The effect of L-dopa and neuroprotective agents on cell replacement therapy for Parkinson's disease.

This thesis is submitted for the degree of Doctor of Philosophy at Cardiff University

by

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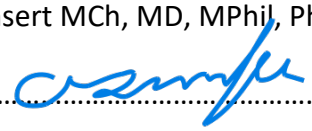
Signed ……  ……(candidate) Date ……23/08/2017……

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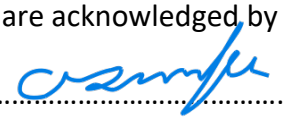
Signed ……  ……(candidate) Date ……23/08/2017……

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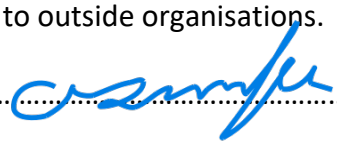
Signed ……  ……(candidate) Date ……23/08/2017……

SUMMARY

Parkinson disease PD is the second most common neurodegenerative disease affecting 1.8% of population aged over 65 years. The current medications that control the symptoms of the disease are associated with limited efficacy and induction of side effects (dyskinesia) at later stages of the disease. One promising future therapy in PD is cell replacement therapy, however clinical trials declared inconsistent outcomes and developing dyskinesia related to the graft. Studies later suggested suboptimal conditions contributed on these outcomes. This thesis builds on this knowledge endeavouring to support cell transplantian therapy in Parkinson disease in models that are more closely aligned to the clinic thorough considering anti-parkinsonian medications in the model. It is addressed the low survival and efficacy problem of the transplanted cells examining neuroprotective agents that have previously shown the ability to protect nigrostriatal dopaminergic neurons against toxic challenges. In addition, this thesis characterises stem cell transplantation, the potential cell source for transplantation that can overcome the many practical and ethical issues surrounding foetal tissue.

In the first part of the thesis, the investigations on finding neuroprotective agents to support graft survival and efficacy was achieved in the unilateral 6-OHDA lesioned rat model, treated with chronic L-dopa, (the gold standard anti- Parkinson medication), prior to, and following, cell transplantation. The results revealed for the first time that Glucagon Like Peptide-1 (GLP-1) receptor agonists (exendin-4 and liraglutide) were capable of improving graft size and the motor and behaviour recovery results from peripheral administration. Importantly, this protection was affected by the presence or absence of L-dopa treatment, as exendin-4 supported the graft only in absence of L-dopa while liraglutide supported the graft only in the presence of L-dopa. While other neuroprotective agents (ghrelin and ghrelin receptor agonist) failed to support graft survival or efficacy in the same animal model. In the second part of the thesis, the characterisation of different source of stem cells derived dopaminergic neurons revealed for the first time that these cells can survive and function in the striatum of 6-OHDA rat model primed with chronic L-dopa treatment and exposed to L-dopa treatment for 16 weeks after transplantation. I show, for the first time, that these cells are capable of ameliorating L-dopa induced dyskinesia.

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ABBREVIATIONS

5-HT	5-hydorxytyptamine or serotonin
6-OHDA	6-hydroxydopamine
AACD	aromatic L-amino acid decarboxylase
AIM	abnormal involuntary movement
AP	anterior posterior
BBB	blood brain barrier
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
COMT	catechol-O-methyl transferase
CRL	crown rump length
DA	dopamine
DA	dopaminergic neurons
DAB	3,3'-Diaminobenzidine
DAT	dopamine transporter
DBS	deep brain stimulation
DCX	doublecortin
DMEM	Dulbecco's Modified Eagle's medium
DV axis	dorsal ventral
ER	endoplasmic reticulum
FACS	fluorescence-activated cell soring
FGF	fibroblast growth factor
F-IHC	fluorescence - immunohistochemistry
GABA	λ amino butyric acid
GCL	granular cell layer
GDNF	glial cell line-derived neurotrophic factor
GHSR	ghrelin receptor or growth hormone secergogus receptor
GID	graft induced dyskinesia
GLP-1	glucagon like peptide-1
GMP	good manufacturing practice
GOAT	ghrelin O acyl transferase
GPCR	G- protein coupled receptors
Gpe	Globus pallidus external
Gpi	Globus pallidus internal
hESC	human embryonic stem cell
hESC-DA	human embryonic stem cell-derived dopaminergic neurons
i.p.	intraperitoneal
ICC	immunocytochemistry
IHC	immunohistochemistry
IL	interleukin
INF-λ	interferon-gamma
iNS	induced neural stem cells
iPSC	induced pluripotent stem cells

LD	L-dopa
L-dopa	L-3,4-hydroxyphenlalanine
LID	L-dopa induced dyskinesia
LRRK2	leucine-rich repeat kinase2
MAO-B	mono amino oxidase-B
MDS-UPDRS	Movement Disorders Society Unified Parkinson's Disease Rating Scale
MFB	medial forebrain bundle
ML	medial lateral
MPTP	1-methyl-1,4-phenyl-1,2,3,6-tetrahydropyridine
NFM	Non-fat milk
NGF	neural growth factor
n.s.	Not significant
NSC	neuronal stem cells
PBS	phosphate buffered solutions
PD	Parkinson disease
PET	positron emission tomography
PINK 1	PTEN-induced putative kinase 1
PLL	poly-L-lysine
ROS	reactive oxygen species
s.c.	subcutaneous
SEM	standard error of the mean
SGZ	Sub granular zone
SHH	sonic hedgehog
SN	substantia nigra
SNpc	substantia nigra pars compacta
SNr	substantial nigra reticulate
SOD	superoxide dismutase
SPECT	single photon emission computered tomography
STN	subthalamic nucleus
T2DM	Type 2 diabetes mellitus
Th1,2	T helper lymphocyte phenotype 1, 2
TNF	tumour necrosis factor
UPDRS	unified Parkinson's disease rating scale
UPR	unfold protein response
VM	ventral mesencephalon

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1 Chapter 1: General Introduction

1.1 Parkinson Disease

Parkinson disease (PD) is a progressive neurodegenerative disease causing a movement disorder, described for the first time by James Parkinson in 1817 (Parkinson 1817). It mostly affects people in later life with an average age of onset of 61±10 years (Marras et al. 2005). The prevalence of the disease is about 1.8% of the population aged over 65; with the rate increasing from 0.6% at ages 65-69 years to 2.6% at ages 85-89 years (de Rijk et al. 2000). It is reported that its incidence in the western world is about 1:10,000 of population per a year (Foltynie et al. 2004).

1.2 Diagnosis

Despite it being two centuries since the first description of PD, it's clinical diagnosis is still dependent upon physical examination and medical history. To diagnose PD, at least two of its four major cardinal features should be established**.** Firstly, resting tremor, which is a highly typical feature of PD, present at the resting state and ceasing during voluntary movement**.** This is usually recognised unilaterally in a hand, jaw and sometimes in the leg (Sonsalla, 1997). The another feature is bradykinesia which refers to extreme slowness of movement and is considered a highly disabling symptom because it affects all motor systems leading to difficulties in facial expression, gesturing, swallowing and blinking; in addition to reduced or absence of arm swing during walking (Jankovic 2008; Sonsalla 1997)**.** The progression of the bradykinesia may lead to cessation of some actions like finger tapping, or cause intermediate immobility (freezing) which specifically occurs in crowded places or narrow doorways (Giladi et al. 1992). The third cardinal feature is rigidity which is an increase in muscle resistance causing a jerky movement described as being like a cogwheel movement (Sonsalla 1997)**.** The last cardinal feature is postural instability which is considered as one of the most severely disabling symptoms in PD because it directly contributes to falls (Nelson et al. 2005)**.** Although PD is predominantly described as a motor disorder, a number of non-motor symptoms can also be detected throughout the progression of the disease including deficits in cognition, mood, autonomic function and sleep (Garcia-Ruiz et al. 2014)**.** This wide spectrum of symptoms between motor and non-motor signs indicates the pathologic complexity of PD which may include more than one region of the CNS or even PNS, despite its main pathologic feature involving dopaminergic neuron degeneration in the basal ganglia.

1.3 Pathophysiology:

The main pathologic feature of PD is the loss of dopamine neurons (DA) of the substantia nigra pars compacta (SNpc). The neurological deficit in PD starts after the loss of around 70-80% of nigral dopaminergic neuron (DA) projections that supply the caudate putamen (Bernheimer et al. 1973)**.** This high threshold level is attributed to the compensatory mechanisms which involve an increased synthesis and release of dopamine by the remaining neurons, in addition to a decrease in DA turnover (Zigmond et al. 1990). The reduction in DA neurons causes a disturbance in the basal ganglia circuitry, consequently leads to decreased activation of the frontal cortex and the existence of the motor symptoms. DA neurons affect this circuit through two pathways. Activation of D1 receptors in the direct output pathway from the putamen leads to release of the inhibitory neurotransmitter GABA and its cofactor substance P to the globus palludius interna (GPi) and substantia nigra pars reticula (SNr). While, the indirect pathway is maintained through the interaction of dopamine with D2 receptor in the caudate putamen. Stimulation of the D2 receptor causes suppression of the release of the GABA and enkephalin, inhibitory neurotransmitters, to the globus pallidus externa (GPe) which cause activation to release of an inhibitory impulse to the Subthalamic nucleus (STN), which in turn causes a decreased level of the excitatory impulses to the GPi. So, the later receives a balanced level of inhibitory signal, from the direct pathway, and excitatory impulses, from the indirect pathway, which determines its transmission of the GABAergic efferent neurons to the ventro-lateral and ventro-anterior thalamus which is sending a a glutamatergic output to the frontal cortex (Alexander GE1 1990; Albin, R. L., Young, A. B., and Penney 1989; DeLong 1990)

In the case of PD, the level of dopamine and the stimulation of D1 and D2 receptors in the putamen is reduced. Consequently, the signals received by GPi from the inhibitory pathway (direct pathway) decline, while they are boosted from the excitatory pathway (indirect pathway) resulting in the stimulation of the inhibitory neurotransmitter to the thalamus which in turn over inhibits the release of glutamate signals in the cerebral cortex (Figure 1).

