNII or UCNIII were administered in place of UCN. In other experiments, the antagonists, α-helical CRF and NBI-27914, were administered prior to UCN, by i.c. and i.p. injection, respectively. Because α-helical CRF was administered i.c., its injection was only 5 minutes before UCN administration. As the highly lipophilic NBI-27914 was administered i.p., it was injected 30 minutes prior to UCN, as that would give it enough time to reach and bind CRF-R1 receptors in the SNc. While α-helical CRF was administered prior to UCN at the time of 6-OHDA lesioning, NBI-27914 was administered seven days after the initial injection of 6-OHDA, where treatment groups were assessed after another 7 days (section.2.1.4.2).

Animal treatments are described in more detail in section 2.1. All results in bar graphs are shown as mean ± SEM. A schematic time bar is provided to clarify times of injections and procedures carried out in each experimental group (diagram 5 for α-helical-CRF, diagram 6 for UCNII & UCNIII and diagram 7 for NBI-27914). In total, 75 rats were used in this section
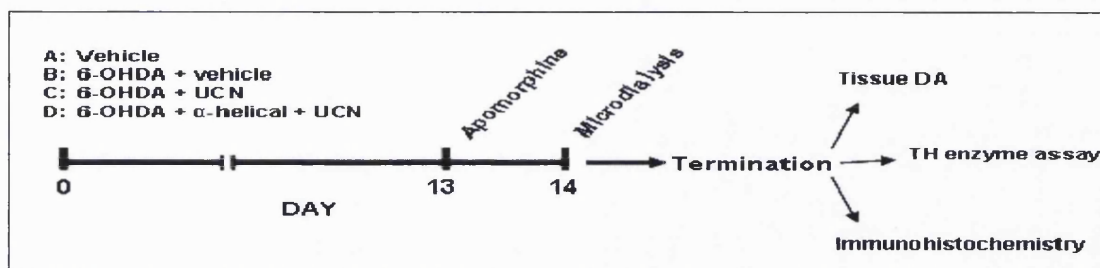
Diagram 5 Schematic time bar indicating times of injections and procedures carried out in each experimental group.
Where A is SHAM group, B is 6-OHDA only group, C is 6-OHDA + UCN group and D is 6-OHDA + α-helical-CRF + UCN only group. Number of rats used in this expirement: n = 22

Diagram 6 Schematic time bar indicating times of injections and procedures carried out in each experimental group.
Where A is SHAM group, B is 6-OHDA only group, C is 6-OHDA + UCN group, D is 6-OHDA + UCNII and E is 6-OHDA + UCNIII group.
Number of rats used in this expirement: n = 25
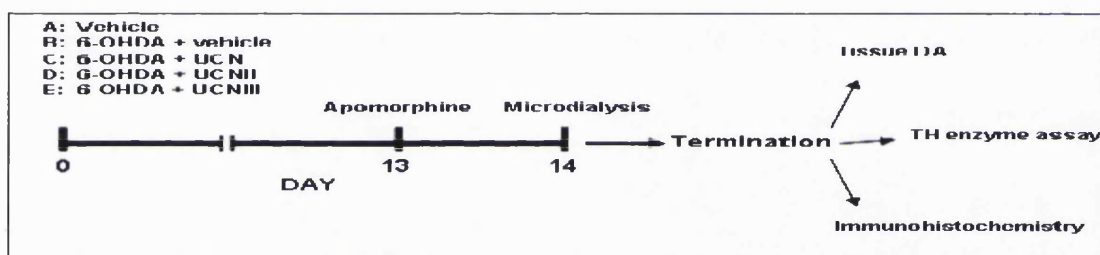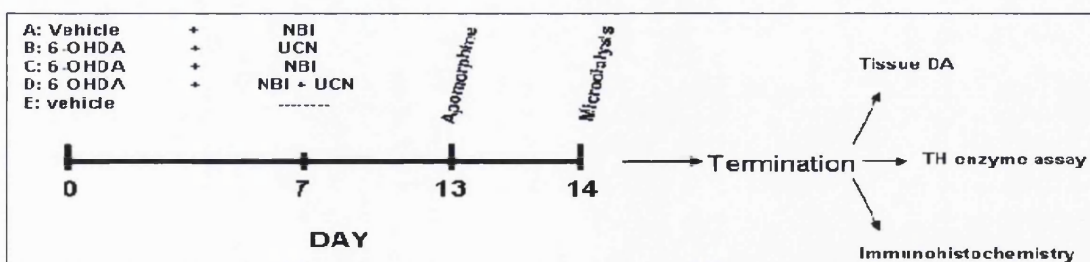

Diagram 7 Schematic time bar indicating times of injections and procedures carried out in each experimental group.
Where A is SHAM group, B is 6-OHDA + UCN at day 7 group, C is 6-OHDA + NBI group, D is 6-OHDA + (NBI + UCN) where NBI was given 30 minutes before UCN and 7 days after initial 6-OHDA treatment, and E is a sham group. Number of rats used in this expirement: n = 28

5.3 Results

5.3.1 Effects of non-selective CRF-R antagonism on UCN-mediated neuroprotection.

5.3.1.1 Apomorphine challenge

When rats were treated with 6-OHDA and vehicle they displayed characteristics of unilateral nigrostriatal degeneration which was apparent after the animals were subjected to the apomorphine challenge where they demonstrated intense, tight contraversive circling which was significantly different to sham controls where no turning was observed (Figure 5.3.1). Again, as shown previously, rats treated with 6-OHDA and co-treated with UCN showed reduced sensitivity to the apomorphine challenge and exhibited a significantly lower number of turns when compared to 6-OHDA + vehicle treated animals, leaving a residual number of turns that was not significantly different to control. When animals were treated with 6-OHDA and, before being treated with UCN, they were treated with the non-selective CRF-R antagonist, α- helical CRF, the effects of UCN were attenuated as indicated by a number of turns significantly higher than in sham controls (Figure 5.3.1).
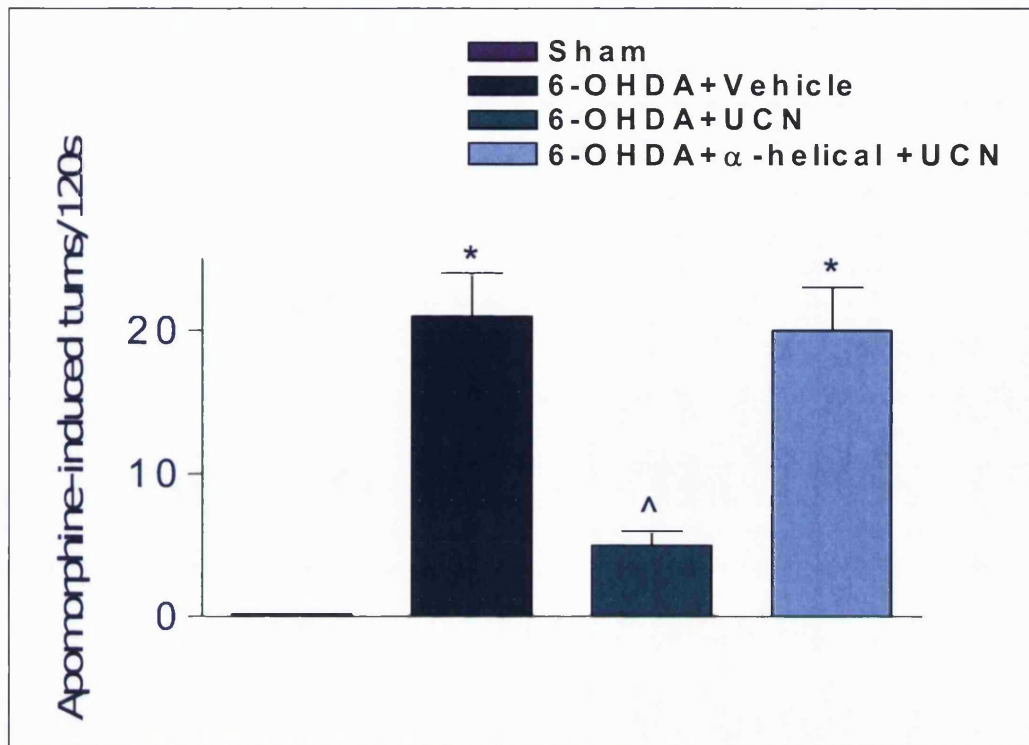
Figure 5.3.1 Effects of UCN in the presence of α-Helical CRF on 6-OHDA-induced contraversive turns in response to apomorphine.
Apomorphine (0.5 mg/kg) was administered through s.c injection. Turns were recorded in a 1.2 metre circular arena for all animals as mean counts / 120 s.
* Significantly different to sham and UCN only treated groups ($p<0.001$),
^ significantly different than 6-OHDA + vehicle ($p<0.001$). 6-OHDA + UCN not significantly different than sham ($p>0.05$). n=6 rats in each treatment group

5.3.1.2 *In vivo* microdialysis

When rats were evaluated for functional integrity of the nigrostriatal pathway through *in vivo* microdialysis, extracellular DA levels in rats treated with 6-OHDA and vehicle disclosed significant reductions when compared to sham-operated animals both at basal levels and at potassium induced levels (Figure 5.3.2), whereas levels estimated in animals treated with 6-OHDA and UCN were comparable with sham equivalents. Animals treated with 6-OHDA followed by the non-selective CRF antagonist α- helical CRF, prior to treatment with UCN showed DA levels that were significantly lower than those in sham controls and similar to values of 6-OHDA +

vehicle treated rats, both at basal levels and at potassium induced levels (Figure 5.3.2). All right brain sides were untreated and displayed extracellular DA levels parallel to those of sham treated animals both at basal and potassium induced levels (Figure 5.3.2).
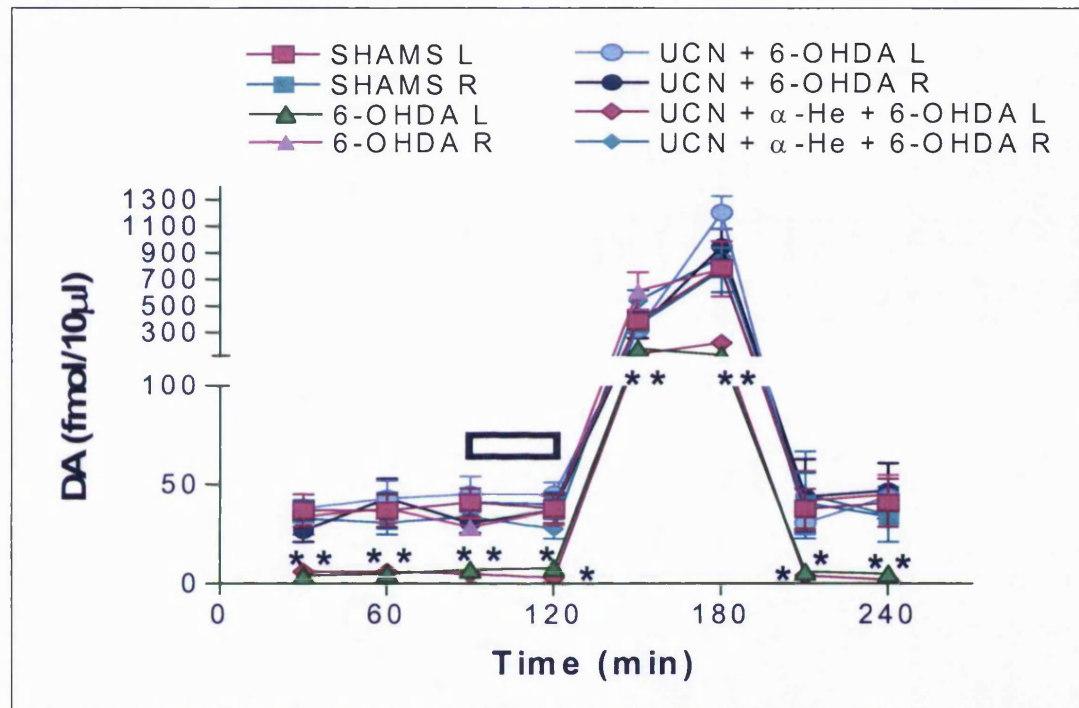

Figure 5.3.2 The effect of UCN with and without α-helical CRF on extracellular striatal DA levels obtained from *in vivo* microdialysis.
Microdialysis was performed over a period of 4 hours both at basal levels, and potassium induced levels where animals were infused with high potassium (ACSF) for 30 min indicated by black rectangle. * Significantly different to sham and UCN only treated animals at both basal and potassium induced levels ($p < 0.01$). 6-OHDA + UCN not significantly different to sham. Bilateral microdialysis was performed with L indicating left or treated side and R indicating right or untreated side. Break in Y axis to facilitate data expression. n=4-5 rats in each treatment group.

5.3.1.3 Measurement of tissue DA

Tissue DA levels were assessed in both the nigra and striata of rats where animals treated with 6-OHDA and vehicle showed dramatic depletion of striatal DA levels which was significantly lower in sham operated animals (Figure 5.3.3). On the other

hand, rats that were co-treated with UCN in addition to 6-OHDA showed almost complete preservation of tissue DA levels. Animals treated with 6-OHDA followed by the non-selective CRF antagonist prior to UCN displayed tissue DA levels that were significantly lower than those in sham controls and comparable to values observed in 6-OHDA + vehicle treated rats (Figure 5.3.3).
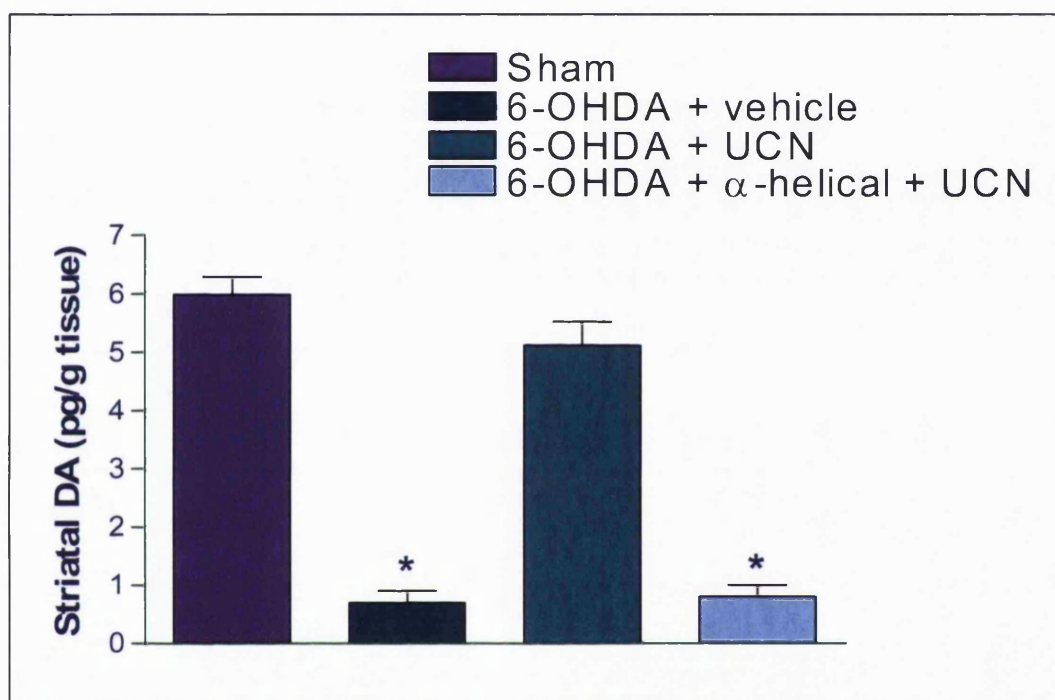
Figure 5.3.3 Effects of UCN with and without α-helical CRF on 6-OHDA-induced tissue DA loss.
DA levels were measured in striata through HPLC-ED. * Significantly different to sham and UCN only treated rats (p<0.001). 6-OHDA + UCN not significantly different than sham (p>0.05). n=5 rats in each treatment group.