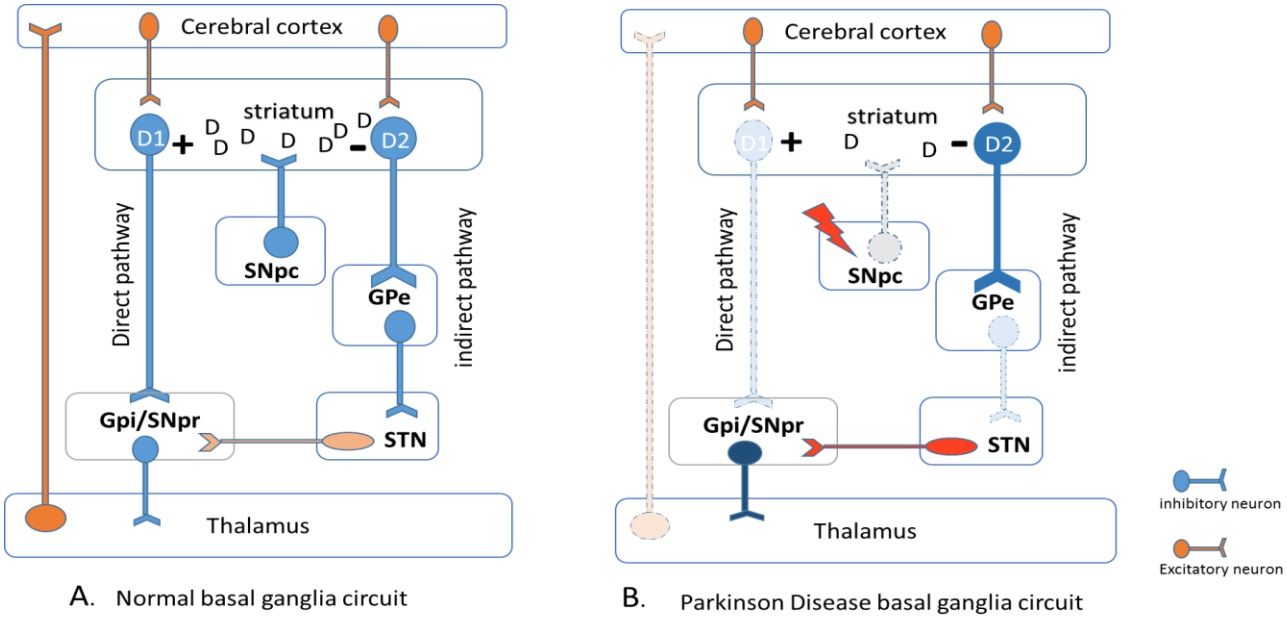


Figure 1: (A) shows a normal physiological role of SNpc DA neurons in the direct and indirect pathways; (B) illustrates the consequences of losing dopaminergic neurons on both pathways (modified from (Nelson et al. 2005)). (abbreviations: D1, D2 dopamine receptors; SNpc, substantia nigra pars compacta; GPi, globus palludius interna; GPe= Globus pallidus externa; SNr, substantia nigra reticula; STN, subthalmic nucleus)

Another hallmark of PD pathology is the inclusion of eosinophilic proteins forming structures known as Lewy bodies. These are found in neurons of the midbrain in addition to their existence in other multiple brain regions like neocortex, diencephalon, spinal cord, and even in peripheral neurons (Gibb et al. 1991; Schulz-Schaeffer 2010). These inclusions are mainly composed of aggregated α-synuclein and other cellular proteins like ubiquitin, elements of the proteasome, and synphilin (Kuzuhara et al. 1988; Ii et al. 1997; Mukaetova-Ladinska & McKeith 2006; Wakabayashi et al. 2000). Although, the biological role of the Lewy body is not well understood yet, there is speculation about its role in damaging, protecting or simply marking neuronal cell damage (Harrower et al. 2005). α-Synuclein, which normally exists as a protein monomer, has a role in neuroprotection and maintaining the function of the dopaminergic synapse (Marques & Outeiro 2012). However, in PD α-synuclein protein is misfolded and exists in oligomers or filaments and its aggregation is suggested to be responsible for the neurodegeneration of the dopaminergic neurons (Schulz-Schaeffer 2010).

Different pathogenic mechanisms have been suggested for the degeneration of the nigral dopaminergic neurons included protein aggregation as described above, increased oxidative

stress, endoplasmic reticular stress, Ca^{++} level dysregulation, mitochondrial dysfunction, neuroinflammation and low trophic support. Misfolded α-synuclein, resulting from gene mutations, interacts with intracellular organelles and membranes and is involved in the increase in oxidative stress and elevation of intracellular Ca^{++} level (Lindholm et al. 2016). Endoplasmic reticulum (ER) controls the misfolded protein levels intracellularly via the Unfold Protein Response (UPR) process. However, the prolonged activation of UPR leads to ER stress and fails to maintain protein folding, ended with inducing the apoptotic cascade and subsequent cell death (Liu & Randal 2011). ER stress is one of the pathological mechanisms of neurodegeneration in PD; and UPR markers were also detected in the Lewy body components of PD patients with dementia (Lindholm et al. 2006; Baek et al. 2016). Dysfunctional mitochondria and impairment of mitochondrial quality control also contributed in the pathological mechanisms. Mitochondrial function normally maintains cell metabolism, intracellular Ca^{++} homeostasis, and the intrinsic death pathway. While in PD, dysfunctional mitochondria have been reported in the pathology of the disease and is the cause of the increase in oxidative stress, reactive oxygen species and alterations in the mitochondrial dynamics (Henchcliffe & Beal 2008). The damaged mitochondria are normally removed from the cells via mitophagy while a failure in clearing the damaged mitochondria leads to the release of cytochrome C, a key cell death component, in to the cytosol and induction of apoptosis (Suen et al. 2008). In familial PD (discussed below 1.4.2), mutations of genes Parkin and PTEN-induced putative kinase 1 (Pink1), which encode proteins required in the ubiquitin-protease system of mitophagy process, give evidence to the involvement of mitochondrial quality control in the pathology of the disease (Truban et al. 2016). Neuroinflammatory reactions and accumulation of activated microglia in the SN were other signs recognized in the pathology of PD (Ouchi et al. 2009). Activated microglia is responsible for the release of pro-inflammatory cytokines like TNFα, IL1β, and INFλ which can exacerbate the oxidative stress and the induction the apoptosis. it has been reported that extracellular α-synuclein participates in stimulation of the neuroinflammatory reaction and the secretion the pro-inflammatory cytokines (Marques & Outeiro 2012).

1.4 **Aetiology**

PD was described for a long time as a prototypic and non-genetic disorder. However, in the early 1980s, 1-methyl-4-phenyl-1, 2, 3, 6,-tetrahydropyridine (MPTP), which has a structure

similar to the herbicide Paraquat, was identified as the cause of PD chronic motor symptoms and selective degenerative of nigral DA neurons in a small group of drug addicts accidentally exposed to this drug. This finding shed light on the role of environmental exposures in the incidence of PD (Langston et al. 1983). Later, identification of inherited forms of PD suggested a genetic role in the pathological mechanism, which is confirmed after distinguishing the role of some of these genes in PD pathology. This is expanded in more details in the following sections

1.4.1 Environmental factors:

Many hospital and population control studies were conducted to validate a correlation between specific categories of pesticide use and the incidence of PD. Some of these studies have reported an increase in the risk of PD with frequent exposure to these chemicals, while others did not report a significant correlation (reviewed in (Wirdefeldt et al. 2011)). Paraquat, which is a herbicide, has been tested on mice and has a dose dependent degenerative effect on the nigral neurons when given intraperitoneally and frequently (McCormack et al. 2002). Rotenone, which is an insecticide, caused degeneration to the nigrostriatal dopaminergic neurons combined with motor activity disorder and existence of Lewy body in the rats (Betarbet et al. 2000). Similarly , Dieldrin, which is an organochlorine pesticide, caused disturbance in the nigral dopaminergic system and increase α-synuclein level in experimental mice (Corrigan et al. 2000; Hatcher et al. 2007). Many other environmental factors have been implicated in the incidence of PD, including drinking well water, rural residence, heavy metal exposure, magnetic field and others; On the other hand, other factors shows a protection against the incidence of PD like intake tea and coffee, smoke cigarettes and utilising non-steroidal anti-inflammatory drugs (NSAIDs) reviewed in (reviewed in (Wirdefeldt et al. 2011)). However, there were no absolute evidence of these factors contribution in the PD aetiology.