5.3.1.4 TH assay

When the functional integrity of the nigrostriatal DA system was evaluated through TH enzyme synthesising capacity, TH activity was significantly reduced in striata obtained from 6-OHDA + vehicle treated rats when compared to sham controls. This loss of TH activity due to 6-OHDA treatment was prevented by co-treatment with

UCN. Importantly, the effect of UCN was ablated when α-helical CRF was injected prior to UCN treatment and after injection of 6-OHDA (Figure 5.3.4).
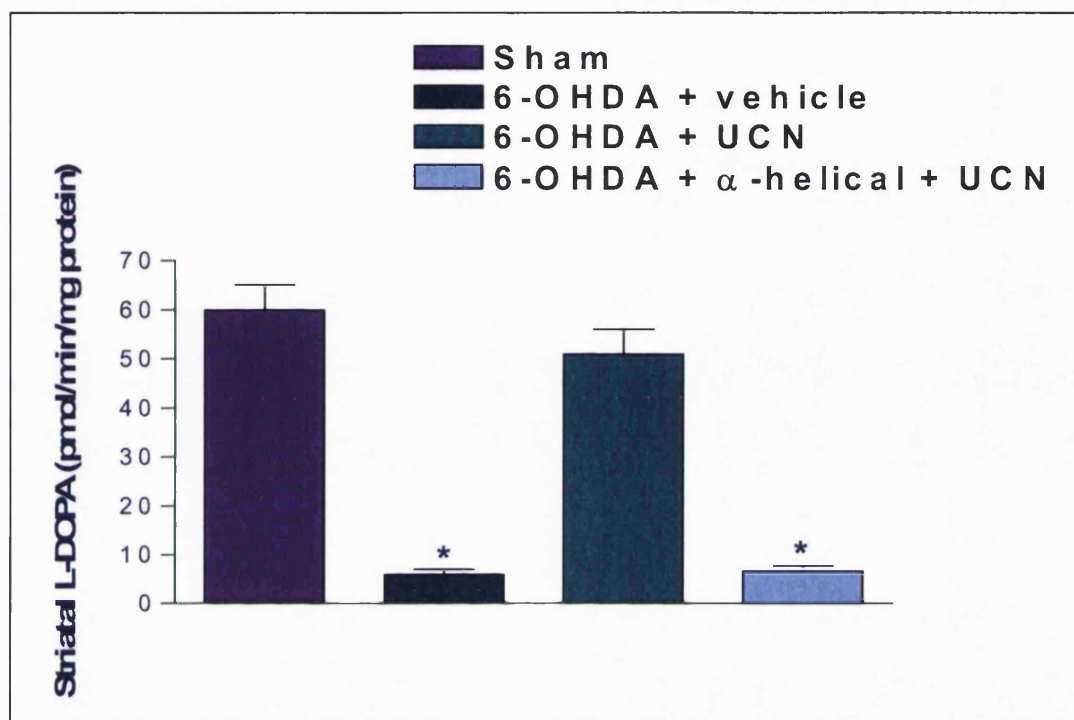

Figure 5.3.4 The effect of UCN with and without α-helical CRF on 6-OHDA-induced TH activity loss.
TH activity was measured as moles L-DOPA produced per min per mg protein after a 10 min enzyme assay using homogenates from rat brain striata. L-DOPA was quantified using HPLC-ED after the termination of the reaction and DA was used as the internal standard. * Significantly different to sham and UCN only treated animals ($p < 0.001$). n=5 rats in each treatment group.

5.3.1.5 Immunohistochemistry

When nigral sections were stained for TH positive neurons, it was clear that there was a marked difference between the TH positive neurons in the 6-OHDA lesioned SNc than in the non lesioned SNc, as illustrated in Figures 5.3.5 b. The effects of UCN can clearly be seen, with preservation of the TH positive cells (comparable to untreated SNc; Figure 5.3.5 d) in comparison to animals that were 6-OHDA-lesioned (Figure 5.3.5 b). Finally, when α-helical CRF was administered before UCN injection it reversed the effects of UCN in preserving TH positive cells (Figure 5.3.5 f). Untreated SNc were used as controls.
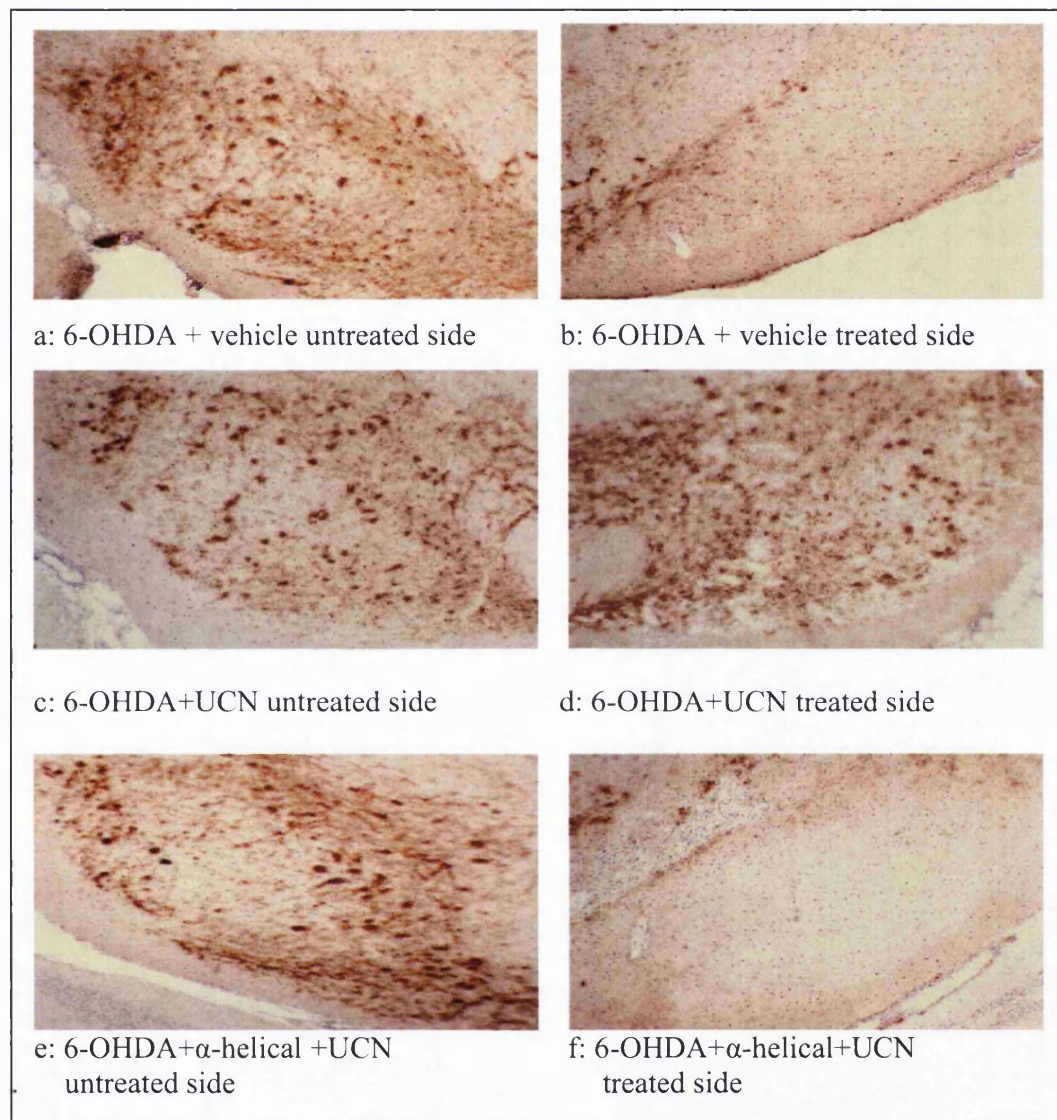
Figure 5.3.5 representative photomicrographic images of a rat brain SNc cross sections showing the effect of α-Helical CRF on reversing the preservative effects of UCN on TH neuron loss from 6-OHDA unilateral lesioning.
In each section a comparison can be made between treated and untreated SNc in an individual rat (image shown is one example from a group of 6), sides labelled treated were treated 6-OHDA+ vehicle (b), 6-OHDA + UCN (d) or 6-OHDA + α-helical + UCN (f). SNc labelled untreated were used as controls. DAB stain (orange brown colour) is indicative of TH positive neurons.

5.3.2 Effects of UCNII and UCNIII in 6-OHDA lesioned

In this section either UCNII or UCNIII was used in place of UCN to examine any neuroprotective effect these peptides may possess in 6-OHDA lesioned animals.

5.3.2.1 Apomorphine challenge

As expected, rats treated with 6-OHDA and vehicle displayed characteristic turning behaviour of unilateral nigrostriatal degeneration (Figure 5.3.6). By contrast, rats treated with 6-OHDA and co-treated with UCN showed reduced sensitivity to the apomorphine challenge with significant reduction in the number of turns in comparison to 6-OHDA + vehicle treated animals. When UCN II or UCN III were used in place of UCN, after 6-OHDA treatment, there was no significant reduction in circling behaviour, when compared to 6-OHDA + vehicle treated animals, and rats exhibited a significantly greater number of turns compared to sham controls and 6-OHDA + UCN treated animals (Figure 5.3.6).
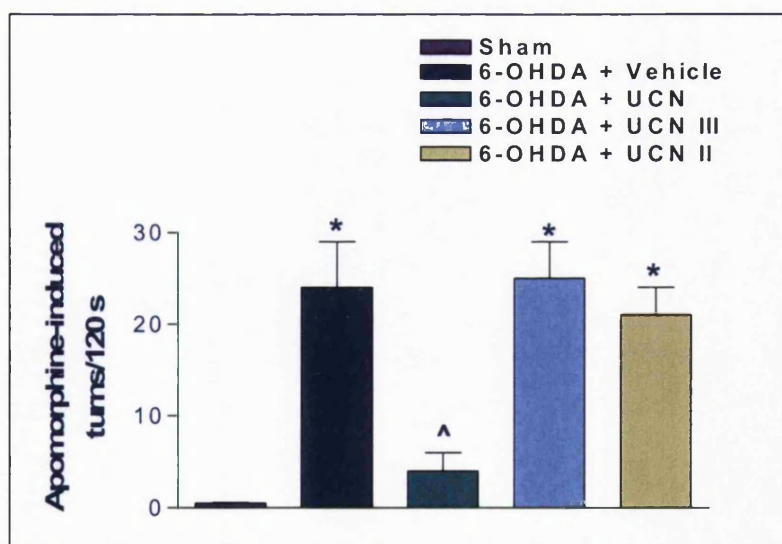
Figure 5.3.6 Effects of UCN on 6-OHDA-induced contraversive turns in response to apomorphine.
Apomorphine (0.5 mg/kg) was administered through s.c injection. Turns were recorded in a 1.2 metre circular arena for all animals as mean counts / 120 s. * Significantly different to sham and UCN treated groups ($p < 0.001$), ^ significantly different to 6-OHDA + vehicle ($p < 0.001$). 6-OHDA + UCN not significantly different to sham ($p > 0.05$). n=4-6 rats in each treatment group.

5.3.2.2 *In vivo* microdialysis

Rats were subject to *in vivo* microdialysis to evaluate the functional integrity of the nigrostriatal pathway through the measurement of extracellular DA levels. These measurements were performed both at basal levels and potassium induced levels (Figures 5.3.7 & 5.3.8). Extracellular DA levels in rats treated with 6-OHDA and vehicle were significantly reduced when compared to sham-operated animals, both at basal levels and at potassium induced levels. Again, levels in animals treated with 6-OHDA and UCN were comparable to sham equivalents. When UCN III (Figure 5.3.7) or UCN II (Figure 5.3.8) were used in the place of UCN after 6-OHDA treatment, there was no significant reduction in lesion severity, as indicated by extracellular DA levels, when compared to 6-OHDA + vehicle treated animals, and rats showed DA levels significantly lower than sham controls and 6-OHDA + UCN treated animals at both basal and potassium induced DA levels (Figure 5.3.7 for UCN III and Figure 5.3.8 for UCN II). All right brain sides were untreated and displayed extracellular DA levels equivalent to those of sham controls both at basal and potassium induced levels.

Figure 5.3.7 The effect of UCN III on loss of extracellular striatal DA levels after 6-OHDA unilateral lesionning. DA levels obtained from *in vivo* microdialysis
Microdialysis was performed over a period of 4 hours both at basal levels, and potassium induced levels where animals were infused with high potassium (ACSF) indicated by black rectangle. * Significantly different to sham at both basal and potassium induced levels (p<0.01). Bilateral microdialysis was performed with L indicating left or treated side and R indicating right or untreated side. Break in Y axis to facilitate data expression. n=4-5 rats in each treatment group.
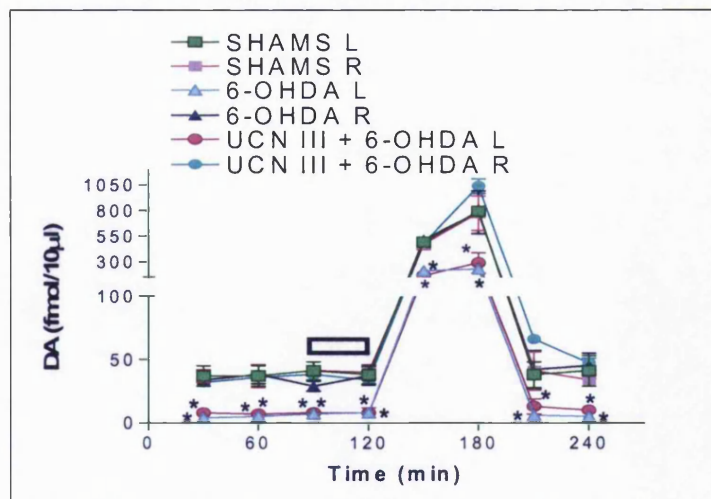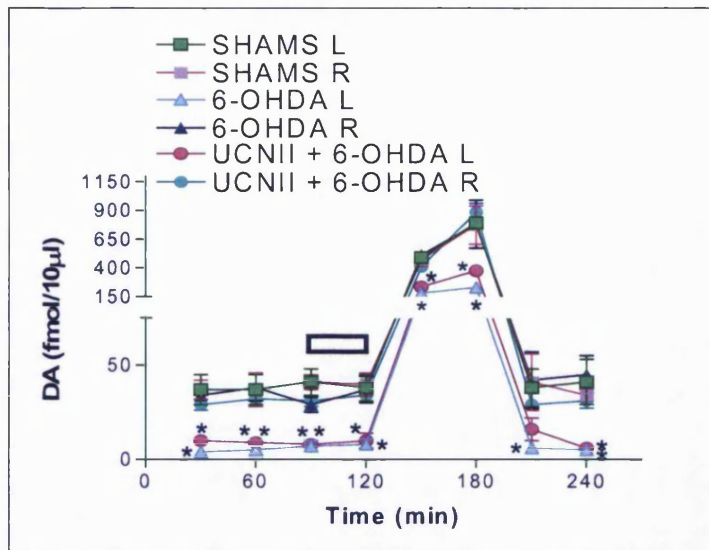
Figure 5.3.8 The effect of UCN II on loss of extracellular striatal DA levels after 6-OHDA unilateral lesionning. DA levels obtained from *in vivo* microdialysis
Microdialysis was performed over a period of 4 hours both at basal levels, and potassium induced levels where animals infused with high potassium (ACSF) indicated by black rectangle. * Significantly different to sham at both basal and potassium induced levels ($p<0.01$). Bilateral microdialysis was performed with L indicating left or treated side and R indicating right or untreated side. Break in Y axis to facilitate data expression. n=4-5 rats in each treatment group.

5.3.2.3 Measurement of tissue DA

When estimating the functionality of the TH enzyme through assessment of striatal tissue DA levels, animals treated with 6-OHDA and vehicle showed dramatic depletion of DA levels in comparison to sham operated animals (Figure 5.3.9). Again, rats that were co-treated with UCN in addition to 6-OHDA showed almost complete preservation of tissue DA levels. However, when UCN II or UCN III were used in place of UCN, after 6-OHDA treatment, there was no significant reduction in lesion severity when compared to 6-OHDA + vehicle treated animals (Figure 5.3.9).