1.4.2 Genetic factors:

In a few families there is clear genetic linkage driving inherited forms of PD. Many family studies have reported that the acquisition of PD from first degree relatives ranged between 1.6 to 10.4% (review, Wirdefeldt et al., 2011). These findings have led to a distinction between the familial form of PD and the sporadic form. However, both of these types closely follow the same clinical and neuropathological features with the exception that familial PD

generally occurs at younger age of onset (Schiesling et al. 2008; Latourelle et al. 2009). Since the late 1990s, several genes were reported to be responsible for inherited forms of the disease. In 1997, α-synuclein was the first gene to be associated with familial PD with a specific A53T substation mutation identified as the pathological trigger (Polymeropoulos et al. 1997). Following this, Kitada and colleagues found that a mutation in the protein Parkin were responsible for a form of autosomal recessive early onset PD (Kitada et al. 1998). Similarly mutations in DJ-1 (Bonifati et al. 2003) and PINK-1 (Valente et al. 2004) cause an early onset autosomal recessive PD. Leucine-rich repeat kinase (LRRK2) is another identified gene responsible on an autosomal dominant late- onset PD and causes the most common feature of the typical PD (Zimprich et al. 2004). Recent findings suggest more genes associated with PD like Vacuolar protein sorting-associated protein 35(VPS35) (Vilariño-Güell et al. 2011) and eukaryotic translation initiation factor 4 Gamma 1 EIF4G1 (Chartier-Harlin et al. 2011). The abnormal expression of some of these genes are not only restricted to familial PD but can also be detected in some cases of sporadic PD (described in the table 1.1). This overlap may be attributed to the gene susceptibility concept which involves gene-gene or gene-environment interaction in sporadic PD (Schiesling et al. 2008). Generally, these mutations exert their pathogenic mechanisms on PD via interfering with the function of the mitochondria, ubiquitin- proteasome system and/or phosphorylation process causing downstream cell death mechanisms like programmed cell death, accumulation of oxidative stress, abnormal protein aggregation or energy depletion (Savitt, J. M. et al. 2007)**.**

Table 1 prevalence of PD cases of the known mutant genes form in the familial and sporadic PD (modified from (Schiesling et al. 2008))

Mutated protein	**Familial PD**	**Sporadic PD**
α-synculein	< 0.5%	Non
Parkin	~10-20%	~20% of early onset PD
PINK1	~1-7%	Rare, but limited data
DJ-1	~1-2%	Unknown
LRRK2	~5-10 %	~2%

1.5 Treatment:

The main medications used currently for the management of PD is dependent on addressing the dopamine deficiency in the putamen by administration of dopamine precursors, dopamine receptor agonists and dopamine breakdown inhibitors (COMT and MAO-B). These medications are not curative and they only relieve the motor symptoms of the disease. Although, these medications show success in controlling most of cardinal features of the disease, they showed limitations on late stages of the disease with developing a disabling side effect. However, new therapeutic approaches have emerged that may carry new possibilities and promises in providing more convenient results for the PD patients like deep brain stimulation, gene therapy, disease modifying approaches and cell replacement therapy. These approaches are currently either in animal models, clinical trials or approved by FDA. Below is a brief description for the classical pharmacological treatments and the new approaches with an emphasis on cell replacement therapy, the cornerstone of this thesis.

1.5.1 Classical treatments

Since the discovery of dopamine deficiency in the putamen as the main cause of PD, a new era of the treatment had been revealed (Marsden 1990). Because dopamine cannot penetrate the blood brain barrier, L-dihydroxyphenylalanine (L-dopa) was the first agent used for replacing the dopamine in 1967 (Cotzias et al. 1967). It showed effectiveness in reliving all the cardinal symptoms of the disease and it is still used effectively today (Nutt & Holford 1996). It is given in combination with peripheral aromatic amino acid decarboxylase (AADC) enzyme inhibitors (benserazide or carbidopa), to avoid its peripheral metabolism, decrease its peripheral side effects and increase its central bioavailability (Sonsalla 1997). L-dopa is converted to dopamine by the action of AADC enzyme then stored and released to the synapses to act on the post-synaptic receptors followed by rapid termination to their action by dopamine transporter DAT. Then, the dopamine is either restored or degraded intracellularly or extracellularly by monoamine oxidase-B (MAO-B) or catechol-o-methyl transferase (COMT) (Sonsalla 1997) (dopamine synthesis, release and metabolism see Figure 2). With progression of the disease, the duration of the effectiveness of L-dopa gradually decreases with fluctuations in effectiveness between a good response “on phase” and bad response “off phase” (Obeso et al. 2000). In addition, with chronic administration a striking set of disabling involuntary movements is developed during the on phase which is called L-

dopa-induced dyskinesia (see below 1.5.2). It was suggested that this decline of L-dopa effectiveness is due to further loss of the dopaminergic neurons and reduction in the pre-synaptic storage capacity of the dopamine. Adjusting the dose of L-dopa to small and frequent doses can sometimes be beneficial to relieve the dyskinesia symptoms. However, with further progression of the disease, this correction to the dose administration becomes more difficult because the lowest dose to avoid existence of dyskinesia is not capable of relieving the PD symptoms (Thanvi et al. 2007). Specifically, the problems with L-dopa are that it has a short half-life and its release is controlled by firstly DA neurons then non-DA neurons causing the problems in later PD. Other anti-parkinsonian medications were developed to overcome these problems with fluctuations include dopamine receptor agonists, MAO-B inhibitors and COMT- inhibitors. Dopamine receptor agonists, like pramipexole and ropinirole, showed effectiveness in alleviating the motor symptoms with fewer fluctuations in their effects and reduced prevalence of dyskinesia due to their longer half-lives. However, they exert a major autonomic and psychiatric side effects. MAO-inhibitors, like rasagiline and selegiline, which inhibit dopamine break down, are also effective in treating PD symptoms as monotherapy in early stages of the disease and it used to prolong the effectiveness of L-dopa with less fluctuation at late stages (Pires et al. 2017). COMT- inhibitors, which inhibit L-dopa metabolism, like tolcapone and entacapone, also show beneficial effects when used as adjuncts to L-dopa at advanced stages, to prolong efficacy and reduce ‘off’ time and fluctuation (Pires et al. 2017).

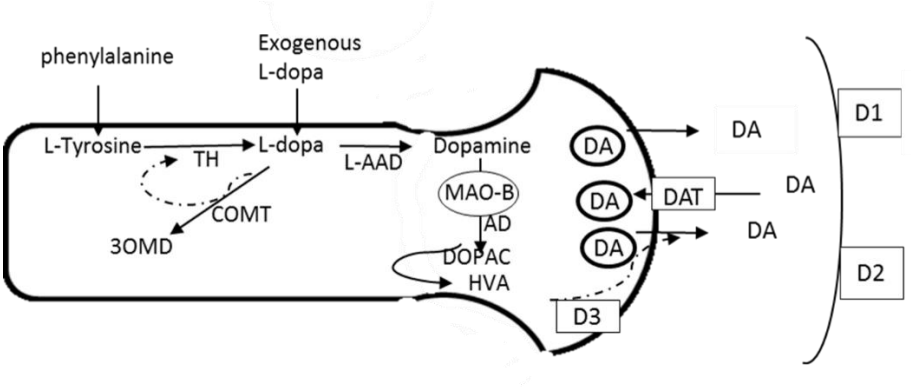


Figure 2: Mechanism of DA synthesis, release, metabolism and reuptake in DA neurons (modified from Nelson et al. 2005). (abbreviations: D1, D2& D3, dopamine receptors; L-dopa, L-dihydroxyphenylalanine; TH, tyrosin hydroxylase; COMT, catechol- o- methyl transferase; 3OMD, 3-O-methyldopa; LAAD, L-amino acid decarboxylase; MAO-B, mono amino oxidase; DOPAC, dihydroxy phenyl acetic acid; HVA, 3 methoxy-4-hydroxy-phenylacetic acid; AD, aldehyde dehydrogenase; DA, dopamine; breaking arrow, feedback regulatory)

1.5.2 L-dopa induced dyskinesia

L-dopa-induced dyskinesia (LID) is characterised by choreiform abnormal involuntary movements affecting the neck, trunk and upper extremities in addition to dystonia affecting the legs. It is classified in two different forms according to their appearance after L-dopa treatment: peak-dose dyskinesia (on phase dyskinesia), appearing at the peak effect of L-dopa (relatively high L-dopa plasma level); diphasic dyskinesia (off phase), appearing at the onset and the decline of L-dopa's effect (Muenter et al. 1977; Thanvi et al. 2007). It has been suggested that L-dopa induced dyskinesia originated from the fluctuation in the synaptic level of dopamine which cause a repetitive pulsatile stimulation to the dopamine receptors. It has been hypothesized also that serotonergic neurons uses L-dopa and release the dopamine as a false transmitter without ability to control its termination due to the lack of the dopamine transporter DAT (Maeda et al. 2005). This pulsatile and excessive stimulation of the D1 receptor causes sensitisation of the receptor and leads to abnormalities in the intracellular signalling of the striatal medium spiny neurons MSN. This abnormality includes changes in the translational and transcriptional downstream molecules that have no direct relation to the dopaminergic transmission, for example extracellular signal regulated kinase (ERK) and mammalian target rapamycin complex (mTORC) (Feyder et al. 2011). Different other hypotheses illustrate the involvement of multiple systems in the development of LID

like the glutamatergic, adenosine and cholinergic, opioid and inflammatory hypotheses reviewed in (Bastide et al. 2015; Niccolini et al. 2014; Carta et al. 2016)

Some therapeutic agents either in animal models or that have progressed to clinical trials have shown the ability to manage LID without affecting the efficacy of L-dopa to alleviate motor symptoms. A double blind placebo controlled clinical study reports that amantadine, which has NMDA antagonist properties, can reduce LID severity by 60% when compared to placebo (Verhagen Metman et al. 1998). In addition, preclinical trial showed that $5HT_{1A/1B}$ receptor agonist can ameliorate LID severity in rodent and non-human primate experimental models of PD (Shin, Tronci, et al. 2012).

1.5.3 New therapy approaches:

1.5.3.1 Deep Brain Stimulation (DBS)

This approach passed phase 3 of clinical trial and was approved by the FDA in 2002. It is based on chronic stimulation of the STN and GPi, by implantation of a small electrode. This therapeutic method showed a remarkable success in ameliorating all the cardinal symptoms of advanced PD without causing dyskinesia or fluctuations in the motor pattern during their use. In addition, the DBS strategy leads to a decrease in the use of regular medications and consequently decrease their side effects, including LID (Benabid 2005). However, it does not have a beneficial effect in relieving non-motor symptoms and could not stop the underlying neurodegenerative progression of the disease (Buttery & Barker 2014)**.** Furthermore, some clinical trial, reported significant side effects, speech and vision problems predominate, in addition to cognitive and psychiatric issues like apathy, depression, emotional stress and suicidal attempts (reviewed in (Pires et al. 2017)).

1.5.3.2 Gene therapy

Gene therapy in Parkinson disease is based on the transference of a gene to a specific area in the brain via viral vectors to induce specific protein expression that can generate mechanism to relieve either the degenerative process or the side effects including LID. Three therapeutic strategies have reached clinical trials targeted gene therapy: First, glutamic acid decarboxylase (GAD) enzyme directed to the STN will increase synthesis of GABA, an inhibitory mediator, trying to achieve the same effects as STN-DBS; second, supplying molecular mechanisms to synthesis DA in the medium spiny striatal neurons. This will enable them to release their own dopamine to stimulate dopaminergic receptors and normalise the

basal ganglia circuit. The last strategy gave attention to halting progression of the disease by providing trophic factors to protect the cells from degeneration (Buttery & Barker 2014). All these strategies illustrated encouraging results in in pre-clinical trials but efficacy ranging from none to moderate in clinical trials, with the exception of the first strategy which illustrated significant improvement in motor symptoms and is currently in phase 3 of clinical trials (LeWitt et al. 2011; Christine et al. 2009; Marks et al. 2008)

1.5.3.3 Disease modifying approaches therapy

Disease modifying therapies are aimed at halting progression of the disease and protect the nigral dopaminergic neurons via targeting the histopathological mechanisms that's caused the degeneration of the dopaminergic neurons like α-synuclein aggregation, ER stress, mitochondrial dysfunction and quality control, neuroinflammation, oxidative stress, in addition to trophic support. Currently some of these strategies showed significant effectiveness in animal models and passed to clinical trials like: hindrance disease progression via immunization strategy to target oligomerization and aggregation of α-synculein (Lindström et al. 2014); protecting nigral dopaminergic neurons with using some anti-diabetic agents like exendin-4 and pioglitazone, this possibly due to an interference with neuroinflammation, mitochondrial function or having anti-apoptotic properties (exendin-4 discussed in details in chapter 4) (Swanson et al. 2011; Aviles-Olmos et al. 2014); or providing trophic factors (delivered through gene therapy). Others agents in pre-clinical trials also showed protection to the dopaminergic neurons like: agonists targeting a non-opioid intracellular receptor in MAM (Mitochondria Associated endoplasmic reticulum Membrane); PACAP (pituitary adenylate cyclase-activating polypeptide) via immunomodulation; MANF (mesencephalic astrocyte-derived neurotrophic factor) trophic factor possibly reduced endoplasmic reticulum stress (reviewed in (Lindholm et al. 2016).

1.6 Cell replacement therapy in PD

Cell therapy is another promising treatment to achieve long lasting relief of the motor symptoms of PD. It is based on transplanting new dopaminergic cells, capable of supplying the putamen with dopamine, to replace that lost through the degenerated nigrostriatal dopaminergic neurons. The first evidence of successful cell transplantation in animal models was published in the 1970s when Bjorklund and colleagues transplanted small pieces of primary tissues (including dopaminergic, serotonergic and noradrenergic neurons) into the

cerebral cortex and hippocampus of intact adult rats. The transplanted cells showed the ability of the cells to survive and produce projecting fibres (Bjorklund A, Stenevi U 1976; Stenevi et al. 1976)**.** At that time, a rat model of PD had been established using the toxin 6-hyrodxydopmaine (6-OHDA) to degenerate nigrostriatal dopaminergic neurons producing motor and behavioural deficits quantifiable through an asymmetric rotational response following administration of amphetamine (Ungerstedt & Arbuthnott 1970). This encouraged two separate groups to conduct experiments to explore the possibility of transplanting pieces of tissue including dopaminergic neurons adjacent to the striatum in rodents (cerebral cortex, lateral ventricle). Both of these studies confirmed the success of the cell transplantation concept with innervation demonstrated in the striatum alongside the recovery of motor deficits (Perlow et al. 1979; Björklund et al. 1980). A couple of years later, papers had been published showing that using a dissociated cell suspension for transplantation at multiple sites inside the striatum lead to much more widespread innervation through the striatum with better recovery not only in the behavioural rotation tests but also in sensorimotor tests (Schmidt et al. 1983; Björklund et al. 1983).

These successes in experimental animal models were then shifted to human clinical trials. In 1987, a clinical study was carried out by auto-grafting a fragment of the patients' adrenal medulla into the right caudate nucleus producing a reported improvement in Parkinson's symptoms (Madrazo et al., 1987). Later in 1989, a multicentre study illustrated that these improvements were minimal and transient (Goetz et al. 1989). In addition, it involved a highly risky procedure which included two major surgeries (abdominal *AND* stereotactic neurosurgery). Subsequently, in the early 1990s, foetal ventral mesencephalic tissue was found to be a more effective source of dopaminergic neurons (Lindvall et al., 1992) compared to autologous adrenal medulla and open label clinical studies were conducted in small groups of PD patients with advanced disease. 18F-DOPA PET scan and the unified PD rating scale (UPDRS) were used to assess graft function and outcomes ranged from 'beneficial' (Levivier et al. 1997; Mendez et al. 2002; Freed et al. 1992; López-Lozano et al. 1997) to 'variable' and 'unsatisfactory' (Defer et al. 1996; Peschanski et al. 1994). In 2001, Freed and colleagues used a sham-controlled double blind study to transplant VM cells and their data initially found no beneficial effect of the graft. Subsequent analysis split the patients into age-based cohorts and identified that the younger patients achieved greater

improvements than older patients. However, in patients who had improvement, many of them experienced a novel form of dyskinesia, which, unusually, was most apparent when patients were assessed in the absence of L-dopa treatment (Freed et al. 2001). These results were supported later by another double blind, controlled-placebo trial conducted in 2003 (Olanow & Goetz 2003). Again, these so-called graft-induced dyskinesia (GID) were also identified in 13 out of 23 patients. In parallel, an EU based open label trial was occurring reported increase dyskinesia level in 6 patients out of 14 during L-dopa off phase post-transplantation (Hagell et al. 2002) (discussed in section 1.6.4). Thus, a heated argument emerged about the validity and safety of cell therapy in the treatment of PD and ended with the stopping of clinical trials. This persisted despite evidence from the PET scans and autopsy studies which illustrated that the transplanted cells survived and were functional in the host brain.

However, studies later suggested that suboptimal conditions in the controlled placebo clinical trials were the reasons for the inconsistent results. For instance, in Freed et al 2001 trial, the cells were stored for up to 4 weeks before transplantation and they did not choose to use any form of immunosuppressant agent after transplantation. In the Olanow *et al* study 2003, they used immunosuppressant agents for only 6 months before withdrawal. An autopsy study performed on 5 patients of Olanow et al trial (two died due to unrelated causes during the study and 3 during 29 months follow up study) detected a significant presence of immunological reaction around the graft which highlighted the possible interference of an inflammatory reaction with graft survival, function and in the induction of GID. Later Piccini and colleagues suggested that the limitations in clinical outcomes could be related to the extent of dopaminergic denervation outside the graft area as the patients who had denervation of dopaminergic neurons in the ventral striatum (area outside the graft site) had no recovery of the graft while patients with no denervation had improvement in clinical assessment. They also showed that long term immunosuppression (for up to 29 months) had no effect on cell survival and function (Piccini et al. 2005). They concluded that although the data itself was not wholly conclusive, a successful outcome appeared to depend upon patient selection (depending on the disease stage) and putting the patients on a longer period of immunosuppression therapy post-transplantation. Clinical trials ceased but preclinical work then continued to uncover what was precipitating GID. The idea of improved

patient selection being key was given weight by a published paper showed that the GID might be linked to the history of LID, suggesting that at selection LID severity history should be considered (Lane et al. 2009). Another clinical key paper came from Politis and colleague clarifying that the patients who experienced GID had high innervation of serotonergic neurons in the graft and the dyskinesia score reduced by using anti-serotonergic release treatments. Although this lacks any control data of patients with successful grafts without GID, this data suggested that the GID may be due to the contamination of graft material with serotonergic neurons as a result of wide VM dissections of the primary tissue (Politis 2010). Importantly for the success of transplantation when effective, a more recent paper of long term patient monitoring showed that transplanted cells had very long term benefits in reliving motor complications up to 18 years post transplantation without need to use anti-parkinsonian medications (Kefalopoulou et al. 2014).