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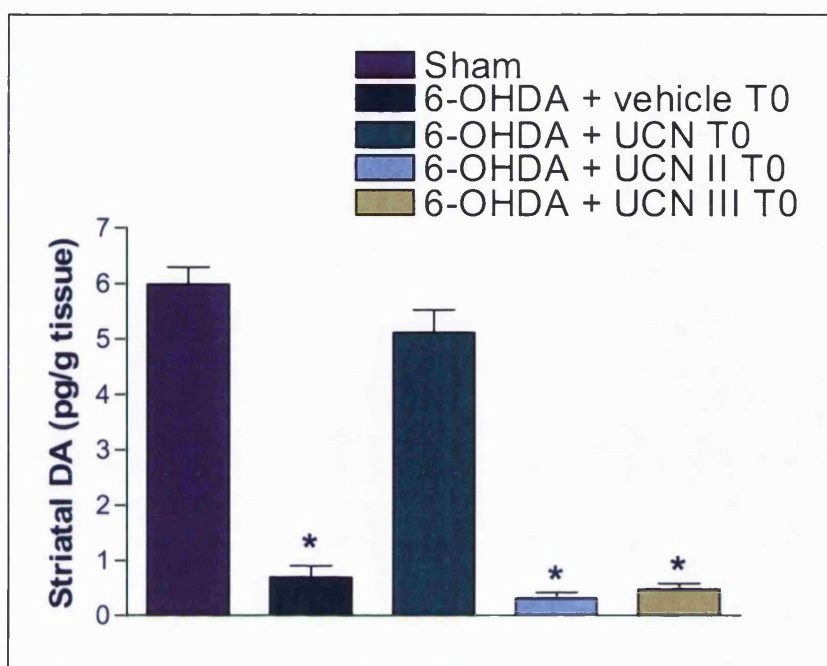
Figure 5.3.9 Effects of UCN, UCN II and UCN III on 6-OHDA-induced tissue DA loss
DA levels were measured for striata through HPLC-ED. * Significantly different to sham and UCN treated rats ($p < 0.001$). 6-OHDA + UCN not significantly different to sham ($p > 0.05$) T0 indicates the UCN injections were made on the first day and about 5 minutes after 6-OHDA. n=4-6 rats in each treatment group.

5.3.2.4 TH assay

When the TH enzyme assay was performed to estimate the functional integrity of the nigrostriatal DA system through the measure of L-DOPA synthesising capacity, TH activity was significantly reduced in striata obtained from 6-OHDA + vehicle treated rats when compared to sham controls. This loss of TH activity due to 6-OHDA treatment was prevented by co-treatment with UCN. When UCN II or UCN III were used in place of UCN, after 6-OHDA treatment, there was no significant reduction in lesion severity when compared to 6-OHDA + vehicle treated animals. 6-OHDA + UCN III or UCN II treated animals also displayed significantly lower TH activity when compared to either sham controls or 6-OHDA + UCN treated animals (figure 5.3.10).
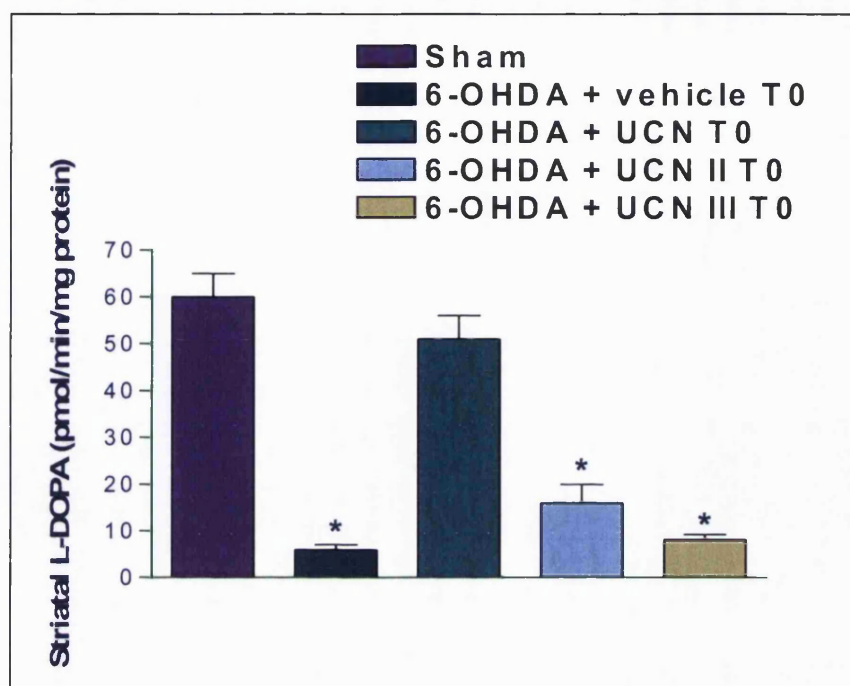
Figure 5.3.10 The effect of UCN, UCN II and UCN III on 6-OHDA-induced TH activity loss

TH activity was measured as moles L-DOPA produced per min per g protein after a 10 min enzyme assay using homogenates from Striata. L-DOPA was quantified using HPLC-ED after the termination of the reaction and DA was used as the internal standard. * Significantly different to sham and UCN treated animals ($p < 0.001$). 6-OHDA + UCN not significantly different to sham. n=4-6 rats in each treatment group.

5.3.2.5 Immunohistochemistry

Nigral sections were subjected to TH staining in order to determine whether the changes in TH activity were mirrored by alterations in the amount of TH in the SNc. It can be clearly seen from the results that there was a marked reduction in TH positive staining in the 6-OHDA lesioned SNc in comparison to non lesioned SNc of the same rat, as illustrated in Figures 5.3.11 a and b, respectively. The effects of UCN treatment led to preservation of normal (comparable to untreated SNc) TH positive staining in the SNc of 6-OHDA treated animals (Figure 5.3.11 d). However, when UCN III or UCN II were co-administered with 6-OHDA, they did not have any preservative effects on TH positive cells (Figure 5.3.11 f and h respectively)

a: 6-OHDA untreated side   b: 6-OHDA treated

c: 6-OHDA+UCN untreated side   d: 6-OHDA+UCN treated side

e: 6-OHDA + UCNII untreated side f: 6-OHDA+ UCNII treated side

g: 6-OHDA + UCNIII untreated side h: 6-OHDA+ UCNIII treated side
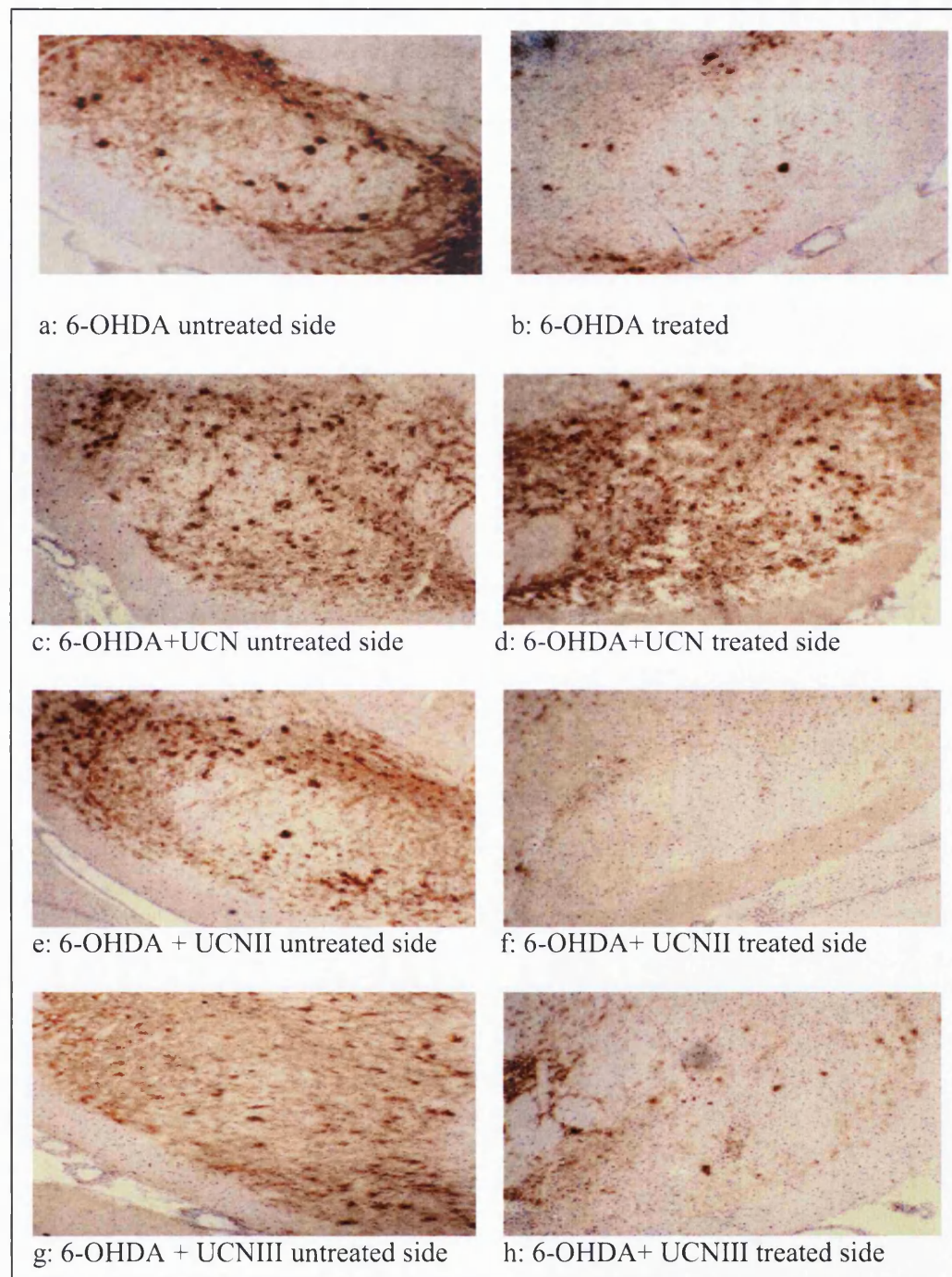
Figure 5.3.11 Representative photomicrographic images of a rat brain SNc cross sections showing the effect of UCN, UCNII and UCNIII on the neuron loss due to unilateral 6-OHDA lesionning.

In each section a comparison can be done between treated and untreated SNc in an individual rat (image shown is one example from a group of 4), sides labelled treated were treated with 6-OHDA + vehicle (b), 6-OHDA + UCN (d), 6-OHDA + UCN II (f) or 6-OHDA + UCN III (h). SNc labelled untreated were treated as controls. DAB stain (orange-brown colour) is indicative of TH positive neurons.

5.3.3 Effect of the selective CRF-R1 receptor antagonist NBI – 27914 on UCN-mediated neuroprotection

In this section UCN was used in conjunction with the selective CRF-R1 antagonist NBI-27914 in the 6-OHDA PD model to determine the effects of this antagonist on the neuroprotective effects of UCN. This antagonist was administered after the lesion had progressed to assess if the CRF-R1 receptors would survive the damage caused by 6-OHDA after seven days; when UCN is administered.

5.3.3.1 Apomorphine challenge

Rats that were treated with 6-OHDA and vehicle displayed characteristics of unilateral nigrostriatal degeneration, in response to apomorphine challenge, as previously. NBI-27914 alone was without effect. By contrast, when the rats were treated with 6-OHDA and co-treated with UCN seven days *after the injection of insult*, reduced sensitivity to the apomorphine challenge was displayed. Animals that were treated with 6-OHDA and seven days later were exposed to the selective CRF antagonist NBI-27914, prior to UCN, turned significantly more than controls (NBI-27914+vehicle) and sham treated (Figure 5.3.12).
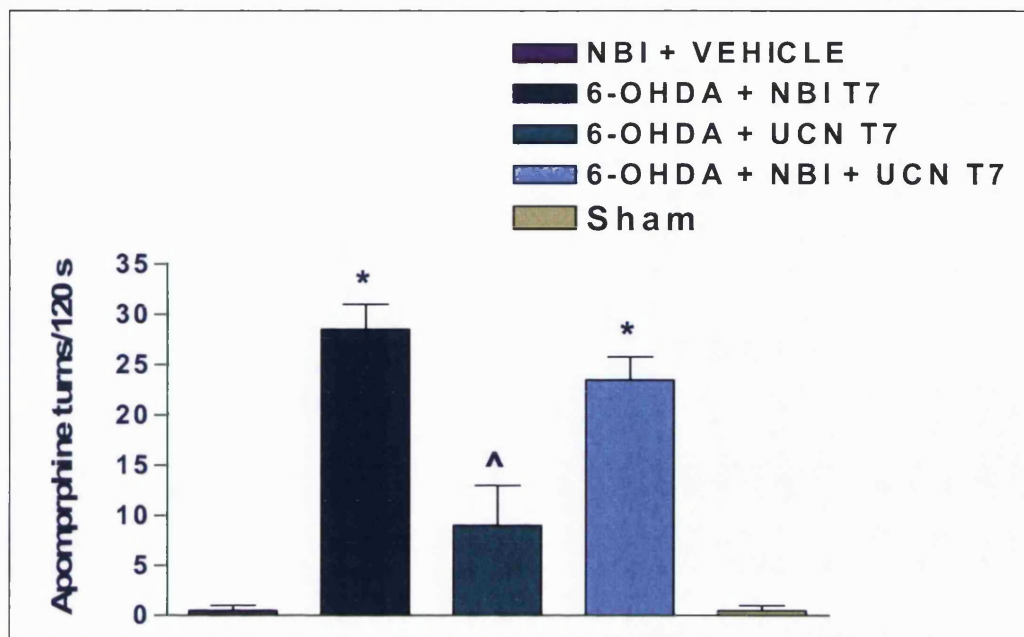
Figure 5.3.12 Effects of NBI-27914 on UCN's attenuation of 6-OHDA-induced contraversive turns in response to apomorphine.

Apomorphine (0.5 mg/kg) was administered through s.c injection. Turns were recorded in a 1.2 metre circular arena from all animals as mean counts / 120 s.

* Significantly different to NBI-27914 + vehicle and sham ($p<0.001$), ^ significantly different to 6-OHDA + NBI-27914 ($p<0.001$). 6-OHDA + UCN T7 not significantly different than NBI-27914 + vehicle ($p>0.05$) but significantly different than 6-OHDA + (NBI + UCN) T7 ($p<0.01$). 6-OHDA + (NBI + UCN) T7 indicates that rats were given both NBI and UCN seven days after injection of 6-OHDA. n=6 rats in each treatment group.

5.3.3.2 *In vivo* microdialysis

In vivo microdialysis was used to evaluate the functional integrity of the nigrostriatal pathway through measuring extracellular DA levels at both basal and potassium induced levels (Figure 5.3.13). Extracellular DA levels in rats treated with 6-OHDA + UCN were significantly lower than the levels in untreated sides of all animals (control) both at basal and at potassium induced levels. In contrast, when the rats were treated with the insults and co-treated with UCN seven days after the injection of insult, extracellular DA levels were restored to control at both basal and potassium induced levels (Figure 5.3.13). Animals that were treated with 6-OHDA and after seven days were administered the selective CRF antagonist NBI-27914 i.p. injection,

30 minutes prior to UCN, showed significant reduction in extracellular DA levels (Figure 5.3.13). Rats treated with 6-OHDA followed by UCN 7 days later showed DA levels that were not significantly different to untreated sides. All right hand sides were untreated and displayed extracellular DA levels equivalent to those of shams both at basal and potassium induced levels (as shown previously) and were thus considered as controls.
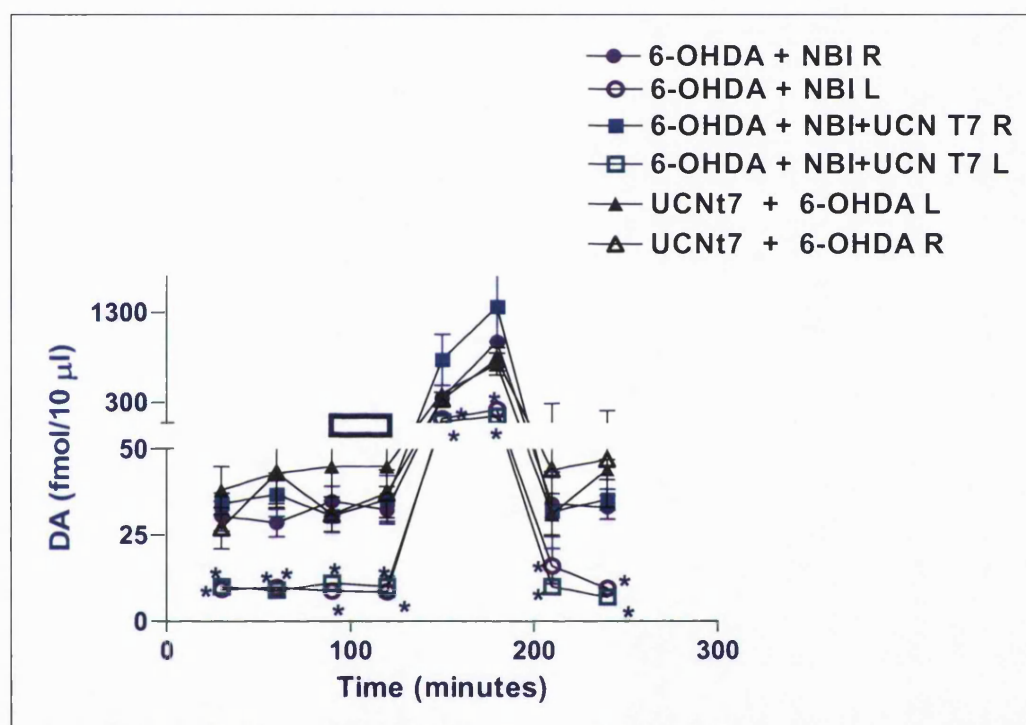
Figure 5.3.13 The effect of UCN with and without NBI-27914 on loss of extracellular striatal DA levels due to unilateral 6-OHDA lesionning. DA levels obtained from *in vivo* microdialysis.