These studies all encouraged the revisiting of the cell therapy approach in clinical trials with considering optimisation the suboptimal conditions. Currently, a new clinical trial has commenced in Europe, under the name of TransEuro, to transplant primary foetal VM dopaminergic neurons into the putamen of PD patients. In this trial an optimised protocol of cell processing with minimized serotoninergic neuron contamination of the transplanted cells has been considered. In addition, patient selection is dependent on stage of the disease with a high expression of LID being an exclusion criteria (www.transeuro.org.uk). The study has not concluded yet and the results will be important to determine the future of cell therapy in PD.

1.6.1 Cell survival and efficacy

The caudate putamen is topographically heterogenous in controlling specific motor tasks (Dunnett & Iversen 1982). This underlines the importance of the graft to have a sufficient fibre innervation capacity to reach across the brain area to reverse motor complications. Studies have shown that both the graft innervation capacity and amelioration of motor complications depend on the number of dopaminergic neurons in the graft (Schierle et al. 1999; Nakao et al. 1995). The clinical and pre-clinical trial clarified that there is a low survival rate of transplanted cells and only small proportions of the dopaminergic neurons remaining in the graft ranged between 1-20% of the transplanted cells, reviewed in (Brundin et al. 2000). Open clinical trials showed that in order to achieve therapeutic efficacy, cells obtained

from 3-5 VM sections were needed for transplantation on each side of the brain (reviewed in (Hagell & Brundin 2001)). This creates a practical hurdle as obtaining several VMs at the time of transplantation is logistically complex. Especially, prolonged periods of cells storage lead to reduces viability and efficacy of the cells. Importantly, as described above, reducing cell loss will improve graft efficiency and the reduce number of cells required for transplantation. The loss then occurs at the point of transplantation and post-transplantation, particularly in the early post-implantation period. The following is a description of the main factors contributing to cell loss and/ or efficacy of the cell transplantation.

1.6.1.1 Pre-transplantation cell loss

Preparing the primary cells for transplantation pass through different steps, starting from collecting the embryos, dissecting out the VM piece and the cells dissociation process. Through this process the cells are exposed to hypoxic, hypoglycaemic and mechanical stress environments resulting in the death of some of the cells. This issue has been addressed by different studies clarifying that using suitable culture media like DMEM (Watts et al. 1998), storing the cells in a cool place (Watts et al. 1998), and reduced mechanical stress on the cells, as well as transplanting fragmented cells suspension rather than single cells suspension (Nikkhah et al. 2009) will lead to significantly improved cell survival. Once this process is complete, further loss is then occurring through loss of cells inside the cannula mainly due to incomplete delivery or traumatic effect on the cells. Using smaller cannula size (inner diameter 0.26 mm) may increase the survival of dopaminergic neurons in the graft compared to larger cannula (inner diameter 0.9) (Steiner et al. 2008). In single cell suspension transplantation, using glass capillary cannula (outer diameter 50 µm) lead to increased cells survival of four times compared to metal cannula (inner diameter 0.26 mm) (Nikkhah et al. 2009). It also has been suggested that the smaller cannula causes less damage to the host cells, consequently this cause less inflammatory and excitotoxicity reaction to the graft. However, the practicalities and safety issues of a glass cannula in patients make this an unlikely development to translate into clinic.

1.6.1.2 Early cell death post-transplantation

Inside the caudate putamen, different factors are suggested to be involved in cell death, including brain injury during the surgical intervention, low trophic factor expression within the diseased adult host environment and inflammatory reactions to the transplanted cells.

Inserting the cannula during surgical intervention causes injury to the host neurons which may induce excitotoxic peptides, oxidative reaction and free radicals to the environment around the graft and this increase cell vulnerability and propensity to induce apoptosis (reviewed in (Patrik Brundin et al. 2000)). A delay in the release of the transplanted cells in the host brain from the cannula by 1 hr after placing them inside the brain may lead to increased cells survival rate by 3 times which suggested that there are acute but fast recovering changes in the host environment (Sinclair et al. 1999). Moreover, other studies illustrate that the majority of cell loss occurs throughout the first week of transplantation (Emgård et al. 1999; Barker et al. 1996). Some studies showed that treating the cells, in cell preparation and prior to transplantation, with neuroprotective agents like Tirilazad Mesylate as anti-oxidants (Björklund et al. 1997) and Ca^{++} blocker to reduce excitotoxicity (Kaminski Schierle et al. 1999) lead to a significant improvements in cells survival.

1.6.1.3 Low trophic factor tone of host environment

The other concern in the death of transplanted cell is the low trophic factor environment of the adult caudate putamen to support the survival of the newly implanted dopaminergic neurons. An *in vitro* study showed that trophic factors released from the striatum can support the survival of dopaminergic neurons at early stages of its development (E15 embryonic VM rats) but they had no effect on its survival at late stage (E17 VM rats) while autocrine acting growth factors (like GDNF and BDNF) improved dopaminergic neuron survival at late stages of VM development (Engele 1998). *In vivo***,** various studies show a significant increase in dopaminergic neuronal survival after enrichment of the host environment with the trophic factor GDNF, using different methods like direct infusion into the striatum (Yurek 1998), engraftment of capsules continually releasing GDNF in the graft (Sautter et al. 1998), co-grafting cells programmed to produce and release the trophic factors (Wilby et al. 1999), or genetically overexpression the GDNF in the striatum itself using viral technology (Yurek et al. 2009). VM transplantation in the striatum of younger rats provides a better cell survival than transplantation in older rats while co-grafted Schwann cells, as a source for trophic factors, in the older group leads to an improvement in survival and behavioural outcomes (Collier et al. 1999). Moreover, it has been suggested that older patients have lower trophic tone than the younger patients, explaining why cells survival is lower in older patients/rats (Thompson & Björklund 2012).

1.6.1.4 Neuroinflammation

The detection of a significant increase of activated microglial around the graft in the Olanow et al double blind clinical study in 2003 shed light on the importance of inflammation on the optimization of graft survival and efficacy (Olanow & Goetz 2003). The innate immune system, as a defence mechanism, reacts to the foreign cells by activation of the microglia to release pro-inflammatory molecules and cytokines like TNF-α, IL-1β, and INF-λ, inducing apoptosis. However, the inflammatory reaction is complex and involves different phases after initiation of the cascade. After the pro-inflammatory phase, the innate immune system passes through a 'resolution and repair' phase to maintain normal tissue homeostasis and this involves alteration of the microglial phenotype from pro-inflammatory microglia to neuroprotective microglia which release neuroprotective cytokines like IL-4, IL-10 and TGF-β (reviewed in (Colton 2009). Immunosuppressant agents gave the first evidence as to the role of inflammation on cell survival**.** As in animal models, their use with xenografts produces a significant improvement in cell survival and prevention of graft rejection. For instance, monoclonal antibodies against CD4 treatments prolong graft survival dramatically (Wood et al. 1996) and the treatment with monoclonal anti-IL2 receptor (anti-CD25 mAb) or cyclosporine A improved survival significantly compared to the control group (Honey & Shen 1999) (Pakzaban & Isacson 1994). Other evidence came from experiments which induced an inflammatory response to challenge the survival of VM allografts showing that the stimulation of a peripheral inflammatory reaction can lead to compromised survival in the brain. For instance, transplanting an orthotropic allogenic skin graft before or after allogenic VM striatal transplantation from the same donor strain caused a significant reduction in dopaminergic neuronal survival in the case of post-grafting immunization whilst rejection of the VM graft was caused by pre-grafting immunization. An alternate approach to exploring this was to inject spleen cells of the donor strain peripherally after transplantation to cause a significant increase in the immune response around the graft (MHC II level) and reduced DA survival by 64% (without significant difference, p=0.09) (Soderstrom & Meredith 2008). Duan and colleagues compared the time course of the inflammatory reaction toward syngeneic, allogenic and xenogeneic grafts in the rat model without adding immunosuppressant agents or peripheral inflammatory stimulation. Increase activated microglia, infiltration of lymphocyte and elevated MHC expression around the syngeneic and allogenic grafts appeared over the first 4 days but most of these inflammatory signs had

disappeared by week 6 while xenografting had a heavier presence of these inflammatory signs after the first 4 days and the grafts had been rejected by week 6 (Duan et al. 1995). Overall this appears to show that the host immune response had negative effect on cell survival and long term use of immunosuppressant agents could have a positive impact on cell survival and graft function. Some clinical trial data now appears to suggest that there is a possible gain to a functional graft of long term immunosuppression of up to 2 years improving the long term motor symptom relief (Kefalopoulou et al. 2014; Piccini et al. 2005).