Microdialysis was performed over a period of 4 hours both at basal levels, and potassium induced levels where animals were infused with high potassium (ACSF) for 30 min indicated by black rectangle. * Significantly different than sham at both basal and potassium induced levels (p<0.001). 6-OHDA + UCN T7 not significantly different to untreated sides. Bilateral micro dialysis was performed with L indicating left or treated side and R indicating right or untreated side. Break in Y axis to facilitate data expression. n=4-5 rats in each treatment group.

5.3.3.3 Measurement of tissue DA

Tissue DA levels were assessed in rat striata where animals treated with 6-OHDA and NBI-27914 exhibited dramatic reductions in striatal DA levels which were significantly lower than those in sham operated and control (NBI + vehicle) treated animals (Figure 5.3.14). Again, rats that were treated with UCN seven days post-6-OHDA showed almost complete restoration of tissue DA levels. These effects were prevented by treatment with NBI-27914 30 mins prior to UCN. Animals receiving only NBI and vehicle did not show any significant change in DA tissue levels in comparison to untreated sides (not shown) and were considered as controls.
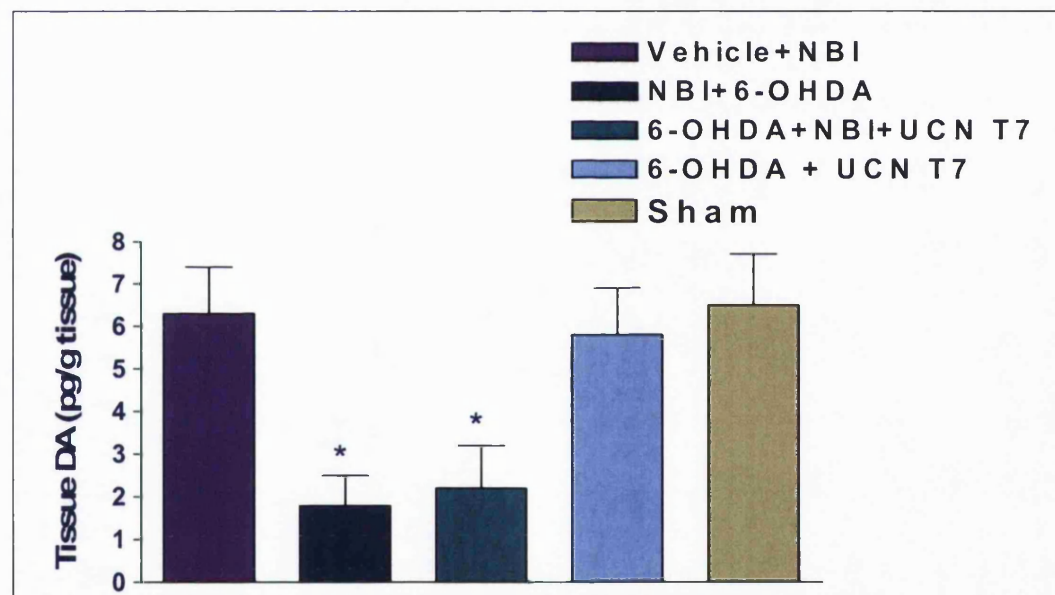

Figure 5.3.14 Effects of UCN with and without NBI-27914 on 6-OHDA-induced tissue DA loss from 6-OHDA lesionning
DA levels were estimated for striata through HPLC-ED. * Significantly different to vehicle + NBI and sham ($p < 0.05$). 6-OHDA + UCN T7 not significantly different to NBI-27914 + vehicle ($p > 0.05$). n=5-6 rats in each treatment group.

5.3.3.4 TH enzyme assay

When the functional integrity of the nigrostriatal DA system was evaluated through the L-DOPA synthesising capacity of the TH enzyme, TH activity was significantly reduced in striata obtained from 6-OHDA + vehicle treated rats when compared to NBI-27914 + vehicle treated animals. This loss of TH activity due to 6-OHDA treatment was prevented by co-treatment of UCN even when administered seven days after the lesion had progressed and TH activity had significantly dropped. This effect of UCN was reversed when NBI- 27914 was injected prior to UCN treatment and seven days after injection of the insult. NBI-27914 + vehicle alone did not have any significant effect on TH activity and was considered as a control (Figure 5.3.15).
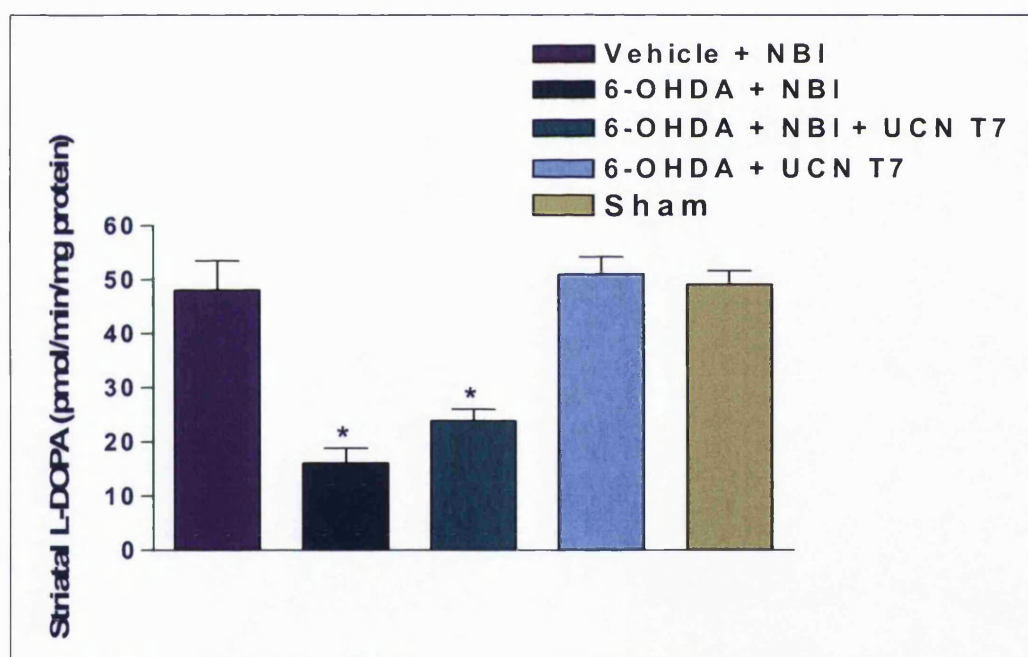
Figure 5.3.15 The effect of UCN with and without NBI-27914 on 6-OHDA-induced TH activity loss due to 6-OHDA unilateral lesionning.
TH activity was measured as moles L-DOPA produced per min per gm protein after a 10 min enzyme assay using striatal homogenates. L-DOPA was quantified using HPLC-ED and DA was used as the internal standard. * Significantly different to (NBI + vehicle) and sham (p<0.001). 6-OHDA and UCN T7 not significantly different to NBI-27914 + vehicle. NBI-27914 + vehicle not significantly different to untreated sides. n=5-6 rats in each treatment group.

5.3.3.5 Immunohistochemistry

When nigral sections were stained for TH positive neurons, it was clear that there was a substantial difference between TH positive staining in the 6-OHDA lesioned SNc than in the non lesioned SNc, where as NBI-27914 did not have any effect on 6-OHDA mediated cell loss, as illustrated in Figure 5.3.5 d when compared to either the non treated side of the same animal (Figure 5.3.16 c) or to NBI-27914 + vehicle treated SNc (Figure 5.3.16 b). When UCN was administered 7 days after 6-OHDA injection its neuroprotective effects can be clearly seen, with preservation of the normal (comparable to controls (NBI-27914+vehicle treated or untreated SNc) TH positive cells in the SNc of lesioned animals (Figure 5.3.16 f). Finally, when NBI-27914 was used prior to UCN injection and seven days after initial 6-OHDA injection, this selective CRF-R1 antagonist reversed the effects of UCN preserving TH positive neurons (Figure 5.3.16 h). NBI-27914 + vehicle alone was without effect and was treated as control (Figure 5.3.16 b).

a: Saline + NBI untreated side

b: saline + NBI treated side

c: 6-OHDA + NBI untreated side

d: 6-OHDA + NBI treated side

e: 6-OHDA+UCN T7 untreated

f: 6-OHDA+UCN T7 treated

g: 6-OHDA+NBI+UCN untreated

h: 6-OHDA+NBI+UCN treated
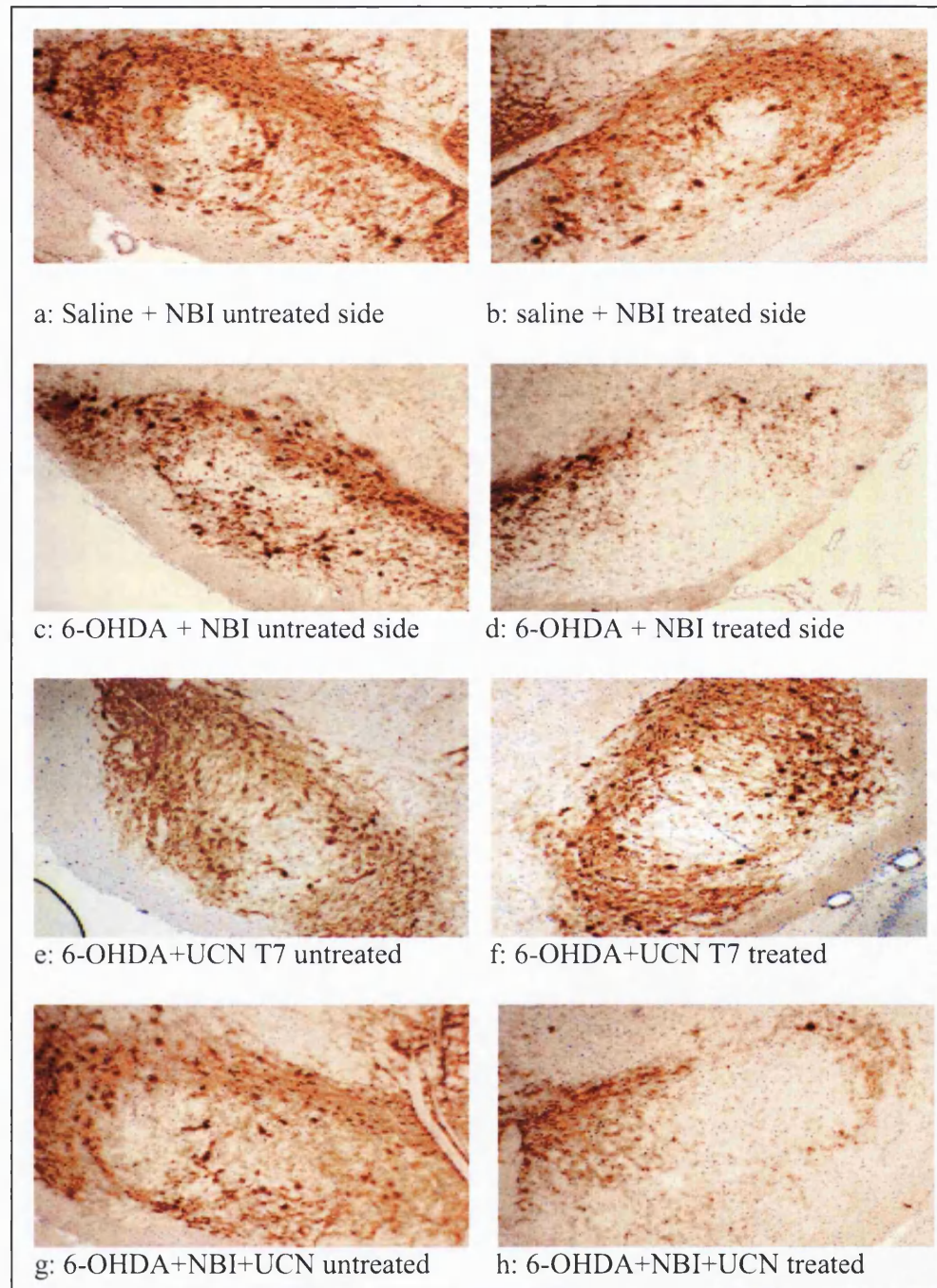
Figure 5.3.16 Representative photomicrographic images of a rat brain SNc cross sections showing the effect of NBI-27914 on reversing the UCN preservative effect on TH neurons after 6-OHDA unilateral lesioning.

In each section a comparison can be made between treated and untreated SNc in an individual rat (image shown is one example from a group of 6), in first example, A was untreated and (b) was treated with vehicle + NBI, and image d shows 6-OHDA+ vehicle treated SNc, image f shows 6-OHDA + UCN T7 treated SNc and finally Image h shows 6-OHDA + (NBI-27914 + UCN) T7. SNc labelled untreated were untreated were used as controls. DAB stain (orange brown colour) is indicative of TH positive neurons.

5.4 Discussion

In order to establish the receptor target through which UCN was mediating its neuroprotective effects, known ligands for CRF-R1 and CRF-R2 sites were utilized. As shown in the results, UCN failed to attenuate either apomorphine-induced circling, DA depletion or loss of TH activity and staining when administered after the injection of the mixed CRF-R1/R2 antagonist, α-helical-CRF9-41 (section 5.3.1). This finding confirms that the UCN elicited effects were CRF receptor mediated and not a result of possible neuroprotection resulting from other non-receptor mediated events, such as the reduction of intracellular Ca^{+2} due to calcium channel blockade (Stefani *et al.*, 1998; Tao *et al.*, 2006). Furthermore, UCN II and UCN III, two selective ligands for CRF-R2 (Reyes *et al.*, 2001) sites had no effect on either apomorphine-induced circling, reduction in tissue DA content or TH activity (section 5.3.2). Similar data were found for preservation of TH positive nigral cells and dialysate DA levels. From these data the logical conclusion is that the observed effects of UCN in this work are being mediated by CRF-R1 receptors, and not through ion mediated target sites indicated in some studies (Tao *et al.*, 2005). Recent findings have shown that UCN promotes the survival of cultured cerebellar GABAergic neurons through the CRF-R1 receptor (Choi *et al.*, 2006). Also, the involvement of the CRF-R1 becomes more probable due to its significant presence in the SNc (Van Pett *et al.*, 2000).

All of these findings were supported by the work using NBI-27914 (section 5.3.3), where this selective CRF-R1 antagonist reversed the preservative effects of UCN on reducing the overall severity of the lesion seven days after insult treatment. This process, which occurs after the lesion has progressed, not only validates that the UCN effects are CRF-R1 mediated, but also indicates that the cells where the receptors are located have also survived the lesion, even after it has significantly progressed. If this situation occurs also in the PD brain, these data would indicate that UCN could possess   potential   utility   in   the   treatment   of   established   PD.

Chapter 6

General Discussion

6 General Discussion

This project started out with the aim to explore the potential usefulness of a CRF related peptide in the treatment of PD. This peptide, UCN, which has been shown to possess anti-inflammatory properties (Brar *et al.*, 2000), would be examined for any positive effects it has on a well established *in vivo* model of PD (the 6-OHDA model). This paradigm which has been used for over four decades would serve as an initial indicator for any value that UCN may hold in the treatment of PD. The manner in which the peptide was evaluated for efficacy was through one direct injection into the SNc, the area containing the neuronal cell bodies of the DA neurons, five minutes after the injection of the 6-OHDA insult. The aim here was to determine if UCN injection would have any preservative effects on the integrity of the 6-OHDA affected nigrostriatal pathway. When this was achieved, it was essential to exclude UCN from being toxin specific and having interacted with 6-OHDA itself, thus preventing it from giving its full toxic effects. To achieve this, another rodent model of PD was used, the LPS model. The advantage of this model was that the means by which it initiates the lesion is through neuroinflammation, which is a different mode of action than that of 6-OHDA. Again UCN achieved similar effect in the LPS model to those using the 6-OHDA one. The findings where UCN gave similar neuro-preservative effects in both models of the disease made it reasonable to assume that the UCN protection was not model specific.