1.6.1.5 Graft location

Graft location is another area that has been considered in cell therapy optimisation. Ectopic transplantation in the striatum has been preferred over intra-nigral transplantation for numerous reasons; the latter shows insufficient striatal innervation and low cell survival compared to intra-striatal transplantation unless external trophic agents are added to support its survival and fibre outgrowth, even then innervation of the human striatum is unlikely (Thompson et al. 2009; Nikkhah, Cunningham, et al. 1994; Nikkhah, Bentlage, et al. 1994). Redmond and colleagues characterised VM cell transplantation at different sites in the striatum and they found there was no difference in cell survival or innervation capacity in the caudate or the putamen in non-human primate model, however they found that putamenal grafts produced better behavioural recovery (Redmond et al. 2008). Transplantation of the graft across multiple sites and more than one deposit has also been shown to yield wider innervation of the striatum and better behavioural improvement in the rat 6-OHDA lesioned rat model (Falkenstein et al. 2009). Interestingly, single site, small graft transplantation induced forelimb-facial dyskinesia stereotypies in one study, contrasting with the case in multiple site transplantation suggested that focal transplantation are inducing factor for the GID (Maries et al. 2006a).

1.6.1.6 Dopaminergic neurons subtype

Dopaminergic neuron subtype is another factor found to have influence on graft efficacy. The transplanted VM cells population includes a mix of midbrain dopaminergic neurons subtypes mainly A9, which is the nigral phenotype innervating the putamen, and the A10 subtype which is the developing VTA (innervating the caudate and frontal cortex) (Mendez et al. 2005; Thompson et al. 2005). A9 dopaminergic neurons are the most vulnerable population in PD with a relative sparing of the A10 population (Damier , Hirsch , Agid , 1999).

Grealish and colleagues found that the A9 subtype in the VM graft is actually the one responsible for reversal of the motor deficits while the A10 subtype may have limited influence. They suggest that the A9 subtype has the exceptional ability to target the motor area of the striatum (dorsolateral area) (Grealish et al. 2010). These finding highlight the importance of the relative proportion of A9 to A10 subtypes to reverse the motor complications and graft efficacy. Later, a study identified that the birth of A9 subtype during ventral midbrain neurogenesis is earlier than the A10 subtype and clarified that transplantation of younger VM cells leads to an A9 enriched graft with larger innervation to the dorsolateral area of the striatum and better motor recovery (Bye et al. 2012). A recent published study showed that the dorsal striatal environment preferentially supports the survival and innervation of the A9 subtype over A10 of younger VM cells (E12) (Fjodorova et al. 2017). These findings support the advice of using a younger VM age for transplantation in the ongoing foetal tissue clinical trials.

1.6.1.7 Synaptic integration

Boosting proper synaptic integration between the graft fibres and the striatal dendrites was another target in the optimisation of graft efficacy. A recent paper showed that administration of estradiol systemically into hemiparkinson rats lead to enhanced synaptic integration of the transplanted dopaminergic neurons in the striatum via stimulation expression of the integrin α5β1 molecule, which is a cell adhesion molecule on the striatal cells and innervated by midbrain dopaminergic neurons. This enhancement in synaptic integration was reflected in a significant improvement in the behavioural test (Nishimura et al. 2016). Similarly, targeted preservation of the host dendritic spine after transplanting VM cells in a rodent model of PD using, the Ca^{++} channel blocker Nimodipine, caused a significant improvement in some behavioural tests compared to controls (Soderstrom et al. 2010).

1.6.1.8 Lewy body existence around the graft

A recent issue raised from an autopsy study for a patient who received a VM graft 24 years prior to his death showed the existence of α-synuclein and ubiquitin positive deposits, appearing with Lewy body-like features around the graft. There was extensive fibre innervation in the putamen after 24 years, however the patients' monitoring history was recorded as a gradual loss of the recovery of motor complications after 14 years post-transplantation. The authors suggested that a wide spread degeneration could lead to the

loss of efficacy of the graft (Li et al. 2016). The presence of these Lewy bodies is however still very low. Cell transplantation in model needs considering the presence of α-synuclein and its degenerative effect on the transplanted cells and evaluation of the efficacy of the graft in this environment.

1.6.2 Neuroprotective agents to support cell transplantation

1.6.2.1 Mechanisms of Cell death

As described above, the cell death in transplantation therapy occurs at different stages of transplantation process either in cell preparations, during transplantation or post-transplantation. Generally, cells death happens in two different patterns either necrosis or apoptosis, each has different features and mechanisms. Necrotic cell death results from acute injury characterised by swelling of the cell plasma membrane and organelle leakage followed by inflammation (Wyllie et al. 1980). Conversely, apoptotic cell death characteristics include shrinkage of the cell and nuclear chromatin condensation, DNA fragmentation, intact plasma membrane with blebs (apoptotic bodies), terminated by phagocytosis, no leakage of cell component and no local inflammation (Raff et al. 1993). The process of apoptosis is stimulated through activation of a caspase cascade, a protease family that mediate cell death. It is activated through two pathways: external activation mediated from direct stimuli to surface receptor lead to activation caspase 9; internal activation, mediated through release cytochrome C from the mitochondria which in turn activate caspase 8. The release of cytochrome C from mitochondria controlled by the Bcl-2 protein family which either inhibit release cytochrome C and suppress the apoptosis like Bcl-2, Bcl-xl and Mcl , or induce release cytochrome C like Bax and Bak proteins and activate apoptosis (Troy & Salvesen 2002; Suen et al. 2008).

Both processes, apoptosis and necrosis, were identified to be involved in the death of transplanted VM cells in animal models. Emgard et al tracked the pattern of VM cell death at different time points post transplantation (90 min, 1, 3, 6 and 45 days). They identified that apoptosis marker (anti-caspases 3) and necrosis marker (calpain activity) were both high at 90 min and first day post-transplantation. They then noticed that necrosis level reduced dramatically on day 3 with the persistence of apoptosis which lowered significantly on day 6 (Emgård et al. 2003). Sortwell et al. also identified that a higher rate of apoptosis (labelled with TUNEL-positive nuclei) in the first week of VM transplantation followed by a significant

reduction (Sortwell 2003; Sortwell et al. 2001; Sortwell et al. 2000). Both studies concluded that the apoptosis happened in 3 stages: early stage loss resulted from cell dissociation, preparation, mechanical stress, hypoxia and hypoglycaemia; second stage cell death related to the host environment including cytokine release from the damaged striatum or low trophic factor tone and oxidative stress; late stage had a low incidence of apoptosis.

1.6.2.2 Neurotrophic factor

Since one of the suggested causes of cell death is low neurotrophic tone in the striatum, several studies have explored the value of suppling external neurotrophic factors to support graft survival. Some of them were identified to have protection for VM cell in cell culture and in animal models. Glial cell line-drived neurotrophic factor (GDNF) illustrated a great influence on increasing survival of VM cells in cell culture and animal model. It can increase survival rate from double to 13 times depending on method of administration, intra-striatal administration post-grafting or in combination during cell processing and transplantation (reviewed in (Brundin et al. 2000). Brain derived neurotrophic factor (BDNF) and neurotrophic factor 4/5 (NT-4/5) are other trophic factors showing the ability to protect VM dopaminergic neurons in cull culture (Hyman et al. 1991; Studer et al. 1995) but they do not support survival *in vivo* (Haque et al. 1996; Yurek et al. 1996). Trophic factors not only support cell survival but also stimulate the axonal outgrowth of the graft which is also important for expanding coverage of the striatum and consequent graft function (Haque et al. 1996; Yurek et al. 1996; Sinclair et al. 1996).

1.6.2.3 Anti-apoptotic agents

Modulating apoptotic mechanisms to prevent cell death was targeted through using caspase inhibitors or modulating the intracellular level of Bcl-2 family. One study showed that inclusion Ac-YVAD-cmk (anti-caspase-1) during VM cell processing and in transplanted vehicle causes double the survival of VM cells transplanted in 6-OHDA parkinsonian rats (Schierle, Hansson, et al. 1999a). However, a study later failed to replicate this effect of using same agent in similar model which diminished its possible effectiveness (Hurelbrink et al. 2001). Other study targeted inhibition of c-Jun-N-terminal Kinase (JNK) to improve VM cells survival. JNK is an activator for caspase and apoptosis under cellular stress and DNA damage. The results showed that infusion of SP600125 , JNK inhibitor, intra-striatal for 4 days post-transplantation lead to increase survival of VM dopaminergic neurons more than double

(Rawal et al. 2007). Using similar model, investigation of overexpressing the anti-apoptotic Bcl-2 molecule in the transplanted VM cells demonstrated limit effect on cell survival but rather it has significance on promoting axonal outgrowth (G S Schierle, Leist, et al. 1999; Holm et al. 2001)

1.6.2.4 Anti-oxidants

As described above one of the expected reasons of death the transplanted cells is influx of reactive oxygen free radicals like superoxide (O_2^*) and nitric oxide (No^*) around the graft due to neural injury. The free radicals can invade different targets intracellularly including proteins and DNA lead to cellular dysfunction (Floyd & Carney 1992). In addition, they can interfere with lipid membranes creating lipid peroxidation chain and lipid free radical formation (Halliwell 1992). Study showed that overexpression of Cu/Zn superoxide dismutase (SOD), used for oxygen free radicals' suppression, in the VM cell before transplantation causes increase cell survival of dopaminergic neurons up to 4 times (Nakao et al. 1995). This followed by studies used lazaroids compounds, lipid peroxidation inhibitors like tirilazad mesylate (Grasbon-Frodl et al. 1996), demonstrated significant support for VM graft survival when administered in cell processing and prior transplantation (Nakao et al. 1994; Grasbon-Frodl et al. 1996; Hansson et al. 2000).