After establishing the effectiveness of UCN in preventing key indices of PD like effects in two distinct models of PD, it was quite evident that this protective effect would be of limited use in the treatment of PD, as in the majority of PD patients the disease would be only diagnosed subsequent to significant neuronal loss in the nigrostriatal pathway. If UCN is to be of any therapeutic value, it would have to prove its effectiveness after significant damage to the nigrostriatal pathway had already occurred. For this to be achieved, a time point at which considerable damage to the nigrostriatal pathway would occur had to be chosen, and at this time point UCN would be administered in the two rodent models of the disease used.

When UCN was proven to be effective in preserving and even reversing neuronal cell loss in two distinct models of PD, even when the lesion had significantly progressed, the therapeutic potential of this molecule was indicated. One major problem however would be the unfavourable physicochemical properties of UCN, which would limit its therapeutic use due to its limited access to the brain.

In order to overcome this obstacle, another molecule that possesses the same neuroprotective effects as UCN but with favourable physicochemical properties would have to be used. To do so, the target sites and the mechanism of action by which UCN provides its neuronal protection would have to be first uncovered.

The first targets expected to be implicated were the CRF receptors, CRF-R1 and CRF-R2. To confirm this theory; a non selective CRF-R antagonist was used prior to the administration of UCN in the 6-OHDA rodent model of the disease. As this antagonist blocked the preservative effects of UCN, it was evident that the UCN effects are receptor mediated, and as previous findings found the CRF-R2 to be responsible for anti inflammatory effects, two specific CRF-R2 agonists were evaluated for their neuroprotective efficacy, these were UCNII and UCNIII (Reyes *et al.*, 2001). When these two peptides did not show any of the observed UCN effects on nigrostriatal damage, it was determined by a simple case of elimination that UCN exerted its neuro protective effects through the CRF-R1; an outcome confirmed when the selective CRF-R1 antagonist NBI-27914 reversed the UCN induced neuroprotection in the 6-OHDA PD model.

Taken as a whole, the presented data reveal that UCN can act as a potent neuroprotective agent in two distinct rodent models of PD at a dose that was found to be effective in a variety of other studies (Brar *et al.*, 2000; Brar *et al.*, 2002; Choi *et al.*, 2006; Facci *et al.*, 2003). Moreover, multiple parameters of nigrostriatal pathway activity and nigral neuronal integrity were assessed. This approach has important strengths over 'single parameter' studies, given that human PD pathology is complex and multi-component (Blum *et al.*, 2001; Gandhi *et al.*, 2005; Tatton *et al.*, 1998). Of particular significance was the finding that UCN was able to arrest and possibly reverse nigrostriatal lesions once the neurodegenerative process has commenced. This

principle, if reproduced in the human brain, would have obvious clinical significance, since patients generally present with symptoms only once nigral neuronal loss reaches 70-80% of the total nigral complement and striatal DA levels have fallen significantly (Abercrombie *et al.*, 1990). Aside from an apparent facility for preservation of cells, it is possible that UCN may act to stimulate DA neurons via several distinct mechanisms. Recent studies have shown that UCN potentiates release of DA from electrically stimulated rat striatal slices (Bagosi *et al.*, 2006) and increases TH mRNA levels expressed by PC-12 cells (Nanmoku *et al.*, 2005), a cell type phenotypically related to midbrain DA neurons. In the present study, UCN appears to 'reverse' loss of TH positive cell bodies and dendrites, since TH staining in rats terminated seven days post-toxin was noticeably lower than for those where UCN was also administered at this time point, but termination occurred at day fourteen. The underlying cellular mechanism responsible for this highly significant finding is as yet, unclear, although it is possible that UCN increases expression of TH protein in remaining cells (Nanmoku *et al.*, 2005), whilst also effecting a re-growth and remodelling of surviving dendrites, as observed by Swinny et al. (2004) using cerebellar Purkinje cells *in vitro*, where UCN was able to induce a dramatic increase in dendritic growth in these cultured cells (Swinny *et al.*, 2004). Interestingly, Scarabelli et al. (2002) also report an apparent restorative action of UCN in damaged cardiac myocytes (Scarabelli *et al.*, 2002). In addition, it is also probable that UCN achieves a neuroprotective effect partly via prevention of apoptotic cell death (Intekhad-Alam *et al.*, 2004; Scarabelli *et al.*, 2002). Furthermore, studies have shown that this CRF like peptide significantly reduces the number of end-stage apoptotic nuclei observed in random nigral fields, following intracerebral 6-OHDA injection (Biggs *et al.*, 2006), a significant finding, since apoptosis is an important mechanism in neurotoxicity (Cutillas *et al.*, 1999) and human PD (Tatton *et al.*, 1998). In the present work, the use of the single-dose LPS PD model (Gao *et al.*, 2003; Herrera *et al.*, 2000) increases the significance of my data, given that recent evidence supports a neuroinflammatory component in the aetiology of human PD (Block *et al.*, 2007; Gao *et al.*, 2003; Whitton, 2007) and that UCN itself possesses anti-inflammatory properties (Gonzalez-Rey *et al.*, 2006). This agrees with the

finding where UCN was observed to protect DA neurons from LPS-induced neurotoxicity in mesencephalic neuron-glia cultures through inhibiting microglial activation (Wang *et al.*, 2007).

The observations that UCN restores both basal and potassium-evoked release of striatal DA, after either 6-OHDA or LPS, suggests that it is able to elicit a functional recovery in nigrostriatal neurotransmission. This recovery in extracellular DA will most probably permit the return of normal motor activity through restoring the D1 and D2 receptor balance in the striatum, a very significant event since the ability of UCN to effect this preservation or restoration of nigrostriatal function takes place once a lesion is established. In contrast, other agents explored for their anti parkinsonian ability were almost always used either at the same time or prior to the neurotoxic insult (Block *et al.*, 2007; Hunter *et al.*, 2007; Whitton, 2007). Unfortunately for PD patients however, the disease only presents after considerable degeneration to the nigrostriatal system has already occurred, so unless there was an early diagnosis of the disease, which is unlikely, most of these agents would be of limited therapeutic benefit.

Overall, the results greatly add to a growing knowledge base, whereby UCN mediates cytoprotection in cardiac tissues (Brar *et al.*, 2000; Brar *et al.*, 2002; Scarabelli *et al.*, 2002) and cultured neurons (Choi *et al.*, 2006; Facci *et al.*, 2003; Pedersen *et al.*, 2002), with the crucial refinement that we have demonstrated efficacy in an *in vivo* model of a currently incurable neuropathology. The apparent ability of UCN to arrest nigrostriatal damage, and possibly stimulate remaining cells, suggests a novel mechanism, which if translated therapeutically would offer a significant advance in PD treatment. This principle has recently been proposed as an essential prerequisite for the basis of a meaningful advance in PD therapy (Meissner *et al.*, 2004).

The precise neuroanatomical site through which UCN exerts its neuroprotective effects is yet unclear, although a local action at the level of nigral cell bodies and dendrites seems likely, given the presence of CRF-R1 immunoreactivity here

(Sauvage *et al.*, 2001) and widespread distribution of UCN in the brain (Vasconcelos *et al.*, 2003; Yamamoto *et al.*, 1998). Studies utilizing cerebellar granule cells and hippocampal neurons (Pedersen *et al.*, 2002) also suggest that engagement of CRF-R1 is necessary for neuroprotection.

The rapidly expanding interest in UCN itself and central CRF receptor pharmacology is supporting the development of novel small molecule agonists, with selectivity for CRF-R1 and R2 receptor subtypes, some of which will likely be suitable for evaluation in pre-clinical and clinical paradigms. It is believed that the presented work is an important first step towards validating UCN and central CRF-R1 receptors as potential future targets in the treatment of human PD.

The pharmacology of these receptors has thus far not been fully investigated (Reul *et al.*, 2002) and as such establishing clearly the identity of CRF receptors expressed by nigral cells is of considerable importance. There is, however, evidence that UCN is able to mediate protection of some neurons via activation of the CRF1 subtype (Choi *et al.*, 2006; Facci *et al.*, 2003; Pedersen *et al.*, 2002), whilst other groups have cited activation of CRF2 splice variants as a key event (Brar *et al.* 2002; Interkhad-Alam *et al*, 2004). The possibility that UCN may have the ability to 'rescue' damaged cells has been postulated in some studies, most notably in isolated cardiac myocytes (Brar *et al*, 2002) and the heart ex vivo (Brar *et al.*, 2000; Scarabelli *et al.*, 2002). Other authors have demonstrated an anti-apoptotic effect mediated by UCN, in response to challenge with well-characterised pro-apoptotic stimuli (Intekhad-Alam *et al*, 2004). UCN is known to interact with a number of intracellular signaling pathways (Brar *et al.*, 2000; Lawrence *et al.*, 2002; Scarabelli *et al.*, 2002), and modulation of one or more of these may mediate sparing of nigrostriatal cells.

The precise mechanism behind UCN's effects were further clarified when the neuroprotective action of UCN was blocked by the non-selective CRF receptor antagonist α-helical CRF, indicating that this was a receptor mediated event and not ion channel mediated (Tao *et al.*, 2005; Verkhratsky *et al.*, 2003), where researchers have indicated that the inhibition of calcium channels and the reduction of

intracellular Ca^{+2} could be responsible for neuroprotection (Stefani *et al.*, 1998; Tao *et al.*, 2006). This finding is of particular importance since researchers have observed that some neurodegenerative disorders like PD were associated with calcium overload (Verkhratsky *et al.*, 2003).

On the other hand, neither UCN II nor UCN III, which are essentially selective for CFR-R2 sites (Reyes *et al.*, 2001), were not effective in reducing the lesion severity after toxin treatment. This left, by a process of elimination, the CRF-R1 site as the target receptor in protecting and/or restoring nigrostriatal function, a finding verified by studies utilizing cerebellar granule cells and hippocampal neurons (Pederson *et al.*, 2002), which also suggest that the engagement of CRF-R1 is necessary for neuroprotection. Facci *et al.* (2003) also confirmed these findings when they observed that potent neuroprtectantion conferred by CRF peptides was entirely mediated by CRF-R1 (Facci *et al.*, 2003). All of these outcomes, including the findings in the present work, were supported by CRF-R1 immunoreactivity at the level of nigral cell bodies and dendrites (Sauvage *et al.*, 2001).

The role of the CRF-R1 was finally validated when the selective CRF-R1 antagonist NBI-27914 was used in conjunction with UCN treatment after toxin injection, where it was evident that the preservative effects of UCN on improving the overall severity of the lesion were reversed by this antagonist. This process, which occurs after the lesion progresses, not only confirms the CRF-R1 as the target for the UCN mediated effects, but also verifies that the speculated means through which UCN was working would also survive after the lesion had significantly progressed, indicating that these possible neuroprotective mechanisms would still be valuable when treating PD patients, which when diagnosed, will most certainly have suffered significant degradation of the nigrostriatal pathway.

However, the specific cellular events beyond this level are as yet unclear, at least in the SNc. It is known that in general both CRF1 and CRF2 receptors are coupled to the generation of cAMP and this appears to be common to both the cardioprotective (Brar *et al.*, 2000; Brar *et al.*, 2002) and the neuroprotective effects of the UCNs (Facci *et al.*, 2003), as elevating intracellular cAMP levels promotes survival of neurons

including DA neurons (Facci *et al.*, 2003; Mena *et al.*, 1995). The mitogen activated protein kinase (MAPK) pathway has been suggested to be central to the protective actions of UCN in cardiac cells, although via the CRF2 receptor (Herrera *et al.*, 2000), but it has been indicated that the involvement of CRF-R1 versus CRF-R2 in mediating the different actions of UCN may be determined by the relative abundance of the receptors at the site of action (Pedersen *et al.*, 2002).

Activation of CRF-R1 receptors has also been suggested to lead eventually to expression of brain derived neurotrophic factor (BDNF), a protein present in the brain which helps in supporting the survival of existing neurons and also encourages the growth and differentiation of new neurons (Russo-Neustadt *et al.*, 2000). Mohapel et al. (2005) also observed that BDNF was able to generate and recruit new neurons into the striatum of 6-OHDA lesioned rats (Mohapel *et al.*, 2005). Endogenous BDNF is produced as a result of cAMP elevation due to adenylate cyclase (AC) activation. The cAMP elevation then activates protein kinase A (PKA), which is known to phosphorylate Ca^{2+} channels, causing an increased Ca^{2+} influx and consequently activating BDNF expression. The activated PKA also phosphorylates cAMP response element-binding protein (CREB) which initiates the expression of BDNF (Bayatti *et al.*, 2005). Whether UCN's neuroprotection is due to the activation of all the cAMP mediated intracellular signalling pathways or a specific one is still not clear, but for the time being my data show that these effects are CRF-R1 mediated.

Some studies have suggested the possibility that UCN is instigating de-novo neurogenesis, presumably from recruited stem cells (Borta *et al.*, 2007). Although it has still not been proven, it is possible that UCN is able to rescue DA neurons that would otherwise have been damaged beyond repair, as well as possibly stabilizing the nigral environment due to its anti-inflammatory properties, as UCN might reduce the massive astrogliosis which arises in the SNc as a result of LPS toxicity (Hirsch *et al.*, 2005), which possibly happens in PD.

In summary, the presented data constitute the first report of a restoration of nigrostriatal damage in two distinct models of PD once the lesion has become

established. It is quite understandable that UCN itself is unlikely to be of therapeutic use due to its physicochemical properties, where UCN is a relatively large molecule with very poor blood brain barrier penetration, so in order to achieve substantial therapeutic relevance, a means by which central CRF-R1 sites can be activated is clearly essential. It is also clear that the global activation of CRF-R1 receptors with an accompanied stimulation of the HPA axis will have potentially unpleasant side effects. Such an action could lead to acute increase in anxiety and, in the long term, depression. Moreover, as UCN itself may be unlikely to be of therapeutic use due to its physicochemical properties, it could be administered in the same manner as GDNF which would probably avert any possible effect on the HPA axis, which would need to be weighed against the severity of PD itself. However, recently, small, lipophillic, CRF-R1 selective antagonists have become available and CRF receptor pharmacology is an expanding field. If CNS permeable CRF-R1 agonists become available they may, therefore, offer new potential therapeutic possibilities in PD treatment. However, HPA axis stimulation would again become a potential issue, which may have to be weighed against the physical severity of PD and the resulting long-term emotional determent caused by the disease itself. To the advantage of UCN however, the observed neuroprotective effects elicited by this peptide in this work were a result of a single reasonably high dose administration rather than repeated smaller doses, this has also been observed with UCN's effect on dendritic growth of cerebellar Perkinje *in situ*, where it was the single high dose of the peptide and not the repeated small doses that resulted in this significant dendritic growth (Swinny *et al.*, 2004).

Comparatively recently it has become apparent that the CNS has some facility for self repair, though not on a scale sufficient to compensate for a disorder as severe as PD. However, stimulation of the CNS to increase its ability for neurogenesis or cytoprotection may offer a significant advance and already neuropeptides such as glial-cell derived neurotrophic factor (GDNF) are being studied as potential treatments in PD (Yasuhara *et al.*, 2007). This self repair process could also in part underlie a possible mechanism for UCN's effects.

Whether UCN possesses the ability to stimulate neurogenesis and self repair is still not known. This possibility, however, warrants further investigation to determine if this is possible and, if so, to what extent.

In this work, UCN was demonstrated to possess neuroprotective and neurorestorative properties which can possibly be employed in the treatment of PD. Whether, ultimately, UCN itself, or a CRF-R1 selective agonist is used for future pre-clinical trials in primate models of PD is still to be determined. Until that becomes possible, one important aspect that merits investigation is the long term effect of UCN; that is, to determine if the observed restorative and protective effects observed in the present work are sustainable.

6.1 Future work

One important aspect that needs to be clarified is whether neurogenesis plays a role in the restorative effects UCN elicits on the nigrostriatal pathway. For this to be achieved, similar experiments as in chapter 4 should be carried out, and initially it would be sufficient to use a single model of PD (e.g. 6-OHDA). In this set of experiments neurogenesis would be investigated via the use of a neurogenesis detector (e.g. Bromodeoxyuridine). The verification of this and its extent would serve as another advantage for UCN in the treatment of PD, and would support further investigation of UCN's potential in primate models of the disease. Also if UCN did prove to be instigating significant neurogenesis, human trials would be possible in end stage PD patients where the destruction of the nigrostriatal pathway is complete.

One other aspect worth investigating is whether the effects observed by UCN are maintained. This would be a very important aspect to evaluate as the value of UCN as a PD treatment would only be attained if the peptide's effect on restoring the nigrostriatal pathway were long lasting. To evaluate this long term experiments would have to be carried out, these would include assessing animals: days, weeks and months after the point of restoration has been reached. If after significant periods of

time UCN's effects were still maintained, this would provide more evidence that not only is this peptide restoring the nigrostriatal pathway, but it also allows for the long-lasting survival of neurons in this pathway.

To confirm the findings in chapter 5, where UCN was found to be exerting its neuroprotective effects through the CRF-R1 receptor, a different approach would be useful, where it would be very interesting to see if UCN still conferred its neuroprotective effects in CRF-R1 Knock out animals that are lesioned with 6-OHDA. When UCN would still confer its neuroprotection this would again reinforce the implication and importance of the CRF-R1 as a possible target for PD treatment.

References

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Appendix

The corticotrophin-releasing factor-like peptide urocortin reverses key deficits in two rodent models of Parkinson's disease

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Abstract

The potential neuroprotective action of the corticotrophin-releasing factor-related peptide urocortin (UCN) was investigated in the rat 6-hydroxydopamine (6-OHDA) and lipopolysaccharide (LPS) paradigms of Parkinson's disease. UCN (20 fmol) was either given at the same time as ($T = 0$) or 7 days after ($T = +7$) intracerebral 6-OHDA or LPS injection. At 14 days after 6-OHDA or LPS injection, circling behaviour was measured following apomorphine challenge. Circling was significantly lower in rats given UCN at either $T = 0$ or $T = +7$ compared with animals given 6-OHDA or LPS and vehicle. Sham-treated rats showed no circling. Consistent with these observations, striatal dopamine concentrations were markedly higher in 6-OHDA/LPS + UCN rats vs. 6-OHDA/LPS + vehicle groups. Additionally, L-dihydroxyphenylalanine production by tyrosine hydroxylase was greatly reduced in the striata of 6-OHDA/LPS + vehicle rats, whereas this was not the case in rats coadministered UCN. Finally, the numbers of tyrosine hydroxylase-positive cells recorded in the substantia nigra of 6-OHDA/LPS + vehicle-treated animals were markedly lower than those of sham-operated or 6-OHDA/LPS + UCN rats. Critically, UCN was effective in reversing lesion-induced deficits when given either at the same time as or 7 days after the neurotoxic insult. To our knowledge, this is the first time that such an effect has been demonstrated *in vivo*. The apparent ability of UCN to arrest the progression of or even reverse nigral lesions once established suggests that pharmacological manipulation of this system could have substantial therapeutic utility.

Introduction

The well-characterized features of Parkinson's disease (PD) are largely the result of a selective degeneration of nigrostriatal neurones, greatly reduced synthetic capacity for dopamine (DA) and a consequent failure to engage striatal DA receptors (Clark & White, 1987). Before the disease presents clinically, death of nigrostriatal neurones occurs 'silently' in the substantia nigra pars compacta (SNc), probably as a result of concurrent apoptotic, excitotoxic and free-radical-mediated events (Vaux & Korsmeyer, 1999). Environmental and toxicological factors, in addition to the expression of candidate 'Parkinsonian' genes, have been proposed in the aetiology of the disease (Vaux & Korsmeyer, 1999; Gandhi & Wood, 2005). Despite four decades of research effort, a therapeutic strategy offering a cure for, or a means of arresting the pathology of, PD remains elusive. Established drug-based therapies are essentially palliative and not effective in all patients. Moreover, the side-effect profiles of most drugs used in PD account for significant morbidity, especially with chronic use, whereas the efficacy of these treatments inevitably diminishes with time as cell loss proceeds (Hurtig, 1997). A need exists therefore for a novel therapeutic approach, which is both affordable and, more importantly,

provides the potential for arresting disease progression as well as ameliorating symptoms. As apoptotic cell death is almost certainly one of the central components in selective nigrostriatal neuronal death (Ochu et al., 1998; Tatton et al., 1998; Schapira, 2001), future therapeutic strategies could involve the targeted use of bio-molecules with 'anti-apoptotic' properties. One such potential group is the urocortin (UCN) family, a relatively recently discovered group of peptides, closely related to corticotrophin-releasing factor (CRF). Thus far, three members have been described (UCN, UCN II and UCN III) (Lewis et al., 2001; Reyes et al., 2001), all with overlapping distribution within the brain and in a number of peripheral tissues (Takahashi et al., 1998; Lewis et al., 2001; Reyes et al., 2001). Although CRF is a key activator of the hypothalamo-pituitary-adrenal stress response, it has been postulated that the UCNs may participate in so-called 'stress coping' mechanisms (Reul & Holsboer, 2002) and cytoprotection. Markers of apoptotic cell death, assays of functional and bio-energetic recovery, and therefore cell survival and tissue integrity are all significantly improved in tissues exposed to specific insults in conjunction with UCN (Brar et al., 2000; Intekhad-Alam et al., 2004). Moreover, UCN exerts a protective effect in tissues that are required to withstand intense physiological stress, even if addition of the peptide is delayed until after cessation of a hypoxic insult (Brar et al., 2000, 2002; Scarabelli et al., 2002). With respect to neuronal cells, UCN appears to protect cultured hippocampal neurones

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(Pederson *et al.*, 2002), cerebellar granule cells (Facci *et al.*, 2003) and GABAergic neurones (Choi *et al.*, 2006). We have extended these studies, using two animal paradigms of Parkinsonism, and have assessed the potential protective action of UCN in the 6-hydroxydopamine- (6-OHDA) (Ungerstedt, 1971) and lipopolysaccharide- (LPS) (Herrera *et al.* 2000; Gao *et al.*, 2003a) treated rat. The latter paradigm for PD is of considerable current interest as neuroinflammation is becoming widely accepted as playing a likely role in the aetiology of PD (Block & Hong, 2005; Block *et al.*, 2007; Whitton, 2007) and UCN has been shown to be anti-inflammatory (Gonzalez-Rey *et al.*, 2006). The investigation involved the measurement of behavioural, neurochemical and histological parameters in sham-operated animals and those receiving 6-OHDA or LPS singly, or in conjunction with UCN. Two distinct animal models were used in order to circumvent the possibility of UCN being uniquely active against a component of one of the paradigms.

Materials and methods

Surgical procedures

Experiments were carried out in accordance with the Animals (Scientific Procedures) Act UK 1986. Male Wistar rats (210–240 g) were group housed and access to food and water was *ad libitum*. Care was taken to minimize animal usage and typically, tissue was used in several different assay paradigms. Prior to surgery, animals to be lesioned with 6-OHDA received pargyline (50 mg/kg, i.p.) and desmethylimipramine (25 mg/kg, i.p.) in order to maximize the selectivity of the toxin for dopaminergic neurones. Animals were anaesthetized with isofluorane (4% for induction, 1.5% for maintenance), secured in a stereotaxic frame (David Kopf, USA) and given injections of 6-OHDA (8 µg/4 µL of saline with 1% ascorbic acid), LPS (2 µg in 2 µL saline) or vehicle (sham injected). The dose of LPS was selected based upon observations that this is sufficient to initiate damage to nigral tyrosine hydroxylase (TH)-positive cells, whilst sparing acetylcholine (ACh) and glutamic acid decarboxylase (GAD) -containing cells (Herrera *et al.*, 2000). 6-OHDA was injected into the right medial forebrain bundle (from bregma in mm: A, −4.3; L, 1.4 and V, 8.2) and LPS was injected into the SNc (from bregma in mm: A, −5.2; L, 2.2 and V, 8.3). Animals cotreated with UCN received injections (20 fmol/2 µL) into the ipsilateral SNc, whereas those not receiving UCN were given vehicle into the ipsilateral SNc. In other groups of rats, UCN was administered into the SNc 7 days after 6-OHDA or LPS treatment, during a second round of surgery. The possible effects of UCN alone in the intact animal were tested by injecting the peptide into the nigra and killing animals 1, 7 or

14 days later by decapitation under isofluorane anaesthesia. Table 1 provides a summary of the different experimental groups and times at which particular procedures were undertaken.

Assessment of nigrostriatal lesion severity following apomorphine challenge

All animals received an apomorphine challenge (0.5 mg/kg s.c.) in order to assess lesion severity as indicated in Table 1. Animals were placed in a circular test arena and, following a short period of acclimatization, injected with the dopaminergic agonist. Contraversive turns were noted 20 min post-injection and recorded over a 120 s observation period. Only complete 'tight' turns were recorded.

Tissue dopamine assay

Animals received pargyline (50 mg/kg) 30 min prior to killing by decapitation under isofluorance anaesthesia. Brains were removed, striata dissected and homogenized in ice-cold phosphate buffer (pH 7.4). All homogenates were split into two equal portions, with one half of each treated with 0.2 M perchloric acid (1 : 10, w/v) containing ascorbic acid (0.2 µM) and EDTA (0.2 µM) to precipitate cell debris. These were then centrifuged at 9000 g for 15 min at 4 °C, supernatant fluids passed through a syringe filter (10 µm pore size) and whole tissue DA levels estimated using high performance liquid chromatography with electrochemical detection (Biggs *et al.*, 1992). Brain blocs containing nigra were rapidly frozen and retained for immunohistochemistry.

Ex-vivo tyrosine hydroxylase assay

Tyrosine hydroxylase activity was measured in the remaining homogenates, using a modification of the method of Naoi *et al.* (1988). Aliquots were incubated with 200 µM L-tyrosine in a total reaction mixture volume of 100 µL. This consisted of the following components: 100 mM sodium acetate/acetic acid buffer (pH 6.0), 2 mM ferrous ammonium sulphate, 1 mM 6MPH4, 10 µg catalase and 1 mM benserazide, an inhibitor of aromatic L-amino acid decarboxylase. 6MPH4 solution was firstly made as 10 mM in 1 M mercaptoethanol. The incubation mixture, except for tyrosine and the pteridin cofactor 6MPH4, was pre-incubated with homogenates at 37 °C for 5 min and the reaction was initiated by addition of the L-tyrosine and 6MPH4. After incubation at 37 °C for 10 min, the reaction was terminated by addition of 100 µL perchloric acid (0.1 M, containing 0.4 mM sodium metabisulphite and 0.1 mM disodium EDTA). The

TABLE 1. Summary of experimental groups and schedule of procedures

Procedure	Experimental groups						
	Sham ($T = 0$ days)	Sham ($T = +7$ days)	Toxin only	UCN only*	Toxin only (killed at +7 days)	Toxin + UCN ($T = 0$ days)	Toxin + UCN ($T = +7$ days)
Surgery no. 1	+	−	+	+	+	+	+
Surgery no. 2	..	−	..	--	−	−	+
Apomorphine, day 1, 7 or 14	14	14	14	1, 7, 14	7	14	14
Killed, day 1, 7 or 14	14	14	14	1, 7, 14	7	14	14
Tissue DA/TH assay	+	+	+	+	+	+	+
IHC	+	+	+	+	+	+	+

Toxin, 6-hydroxydopamine or lipopolysaccharide; DA, dopamine; IHC, immunohistochemistry; TH, tyrosine hydroxylase. *T*, time in days after intracerebral 6-OHDA or LPS injection. *Three groups of urocortin (UCN) only-treated animals.

sample was vortexed and left to stand on ice for 10 min, and then centrifuged at 1000 *g* for 10 min. The supernatant fluid was diluted to 1 in 1000 with mobile phase (Biggs *et al.*, 1992) and then analysed using high performance liquid chromatography-ED (electrochemical detection) to measure the amount of L-dihydroxyphenylalanine. As blank, a similar reaction mixture containing D-tyrosine instead of the L-isomer and 100 μM 3-iodo-L-tyrosine was used (Naoi *et al.*, 1988).

Western blot analysis

Tissue samples (striatum) were homogenized in 1 mL of phosphate buffer containing 10 μL protease inhibitor cocktail. The samples were then centrifuged (1000 *g* for 10 min) and the supernatant fluids were mixed with their same volume of 2 × sodium dodecyl sulphate–polyacrylamide gel electrophoresis sample buffer containing 100 mM dithiothreitol and then the whole mixture was boiled for 5 min. Once cooled and recentrifuged, samples were then loaded (15 μL/lane) and separated on pre-cast polyacrylamide gels (12%; Invitrogen). Precision Plus® protein standard molecular weight markers (Bio-Rad, Hercules, CA, USA) and a TH standard were also loaded to assure complete electrophoretic transfer and to estimate the molecular weight of the bands of interest. The gels were transferred to nitrocellulose membrane (Whatman) for 1.5 h with a constant voltage of 15 V. Membranes were blocked for 30 min at room temperature (21°C) with 5% powdered skimmed milk in phosphate-buffered saline (PBS)-Tween and then probed overnight at room temperature using primary antibodies for total TH (1 : 500 dilution). Membranes were washed with PBS-Tween buffer (3 × 5 min) and then incubated with horseradish peroxidase-conjugated donkey anti-goat IgG (1 : 5000; Santa Cruz). Proteins were visualized using a chemiluminescence ECL kit (Amersham). Representative bands are shown in Figs 1d and 2d.

Immunohistochemistry

Slide-mounted 10 μm cryostat sections from flash-frozen rat brain blocs were removed from the freezer and allowed to equilibrate to room temperature for 30 min, prior to post-fixation in 4% paraformaldehyde containing 1% glutaraldehyde for 5 min at 0 °C. Following rinsing in 0.1 M PBS for 5 min, sections were dehydrated through graded alcohols and endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in methanol for 10 min. The sections were then rehydrated and non-specific immunoreactivity was blocked with 10% swine serum in PBS for 10 min. Sections were then incubated in primary antibody (rabbit anti-rat TH IgG) at 1 : 700 in PBS for 16 h at 4 °C. After rinsing, the sections were incubated sequentially in biotinylated swine anti-rabbit antibody (1 : 250 in PBS) (Dako, Denmark) for 30 min at room temperature and ABC complex following the manufacturer's instructions. Immunoreactivity was visualized through incubation in 0.5 mg/mL 3-diaminobenzidine containing 0.009% H_2O_2 for 2 min at room temperature. The sections were counterstained in Harris haematoxylin, dehydrated, cleared and mounted for microscopic examination. Sections were viewed under light microscopy (× 40 magnification). Digital images were captured using a Leica DC500 system and the manufacturer's software. Representative sections are shown in Figs 3 and 4. For each animal, four successive nigral sections were selected from both the treated and contralateral side, taken from a starting point of −5.5 mm relative to bregma, using a total of six rats per group.

Data handling and analysis

Data obtained from apomorphine challenge, whole tissue DA and TH assay studies were expressed as mean values ± SEM. Data were subjected to one-way ANOVA to identify overall trends, with a post-hoc Bonferroni's multiple comparison test used to establish significant differences between the groups. Statistical analysis was performed