1.6.2.5 Excitotoxicity inhibitors

It was also hypothesised that the surgical intervention may cause neural injury, leading to release of glutamate that may possibly induce excitotoxity in the transplanted cells. However, a study demonstrated that treating the VM cell before transplantation with the NMDA receptor antagonist (MK- 801) has no effect cell survival. This study weakened the hypothesis of a role for glutamate in cell death in these circumstances (Schierle et al. 1998).

1.6.2.6 Calcium channel blocker

Other studies explored whether Ca^{++} channel blockade can help to protect transplanted dopaminergic neurons. Neuro injury associated with excessive influx of Ca^{++} intracellularly leads to mitochondrial damage, increase free radical formation, and enzymatic activation which participate on cell death (Orrenius et al. 1989). A study treated VM cells during cell processing with flunarizine, a voltage dependent Ca^{++} channels blocker, which lead to a doubling of cell survival of the transplanted VM dopaminergic neurons (Kaminski Schierle et al. 1999).

Although the above neuroprotective agents demonstrated effectiveness in improving survival of transplanted VM dopaminergic neurons in animal models, they only partially solve the problem and the largest proportion of the cells still die. As described above, the survival rate of VM transplantation ranges from 1-10% and 4-5 foetus are needed to produce an effective graft. Thus, even with these neuroprotective agents which improved dopaminergic neuronal survival by double or triple in most cases, multiple VM donors would still be required. The other concern with these neuroprotective agents is the practical difficulty of using them in clinic. The method of delivery of these agents required surgical intervention with each dose administration. In addition, although they produced effectiveness from single or short term administration. However, the presence of long term of inflammation around the graft and the long-time requirement for graft maturation (several months), this may limit their usefulness.

1.6.3 Rodent models for assessment of cell therapy in PD

In order to explore these therapies thoroughly, it has been necessary to employ the use of *in vivo* animal models. PD has complex etiological and pathological features, which make the creation of an ideal animal that mimics all the features of the disease in one model difficult to obtain. Current models depend mainly on inducing the main hallmark of the disease, the loss of nigrostriatal dopaminergic neurons. This lesion, combined with scalable motor and behavioural deficits form the essence of many of the commonly used models. Different factors can be used to degenerate the nigrostriatal dopaminergic neurons including neurotoxins and environmental and genetic etiological factors depending on the experimental intention. In cell transplantation models, the most commonly used factors are 6-hydroxydopamine (6-OHDA) and MPTP neurotoxins. MPTP is a neurotoxin that is able to selectively deplete nigral dopaminergic neurons in many mammalian species when administered peripherally and reproduces the disease's cardinal features. However, rats have a low sensitivity to the toxin and this makes it less favourable for use in this species (Giovanni et al. 1994; Przedborski et al. 2001). 6-OHDA, another neurotoxic agent, is the most commonly used in rodent models. It has been utilised to degenerate catecholaminergic neurons since 1968 (Ungerstedt 1968). It has a chemical structure similar to dopamine and crosses the cell wall via the dopamine transporter system causing an elevation of the oxidative stress levels and subsequent cell death (Blum et al. 2001). Because it is unable to

cross the blood brain barrier, it is administered stereotaxically into the site of the required lesion. Unilateral lesion rather than bilateral is preferred in the assessment of cell transplantation efficacy. Unilateral administration provides good welfare for the animals (bilateral lesion can affect the ability to eat and drink) (Marshall et al. 1974) and offers an innate control for each animal as motor activity between the lesioned and intact sides can be compared. Moreover, the imbalance in dopamine between the intact and lesioned hemispheres allows for exploitation in the rotational behavioural test (Ungerstedt & Arbuthnott 1970). Many studies show that the most suitable target for inducing complete and fast lesions in the nigrostriatal dopaminergic neurons is the median forebrain bundle (O'Keeffe et al. 2008; Lane, Brundin, et al. 2009; Torres et al. 2011).

The evaluation of the extent of the nigral dopaminergic neuron loss and the functionality of the graft is indicated by the use of several behavioural and motor function tests. The rats have postural bias toward the ipsilateral side of the depleted striatum which is evident in lesioned animals but which is exaggerated by the administration of amphetamine. Amphetamine boosts release of dopamine in the intact striatum and the resulting imbalance in dopamine drives rotational motor behaviour measurable using automated rotometers. Apomorphine, a non-selective dopamine receptor agonist, also causes rotation of the rats but toward contralateral side. This is due to the supersensitivity of dopamine receptors on the lesion side which increases responding to sub threshold levels of receptor stimulation (Ungerstedt & Arbuthnott 1970; Ungerstedt 1971a; Ungerstedt 1971b). The number of rotations is correlated with the severity of the nigral dopaminergic cell loss and striatal dopamine depletion (Hefti et al. 1980). The behavioural rotation assessment depends on exogenous stimuli to trigger behavioural response, while naturally PD symptoms exist spontaneously. So, some studies are designed to assess the natural asymmetric motor deficits that appear in unilateral 6-OHDA lesioned rats. These tests depend on the reduced ability or neglect of the unilaterally lesioned rats to use the contralateral forelimb. Examples of this include the vibrissae test where contacting the rats' whisker with a bench edge in the ipsilateral side, followed normally by paw placing reflex over bench surface; while touching the whiskers of the contralateral side accompanied by limit or no reaction (Dunnett 2005). The evaluation of dopaminergic graft efficacy is dependent on the ability to reverse the observed deficits in these motor and behavioural tasks. For instance, the ability of the graft

to release dopamine in the depleted side is measured by its efficiency in reducing the ipsilateral rotational response to amphetamine or even sometimes evoking a contralateral response when a big graft is yielded. This demonstrates the ability of the transplanted cells to release dopamine, whilst the reversal of the naturalistic motor asymmetric behaviour gives additional evidence of the therapeutic value to improve complex motor complications.

1.6.4 Graft induced dyskinesia

1.6.4.1 Clinical evidence

GID are choric and dystonic movements which exist on the face, upper limb or generalised over the body and appeared in PD patients transplanted with human foetal VM. Importantly, the movements were observed in the absence of L-dopa in the clinically defined 'off phase', suggesting that GID is not related to L-dopa, unlike LID, but resulted directly from the graft. This was reported for the first time in the first controlled, double blind clinical study of foetal cell transplantation where it affected 15% of the transplanted patients (Freed et al. 2001). Then, in the Lund open clinical trial, 14 patients had VM cell transplantation for 11 years retrospectively analysed. The post-transplantation data demonstrated a significant increase in abnormal involuntary movements during off phase in 6 patients (Hagell et al. 2002). The second double blind clinical trial confirmed the existence of GID, they reported that 13 out of 23 patients had stereotypic rhythmic movements in the either in the upper or lower extremities during the off phase (Olanow & Goetz 2003). Initially, it has been suggested that hyper-innervation of the transplanted grafts in the putamen may be the cause for GID (Freed et al. 2001). This theory was weakened by a follow up study using [^{18}F] fluorodopa (FDOPA) and positron emission tomography (PET) scan to compare FDOPA uptake by the graft in the GID and non GID developed patients. The results illustrated that size of these grafts had not reached levels of overgrowth in any of the affected patients. However, it has been suggested that even in the incomplete extent of re-innervation, there may be a threshold of the graft size to precipitate dyskinesia (Ma et al. 2002). In the same study, instead of hyper-innervation theory, a correlation of GID with the site of the transplantation and unbalanced dopamine release within the transplanted putamen region was suggested (Ma et al. 2002). However, in the Olanow et al study, no evidence was found of a regional imbalance in dopamine release in the grafted striatum was detected using FDOPA and PET scan. They rather suggested incomplete or aberrant striatal innervation lead to the existence of GID which may have resulted from suboptimal graft conditions like graft variability, uneven

striatal innervation, inflammatory reaction and aberrant synapse formation. They explain that the inhomogeneity of innervation causes dopamine pulsatile stimulation to adjacent supersensitive receptor area leads to exist the GID. (Olanow & Goetz 2003).