FIG. 1. Effects of urocortin (UCN) administration on various indicators of nigrostriatal pathway function in 6-hydroxydopamine (6-OHDA)-treated rats. (a–c) Key indicates experimental groups in histograms. UCN (20 fmol/2 μL) was administered concomitantly with toxin ($T = 0$) or 7 days post-toxin ($T = +7$). (a) Contraversive turns in response to apomorphine (APO) (0.5 mg/kg). Results of one-way ANOVA: $F = 13.64$. (b) Striatal whole tissue dopamine (DA) levels, estimated using high performance liquid chromatography (HPLC) followed by electrochemical detection. Results of one-way ANOVA: $F = 12.79$. (c) Striatal homogenate tyrosine hydroxylase (TH) activity, assayed by L-dihydroxyphenylalanine (L-DOPA) formation, estimated using HPLC followed by electrochemical detection. Results of one-way ANOVA: $F = 20.90$. (a–c) *$P < 0.01$ with respect to toxin + vehicle. Data are mean ± SEM of six rats per group in all cases. (d) Western blot analysis of TH protein content in striatal homogenates. Representative blots are shown. Lanes: 1, TH standard; 2, sham; 3, toxin + vehicle; 4, toxin + UCN ($T = 0$); 5, toxin + UCN ($T = +7$).
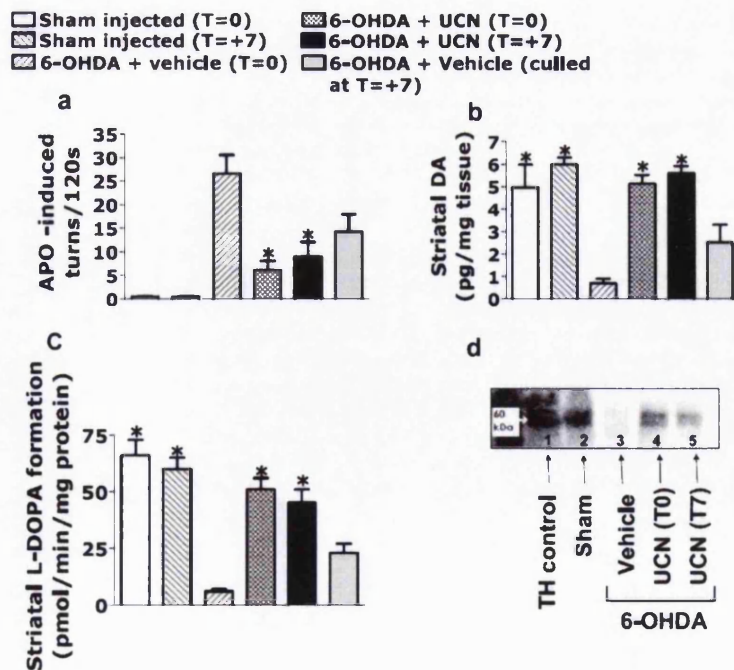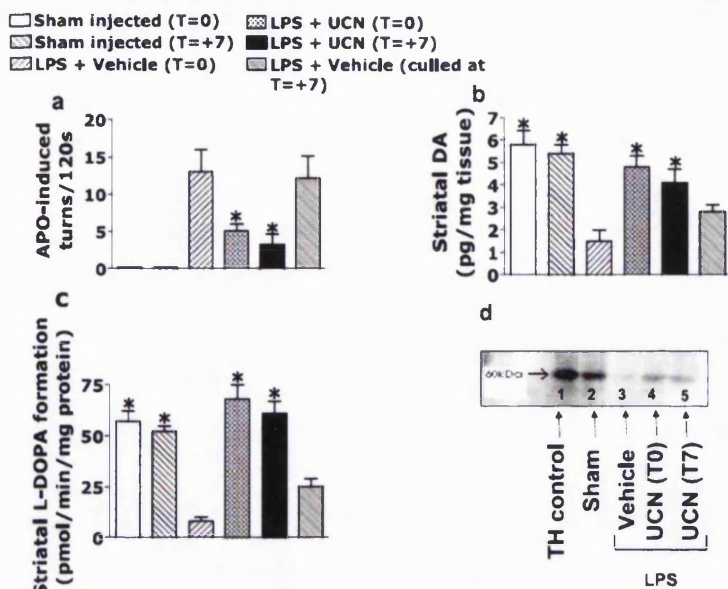
FIG. 2. Effects of urocortin (UCN) administration on various indicators of nigrostriatal pathway function in lipopolysaccharide (LPS)-treated rats. (a–c) Key indicates experimental groups in histograms. UCN (20 fmol/2 μL) was administered concomitantly with toxin ($T = 0$) or 7 days post-toxin ($T = +7$). (a) Contraversive turns in response to apomorphine (APO) (0.5 mg/kg). Results of one-way ANOVA: $F = 8.434$. (b) Striatal whole tissue dopamine (DA) levels, estimated using high performance liquid chromatography (HPLC) followed by electrochemical detection. Results of one-way ANOVA: $F = 11.14$. (c) Striatal homogenate tyrosine hydroxylase (TH) activity, assayed by L-dihydroxyphenylalanine (L-DOPA) formation, estimated using HPLC followed by electrochemical detection. Results of one-way ANOVA: $F = 22.75$. (a–c) *$P < 0.01$ with respect to toxin + vehicle. Data are mean ± SEM of six rats per group in all cases. (d) Western blot analysis of TH protein content in striatal homogenates. Representative blots are shown. Lanes: 1, TH standard; 2, sham; 3, LPS + vehicle; 4, LPS + UCN ($T = 0$); 5, LPS + UCN ($T = +7$).

using a proprietary software package (GraphPad PRISM™). The numbers of animals used in experiments are detailed in the figure legends. In all cases, comparisons were made with respect to toxin/vehicle values. Statistical significance was set at $P < 0.05$.

Materials

Desmethylimipramine, rat UCN, LPS, pargyline, catalase, L-tyrosine, ferrous ammonium sulphate, benserazide, 6MPH4, 6-OHDA, protease inhibitor cocktail, apomorphine hydrochloride, 3-iodo-L-tyrosine, D-tyrosine and TH were all obtained from Sigma (UK). Apomorphine and 6-OHDA were dissolved in 2.0% w/v ascorbic acid, whereas UCN solid was initially dissolved in 70% ethanol and further diluted in saline to 1×10^{-8} M stock concentration. All drugs, apart from UCN, LPS and 6-OHDA, were injected in a volume of 0.1 mL/100 g body weight. Rabbit polyclonal anti-rat TH IgG was obtained from Calbiochem. Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Cell Signalling (MA, USA). Biotinylated swine anti-rabbit IgG was obtained from Dako (Denmark). ABC complex was purchased from Vector Laboratories (UK). All other reagents were of Analar or high performance liquid chromatography grade.

Results

Apomorphine-induced turning

Apomorphine-induced circling is regarded as a quantitative index of nigro-striatal (NS) lesion severity (Ungerstedt, 1971) and thus, an attenuation is predictive of potential anti-parkinsonian activity. Our findings reveal that tight contralateral circling was clearly evident in 6-OHDA- and LPS-treated rats but this was greatly attenuated when UCN was injected concomitantly into the ipsilateral SNc and also 7 days after either 6-OHDA or LPS (Figs 1a and 2a). Note that lesions were already well advanced at 7 days post-injection (Figs 1a and 2a for contralateral circling; Figs 3f and 4f for histology). Whole tissue DA levels estimated in the ipsilateral striata of toxin/vehicle-treated animals disclosed dramatic depletion when compared with sham-operated animals, whereas levels estimated in toxin/UCN-treated

animals were similar to those of shams (Figs 1b and 2b) and this was irrespective of whether UCN was administered at $T = 0$ or $T = +7$ days. In all cases, sham-treated animals ($T = 0$) and sham-treated animals given a second round of surgery ($T = +7$) were nearly identical with respect to circling behaviour, striatal DA concentrations and TH activity (Figs 1 and 2).

Effect of urocortin on striatal tyrosine hydroxylase activity and protein level

In order to estimate the functional integrity of nigral neurones ipsilateral to injection sites, we measured the L-dihydroxyphenylalanine synthetic capacity of striatal homogenates. *Ex-vivo* TH activities were greatly reduced in striata of 6-OHDA- and LPS-treated rats when compared with sham-treated rats and in stark contrast to those treated with UCN at both the $T = 0$ and $T = +7$ time points, where TH activities were near normal (Figs 1c and 2c). We established that TH activities in homogenates correlated closely with the relative intensity of immunostaining for the enzyme; western blots revealed a distinct loss of TH immunoreactivity in homogenates after 6-OHDA or LPS only but this was much less apparent in any of the UCN-treated groups, where band densities were more similar to those of shams (see Figs 1d and 2d for representative blots).

Effect of urocortin on survival of tyrosine hydroxylase-containing nigral cells

Having established that intracerebral administration of UCN prevents the loss of NS function post-toxin in both rodent models, we needed to ascertain whether this could be explained by preservation of discrete nigral cells or alternatively achieved through a substantial up-regulation of TH protein in surviving cells. In order to test these possibilities, we stained cryopreserved brain sections for TH immunoreactivity and counted nigral cell bodies in the substantia nigra. As shown in Figs 3 and 4, 6-OHDA or LPS treatment resulted in a near-complete loss of TH-positive cell bodies, with very few accompanying dendrites remaining (Figs 3b and 4b). Rats
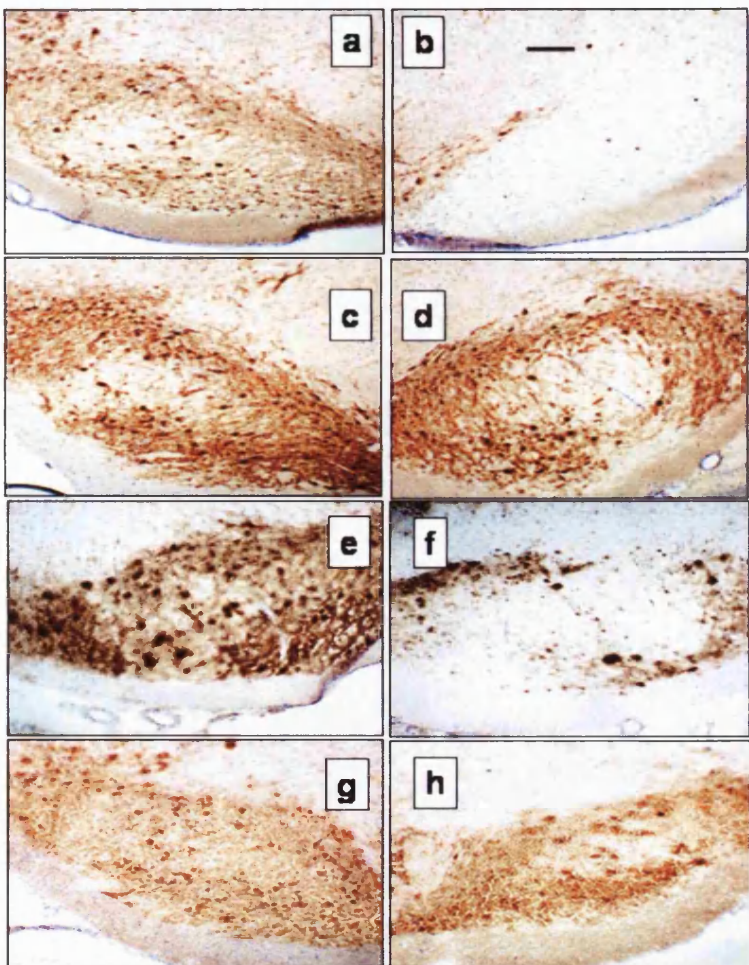
FIG. 3. Photomicrographs of selected rat nigral sections, immunostained for tyrosine hydroxylase. Nigrostriatal cell bodies and dendrites appear dark brown/brown in sections. (a, c, e and g) Contralateral (untreated) nigra for comparison with ipsilateral (treated) nigra (b, d, f and h). (b) 6-Hydroxydopamine (6-OHDA) + vehicle; (d) 6-OHDA + urocortin (UCN) ($T = +7$); (f) 6-OHDA + vehicle (killed at $T = +7$); (h) 6-OHDA + UCN ($T = 0$). Bar, 100 μm.
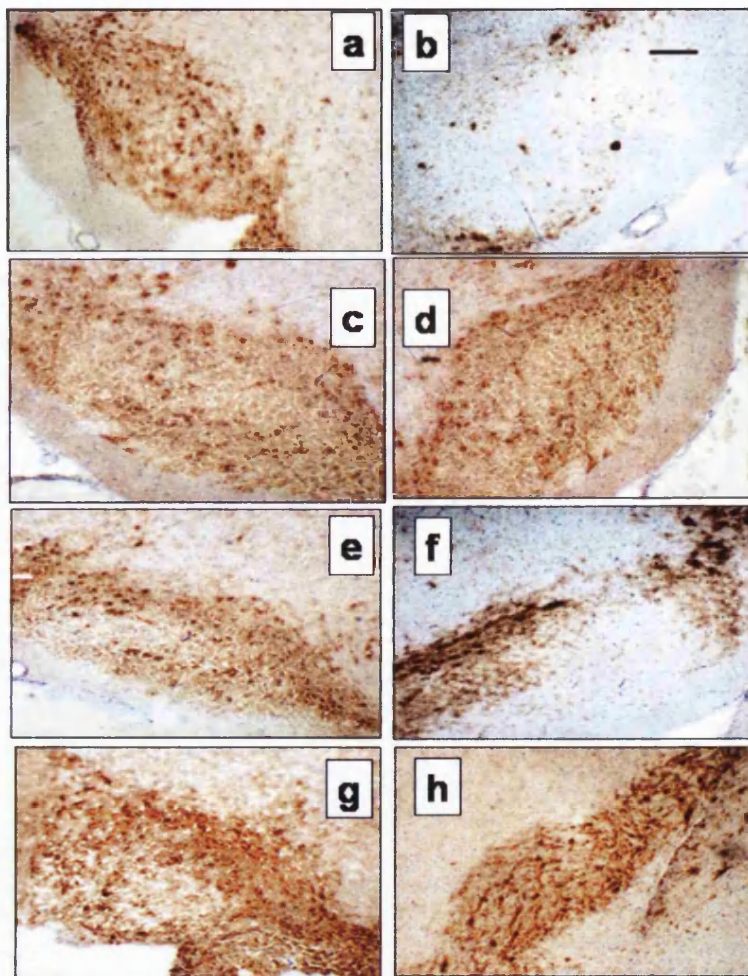
FIG. 4. Photomicrographs of selected rat nigral sections, immunostained for tyrosine hydroxylase. Nigrostriatal cell bodies and dendrites appear dark brown/brown in sections. (a, c, e and g) Contralateral (untreated) nigra for comparison with ipsilateral (treated) nigra (b, d, f and h). (b) Lipopolysaccharide (LPS) + vehicle; (d) LPS + urocortin (UCN) ($T = +7$); (f) LPS + vehicle (killed at $T = +7$); (h) LPS + UCN ($T = 0$). Bar, 100 μm.

similarly treated but killed at +7 days disclosed a more modest loss of both NS cell bodies compared with 14 day exposure (Figs 3f and 4f). As can be clearly seen in Figs 3d or h and 4d or h, UCN almost completely protected against loss of TH-positive cells when administered at the same time as or 7 days following 6-OHDA or LPS injection.

Discussion

In the present study we have used a variety of measures, behavioural, neurochemical and histological, that indicate a clear protective role for UCN against 6-OHDA- or LPS-mediated nigrostriatal lesions. These findings support a potential protective role for UCN in these two paradigms of PD and clearly demonstrate that, when UCN is given concomitantly with either 6-OHDA or LPS, a comprehensive reversal of all selected markers of nigrostriatal cellular loss results. Importantly, the effects of UCN were not restricted to when it was coadministered with 6-OHDA or LPS but were also manifest when given 7 days after toxin injection when neurotoxic damage is already becoming well established (present data; He *et al.*, 2000). This suggests that UCN is able to rescue dopaminergic neurones once damage is established. Taken as a whole, our data reveal that UCN can act as a potent neuroprotective agent in two distinct rodent models of PD and at a dose that was found to be effective in a variety of other studies (Brar *et al.*, 2000, 2002; Facci *et al.*, 2003; Choi *et al.*, 2006). Moreover, we have assessed multiple parameters of NS pathway activity and nigral neuronal integrity. This approach has important strengths over 'single-parameter' studies, given that human PD pathology is complex and multicomponent (Tatton *et al.*, 1998; Blum *et al.*, 2001; Gandhi & Wood, 2005). Of particular significance is our finding that UCN is able to arrest and possibly reverse NS lesions once the neurodegenerative process has commenced. This principle (if reproduced in the human brain) would have obvious clinical significance as patients generally present with symptoms only once nigral neuronal loss reaches 70–80% of the total nigral complement and striatal DA levels have fallen significantly (Abercrombie *et al.*, 1990). Apart from an apparent facility for preservation of cells, it is possible that UCN may act to stimulate dopaminergic neurones via several distinct mechanisms. Recent studies have shown that UCN potentiates the release of [^{3}H]DA from electrically stimulated rat striatal slices (Bagosi *et al.*, 2006) and increases the TH mRNA levels expressed by PC12 cells (Nanmoku *et al.*, 2005), a cell type phenotypically related to midbrain dopaminergic neurones. We investigated the possible effects of UCN on TH in intact unlesioned rats by injecting the peptide alone into the SNc. Rats studied 1, 7 and 14 days later showed no significant effect on tissue DA, TH activity or protein levels (data not shown). In the present study, UCN appears to 'reverse' the loss of TH-positive cell bodies, as sections taken from rats killed 7 days post-6-OHDA or post-LPS clearly show fewer TH-positive cells than those treated with UCN at the 7-day time point (Figs 3d and 4d *cf.* 3f and 4f).

The underlying cellular mechanism responsible for this highly significant finding is, as yet, unclear although it is possible that UCN increases expression of TH protein in PC12 cells (Nanmoku *et al.*, 2005). No such effect was observed in the present study *in vivo*. It is possible that UCN effects a regrowth and remodeling of surviving dendrites as observed by Swinny *et al.* (2004) using cerebellar Purkinje cells *in vitro*. Interestingly, Scarabelli *et al.* (2002) also reported an apparent restorative action of UCN, albeit observed in cardiac myocytes. In addition, it is probable that UCN achieves a neuroprotective effect partly via prevention of apoptotic cell death

(Scarabelli *et al.*, 2002; Intekhad-Alam *et al.*, 2004). Our own studies have shown that the peptide significantly reduces the number of end-stage apoptotic nuclei observed in nigral sections following intracerebral 6-OHDA injection (Biggs *et al.*, 2006), a significant finding as apoptosis is an important mechanism in 6-OHDA neurotoxicity (Cutillas *et al.*, 1999) and human PD (Tatton *et al.*, 1998; Mochizuki *et al.*, 1996). Our use of the single-dose LPS PD model (Herrera *et al.*, 2000) increases the significance of our data, given that recent evidence supports a neuroinflammatory component in the aetiology of human PD (Gao *et al.*, 2003b; Block *et al.*, 2007; Whitton, 2007) and that UCN itself possesses anti-inflammatory properties (Gonzalez-Rey *et al.*, 2006). It is possible that UCN is instigating *de-novo* neurogenesis, presumably from recruited stem cells, as has been postulated in other studies (Borta & Hoglinger, 2007). The determination of this is, however, beyond the scope of the present study. Although it is yet to be proven, we would be inclined to the idea that the peptide is able to rescue DA neurones that would otherwise have been damaged beyond repair as well as possibly stabilizing the nigral environment due to its anti-inflammatory properties. The precise neuroanatomical site at which UCN acts is, as yet, unclear although a local action at the level of nigral cell bodies and dendrites seems likely, given the presence of CRF$_1$ immuno-reactivity here (Sauvage & Steckler, 2001) and widespread distribution of UCN in the brain (Yamamoto, 1998; Vasconcelos *et al.*, 2003). Studies utilizing cerebellar granule cells and hippocampal neurones (Pederson *et al.*, 2002) also suggest that the engagement of CRF$_1$ is necessary for neuroprotection. It has recently been shown that UCN II, which has a high selectivity for the CRF$_2$ receptor subtype, is able to block voltage-gated calcium channels in PC12 cells (Tao *et al.*, 2006). These authors have proposed that this could be the basis for neuroprotection. Whether this applies to UCN and could be shown *in vivo* remains to be seen.

Overall, our results add significantly to a growing knowledge base, whereby UCN mediates cytoprotection in cardiac tissues (Brar *et al.*, 2000, 2002; Scarabelli *et al.*, 2002) and cultured neurones (Pederson *et al.*, 2002; Facci *et al.*, 2003; Choi *et al.*, 2006), with the crucial refinement that we have demonstrated efficacy in two distinct *in-vivo* models of a currently incurable neuropathology. The apparent ability of UCN to arrest NS damage and possibly stimulate the remaining cells suggests a novel mechanism that, if translated therapeutically, would offer a significant advance in PD treatment. This principle has recently been proposed as an essential prerequisite for the basis of a meaningful advance in PD therapy (Meissner *et al.*, 2004). The rapidly expanding interest in UCN itself and central CRF receptor pharma-cology heralds the development of novel small molecule agonists with selectivity for CRF$_1$ and CRF$_2$ subtypes, some of which will probably be suitable for evaluation in pre-clinical and clinical settings. We believe that our studies are an important first step towards validating UCN and central CRF receptors as potential future targets in the treatment of human PD.

Acknowledgements

Abbreviations

CRF, corticotrophin-releasing factor; DA, dopamine; LPS, lipopolysaccharide; NS, nigrostriatal; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; PD, Parkinson's disease; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; UCN, urocortin.

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Short report

Urocortin, a CRF-like peptide, restores key indicators of damage in the substantia nigra in a neuroinflammatory model of Parkinson's disease

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Abstract

We have recently observed that the corticotrophin releasing hormone (CRF) related peptide urocortin (UCN) reverses key features of nigrostriatal damage in the hemiparkinsonian 6-hydroxydopamine lesioned rat. Here we have studied whether similar effects are also evident in the lipopolysaccaride (LPS) neuroinflammatory paradigm of Parkinson's disease (PD). To do this we have measured restoration of normal motor behaviour, retention of nigral dopamine (DA) and also tyrosine hydroxylase (TH) activity. Fourteen days following intranigral injections of LPS and UCN, rats showed only modest circling after DA receptor stimulation with apomorphine, in contrast to those given LPS and vehicle where circling was pronounced. In separate experiments, rats received UCN seven days *following* LPS, and here apomorphine challenge caused near identical circling intensity to those that received LPS and UCN concomitantly. In a similar and consistent manner with the preservation of motor function, UCN 'protected' the nigra from both DA depletion and loss of TH activity, indicating preservation of DA cells. The effects of UCN were antagonised by the non-selective CRF receptor antagonist α-helical CRF and were not replicated by the selective CRF_2 ligand UCN III. This suggests that UCN is acting via CRF_1 receptors, which have been shown to be anti-inflammatory in the periphery. Our data therefore indicate that UCN is capable of maintaining adequate nigrostriatal function *in vivo*, via CRF_1 receptors following a neuroinflammatory challenge. This has potential therapeutic implications in PD.

Findings

Parkinson's disease (PD) is largely the result of a degeneration of nigrostriatal neurons. Before the disease presents clinically, death of dopamine (DA) neurons occurs in the substantia nigra pars compacta (SNc) asymptomatically. This has traditionally been ascribed to concurrent apop-

totic, excitotoxic and free-radical mediated events [1,2]. Recent evidence suggests that both pre- and postnatal neuroinflammation may play a crucial predisposing or causative role in the aetiology of PD [3,4]. Prevention of nigrostriatal neuronal destruction once established, or prior to lesion development, represents an ideal future

therapeutic goal in PD. Urocortin (UCN), a cortico-trophin releasing hormone (CRF) related peptide has recently been proposed as a cytoprotectant. Evidence for this exists in a range of tissues including neuronal cells [5,6]. Interestingly UCN, acting via CRF₁ receptors, is anti-inflammatory in the periphery [7]. We have recently observed that UCN arrests the development of Parkinso-nian like features in the 6-hyroxydopamine lesioned hemiparkinsonian rat [8]. UCN substantially reverses apomorphine-induced circling, loss of tissue DA, loss of nigral and striatal tyrosine hydroxylase (TH) activity and loss of TH protein levels [8]. Although the 6-OHDA model of PD is well established it has significant physio-logical limitations. In contrast, lipopolysaccaride (LPS) is an established product of bacterial infection, including relatively common conditions such as bacterial vaginitis. Significantly, evidence suggests that systemic inflamma-tion can predispose or be causative in the genesis of PD [3,4]. Therefore, exposure to conditions leading to neu-roinflammation, a condition to which the SNc in particu-larly susceptible, constitutes a realistic mechanism by which the disease may be initiated. Here we have investi-gated the potential protective effects of UCN in the LPS paradigm of PD. Additionally, we have studied the effects of the non-selective CRF receptor antagonist α-helical CRF and also urocortin III (UCN III), a selective CRF₂ agonist [9], on indices of nigral DA neuronal integrity to deter-mine whether effects of UCN are receptor mediated and the likely subtype.

UCN, UCN III, LPS, α-helical CRF and apomorphine were all obtained from Sigma, UK. The latter agent was dis-solved in 0.2% w/v ascorbic acid, whilst LPS, α-helical CRF, UCN and UCN III were initially dissolved in water and further diluted in saline. The concentration of UCN chosen is identical to that used in previous investigations [8,10,11]. Apomorphine was injected in a volume of 0.1 ml per 100 g bodyweight. Experiments were performed in accordance with the Animals (Scientific Procedures) Act, UK (1986). Male Wistar rats (Charles River, UK; 210–240 g) were group housed with food and water *ad libitum*. Ani-mals were anaesthetised, secured in a stereotaxic frame and given injections of LPS (2 µg/2 µl) into SNc (from bregma in mm; A -5.2, L 2.2 and V 8.3). Animals co-treated with UCN, UCN III or α-helical CRF received injections (20 fmols/2 µl) directly into the ipsilateral SNc whereas those not receiving UCN were given vehicle. UCN itself was administered either concomitantly with LPS or 7 *days later* (i.e. once lesions were clearly evident; Fig. 1). All intracerebral injections were performed using a stereotaxic frame mounted microsyringe (Hamilton, US) over approximately four minutes (0.5 µl/min.) and the needle left in place for five min post-injection. Fourteen days after toxin administration (seven days in certain experiments, Fig. 1) rats were given apomorphine (0.5

mg/kg, s.c.) and rotations measured 30 min later for 2 min in a circular 'arena', approximately 1 metre in diame-ter, to estimate lesion severity. Rats were then lightly anaesthetised, brains removed and their substantia nigra dissected on ice. DA was estimated as previously described [12] and TH activity estimated as outlined previously [8]. Data were subjected to one way ANOVA with a *post hoc* Dunnett's test.

Rats treated with LPS and vehicle displayed characteristic intense, 'tight' contraversive circling, following apomor-phine (Fig 1) while those co-treated with intracerebral UCN displayed much reduced apomorphine sensitivity. UCN III did not attenuate apomorphine-induced circling in lesioned rats (Fig 1). LPS treatment produced drastic decreases in nigral DA which were substantially attenu-ated when UCN was injected at the same time as the LPS (Fig. 1) and critically, when UCN was given seven days *fol-lowing* LPS injection. A similar pattern of reduction was seen in nigral TH activity following LPS, and this was also reversed by UCN either given at the same time and also seven days post LPS injection (Fig. 1). In none of the experiments did UCN III effect any decrease in LPS-induced loss of tissue DA or TH activity. In order to estab-lish whether these apparent reductions in 'Parkinsonian-like' pathology were mediated by CRF receptors we co-administered LPS and UCN with the non-selective CRF receptor antagonist, α-helical CRF. α-helical CRF reversed the 'protective' effects of UCN against LPS-induced loss of DA, TH activity as well as apomorphine-induced circling (Fig. 1). This clearly indicates that CRF receptors mediate the actions of UCN. However, the CRF₂ selective analogue of UCN, UCN III was without effect. This logically indi-cates that the protective actions of UCN are mediated by CRF₁ receptors.

The current findings demonstrate that UCN greatly atten-uates the development of PD-like pathology in a recently proposed [3,4] paradigm of the illness (LPS). The validity of this model is being increasingly appreciated as the role of neuroinflammation as a factor in the aetiology of PD is gaining substantive support in patients and animal-mod-els [3,4,13]. The ability to restore these indices of PD-like damage in dopaminergic nigral neurons seven days after administration of LPS is particularly significant. We have determined that at this time point in our models the lesion has become established but is evidently still unsta-ble since degeneration continues to proceed for a least a further seven days [8,14], Fig. 1. This is reasonably analo-gous to the predicament of PD patients, where degenera-tion proceeds until the nigrostriatal system is to all intents destroyed as the illness reaches its terminal phase. Current treatments are of limited, and purely symptomatic value, becoming ineffective as the neurodegeneration proceeds. What is clearly required is some treatment strategy which
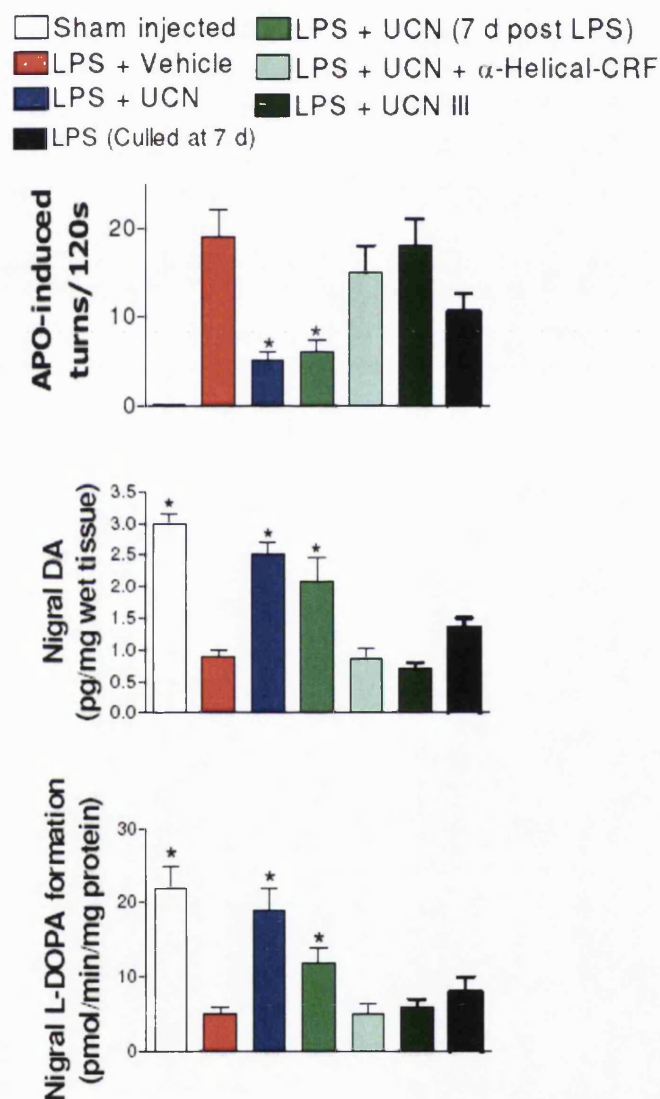
Figure I
Effect of the CRF-like peptide UCN on indices of nigrostriatal damage induced by intranigral injection of LPS. Data were taken from rats 14 days after injection of LPS and UCN except where indicated (7 d post LPS) in which case UCN was given seven days *after* LPS injection. In some experiments rats were culled seven days after administration of LPS alone to indicate the development of lesion severity at this point. Indices assessed were as follows: upper panel, circling behaviour in response to the DA agonist apomorphine (one way ANOVA $F = 7.763$, $p < 0.001$); middle panel, nigral tissue DA concentration ($F = 22.77$, $p < 0.001$), lower panel, nigral tissue TH activity ($F = 11.47$, $p < 0.001$). **Each group comprised 6–8 rats.** In each case differences between groups were assessed using Bonferonni's multiple comparison test. *$p < 0.05$ versus groups treated with either LPS and vehicle or LPS and UCN III.

either stabilizes the hostile conditions prevailing within the SNc, or better, effects some degree of neuronal restoration. Our data suggest that UCN may be able to achieve this, probably acting via CRF$_1$ sites. The observations with UCN suggest that under neuroinflammatory conditions it is able to elicit a functional recovery in nigrostriatal neurotransmission. We have previously found UCN I to be effective in restoring both striatal TH activity and DA content following either LPS or 6-OHDA-induced lesions [8,14]. Futhermore, we have found that UCN I also reverses loss of extracellular DA in the striatum of freely moving rats (unpublished data). The resulting recovery in nigral DA neurons presumably allows for a restoration of D$_1$ and D$_2$ receptor balance in the striatum which would logically underlie the recovery in 'normal' motor activity (loss of circling) seen after apomorphine treatment. However, a determination of actual DA receptor population would be required to prove this.

Evidence has shown that UCN protects some neurons via activation of the CRF$_1$ subtype [5,6], whilst activation of CRF$_2$ sites has been cited as important [10,15]. The possibility that UCN can 'rescue' damaged cells has been postulated in some studies, especially cardiac myocytes [10] and the heart ex vivo [11,16]. We are unclear as to the precise mechanism by which UCN I exerts its protective effect. Our unpublished data indicates that UCN I treatment leads to a preservation or restoration of TH+cells in the SNc. Whether this is the result of cytoprotection, such as might occur due to an anti-inflammatory action, or a stimulation of neurogenesis remains to be determined. One possibility could be that UCN I might reduce the massive astrogliosis which arises in the SNc as a result of LPS toxicity [17]. Additionally, the potential contribution of the SNc relative to the ventral tegmental area in restoring nigrostriatal function is also unclear, although we intend to investigate this.

In summary, our data constitutes the first report of a restoration of key indicators of nigrostriatal damage in a neuroinflammatory model of PD *after* the lesion has become established by a molecule known to have antiinflammatory properties [7]. Although activation of the HPA axis by a CRF agonist might have potentially deleterious side effects, evidence suggests that these may be averted. Thus, CRF and UCN both reduced weight gain in rodents but CRF was much more effective than UCN in this respect and only CRF produced effects consistent with increased sympathetic activity[18]. In order to achieve substantial therapeutic relevance a means by which central CRF$_1$ sites can be activated is clearly essential as may be refinement to ensure an appropriate pharmacological response. While UCN is a relatively large molecule with poor blood brain barrier penetration, recently small, lippophillic, CRF$_1$ selective antagonists have become available and CRF

receptor pharmacology is a rapidly expanding field. As such we consider it highly likely that CRF$_1$ agonists will become available offering new possibilities in the study of UCN mediated neuroprotection as well as being of potential therapeutic value in PD.

Abbreviations

Dopamine (DA), corticotrophin releasing factor (CRF), hypothalamopituitary-adrenal (HPA), lipopolysaccaride (LPS), Parkinson's disease (PD), tyrosine hydroxylase (TH), urocortin (UCN).

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

AA, AH, CSB and PSW were responsible for the planning and actual experimentation involved in this study. RL contributed to the interpretation of the data and writing of the manuscript.

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