Later, another theory was suggested based on the heterogeneity of the transplanted cells themselves. Two transplanted patients which showed GID underwent PET and single photon emission computed tomography (SPECT) scans to determine the amount of 5-HT in the striatum and serotonin/dopamine reuptake transporter ratio. The study produced indications of a hyper innervation of serotoninergic neurons around the grafted area and an increase in the serotonin: dopamine reuptake transporter ratio. Furthermore studies by the same group also demonstrated that administration of buspirone, a $5HT_{1A}$ agonist, reduced expression of GID, postulated as being a direct result of reduced serotonergic activity (Politis et al. 2011; Politis 2010). The hypothesis on the role of 5-HT in GID supposes that serotonergic neurons are capable of using the secreted dopamine from the transplanted dopaminergic neurons and release it as a false transmitter. Without regulated turnover of the neurotransmitters, stimulation of the supersensitive striatal dopamine receptors could then trigger abnormal movements (Shin, Tronci, et al. 2012). Importantly however, transplanted patients without GID have never been scanned in the same way to specifically implicate 5-HT in the generation of GID, the data only supports the idea that there is significant 5HT neuronal content in the grafts. In addition, buspirone (the 5-HT1A agonist) also has partial dopamine D2 receptor agonist activity (meaning that it can behave as an antagonist in the presence of the full agonist for the dopamine receptor). It is entirely possible that the observed reduction in GID is through the action of the drug on supersensitive dopamine receptors (described below).

The appearance of inflammatory signs around the grafted area and the existence of GID symptoms after withdrawal of the immunosuppressant agents also implicates a role for inflammation in the development and existence of this motor side effect (Olanow & Goetz 2003; Piccini et al. 2005). In the Lund-based open label clinical trial, immunosuppressant combination of cyclosporine, azathioprine and prednisolone was administered for 2 years. The study reported that the GID increased at the time of immunosuppression withdrawal (Piccini et al. 2005). In Olanow et al, cyclosporine-A was used as an immunosuppressant for 6 months before withdrawal. Even this study did not state an obvious association between

GID and immunosuppression withdrawal, however they reported that the GID were first identified in the period between 6-12 months post-transplantation (ie during the withdrawal period). Olanow and colleagues also reported the detection of inflammatory signs around the graft and they suggest that inflammation could have a role in developing the GID (Olanow & Goetz 2003). This may suggest that immunosuppression may reduce the existence of GID possibly through reduction of the inflammatory response and protection of the graft.

1.6.4.2 Evaluation of GID in animal models

To explore the different hypotheses that have emerged as to the cause of GID, an animal model was needed. However, graft assessment in unilateral 6-OHDA rat model showed that observing spontaneous abnormal involuntary movements produced by the graft comparable to those in clinical trials was sporadic with minor severity. This reduced the effectiveness of the model on assessing spontaneous GID (Lane et al. 2006). Instead, a fortuitous observation found that some grafted animals expressed AIMs in response to amphetamine which was used in this model to evaluate GID in both rats and mice. Amphetamine causes release the dopamine of the graft and reduce dopamine re-uptake. Its administration to the grafted rats leads to produce abnormal involuntary movements (AIMs) similar to those induced by L-dopa but directly related to the graft (Lane et al. 2006). Other assessment methods have been reported and rely on observing forelimb-facial stereotypies and forelimb tapping stereotype movement in response to L-dopa administration. These movements observed after allogenic graft transplantation and disappeared after rejection the graft suggested its relation to the graft (Steece-Collier et al. 2009).

Two early studies explored the key features that might relate to GID that emerged from the clinical trials, graft size and graft location. Lane and colleagues pointed to the importance of graft size in developing GID showing in a rodent model that larger grafts produces more striking AIMs compared to small grafts in response to amphetamine administration (Lane et al. 2006). Carlsson and colleagues used the same model to investigate the relationship between GID and the site of transplantation in the striatum. They found that the transplantation of VM in the caudal part of the head of the striatum triggered the appearance of GID and that severity of GID was correlated with the amount transplanted in the caudal compared to those in the rostral part (Carlsson et al. 2006).

Another animal study investigated the effect of serotonergic neurons agonists on developing the GID. The study followed the clinical trial data described above and demonstrated that administration of buspirone and another more selective 5-HT_{1A} agonist lead to reduce levels of AIMs induced by amphetamine regardless of the contamination of the VM graft with serotonergic neurons. However, lesion of the endogenous serotoninergic neurons with specific toxin halts the effect of the selective 5-HT_{1A} agonist but not buspirone on reducing the AIMs. The study showed that buspirone action is also not affected by administration of 5-HT_{1A} antagonists. This suggested an important role of the endogenous serotonergic system on modulation GID and illustrated that the 5-HT_{1A} agonist drug buspirone may maintain its effect by blocking the D2 receptor instead of 5-HT_{1A} receptors (Shin, Garcia, et al. 2012). Lane and colleague had previously demonstrated that both $5HT_{1A}$ agonists and dopamine receptors antagonist reduced GID driven by amphetamine stimulation (Lane, Brundin, et al. 2009).

An important link between LID and GID was identified in two studies highlighting that the severity of LID before transplantation was a risk factor for the development of GID (García et al. 2011b; Lane, Vercammen, et al. 2009). The reason behind this may relate to the changes evoked in dopamine receptor signalling in LID. Once LID is established, alterations in the intracellular signalling molecules companied by increases in Δ*fos*β immediate early gene levels, which act as transcriptional activator, increase cellular responding to dopamine levels and trigger LID (Feyder et al. 2011). Importantly this change intracellular Δ*fos*β level and responses to dopamine in the caudate putamen is sustained even after long periods of withdrawal from L-dopa (Andersson et al. 2003). Another study also pointed to the involvement of D_1 and D_2 dopamine receptors in the existence of GID, when low doses of dopamine receptor antagonists nearly abolished all GID symptoms in rats (Lane, Brundin, et al. 2009; Shin et al. 2014; Shin, Tronci, et al. 2012; Shin, Garcia, et al. 2012).

The role of inflammation on inducing GID was explored in two studies used different probe for assessing the graft related dyskinesia. Lane et al used amphetamine to stimulate the graft related dyskinesia. In their experiments, they did not find a correlation between the inflammatory response and GID when IL-2 was used as a pro-inflammatory mediator, despite confirming the inflammatory signs around the graft (Lane et al. 2008). Conversely, Soderstrom and colleagues depended on the stereotypic motor abnormality in response to

L-dopa (described above) as a proxy for GID, to assess the appearance and severity of the dyskinesia. They used a peripheral injection of spleen cells from the same donor as the allogenic neuronal graft to stimulate a generalised inflammatory response. They found that the total GID was transiently augmented after spleen cell injection with only tapping forelimb dyskinesia behaviour significantly increased after a second spleen injection. In this study also, the ultra-structural analysis clarified that the grafted dopaminergic cells make atypical synapses with their targets and these aberrant synapses are increased in the spleen cell injected subjects and positively correlated with GID (Soderstrom & Meredith 2008). The difference in the results of the two studies suggested that could be the abnormal movements produced by the graft due to the effect of inflammation can be detected by L-dopa induced movements but not with amphetamine induced AIMs (Lane & Smith 2010).

A definitive cause of the emergence of GID is still yet to identified. However, the clinical and animal studies provided important evidence to predict possible risk factors for GID. These led to confidence in protocol adaptations in the design of the current clinical trial (TransEuro) aiming to avoid or reduce possible incidence of GID which included reduction of grafted cells contamination with serotonergic neurons, selection of patients with none or low severity of LID, and optimise transplanted cells innervation in the striatum.

1.6.5 Stem cells derived dopaminergic neurons for cell therapy in PD

Despite the issues of GID, early clinical trials in individuals showed the potential benefits of cell transplantation as a therapeutic approach. However, there are significant sustainability issues in using foetal cell transplantation as a main stream therapy. More than one piece of VM and therefore more than one foetus is needed to transplant into the striatum of one patient, 4-6 pieces per hemisphere is typically required. There are logistical problems to obtaining enough VM pieces with the required properties like the appropriate age, quality, and availability at the same time (Brundin et al. 2010). In addition, changes in the process of many terminations from largely surgical to medical involving the use of hormone based medications is used. The impact of this shift in procedure on the viability of VM dopaminergic neurons was uncertain. However, specific study have been carried out to confirm that products from medical terminations of pregnancy can be used (Kelly et al. 2011). Moreover, based on religious and ethical reasons, some countries have banned the use of foetal tissue while others, who have accepted their use, have introduced legislation (the EU Human Tissue

Act) which adds significant logistical hurdles to obtaining the tissue, around consent, management and tracking of the tissue. In addition, the great demand for using foetal tissue, that would emerge from a more widely available therapy, raises concerns about another crucial moral issue, the motivation for elective abortions (Boer & Peschanski 1994).

Alternative sources of dopaminergic neurons are therefore being explored. Pluripotent and multipotent stem cells have been looked to produce dopaminergic neuros that can replace VM cells transplantation. Embryonic stem cells (ESCs) is one of the pluripotent stem cells that is effectively generates dopaminergic neurons for transplantation (Kirkeby et al. 2012)(Kriks et al. 2011). These cells are created from inner mass of the blastocyst of early stage embryos and have proliferative capacity for extended periods of expansion with the maintenance of karyotypic stability *in vitro* (Amit et al. 2000). These cells can theoretically therefore provide a limitless number of cells for transplantation (Xiao et al. 2006). Protocols have been established to produce the ESCs under good manufacturing practice (GMP) and for cell banking (Tabar & Studer 2014). Another type of pluripotent stem cell successfully using in the generation of dopaminergic neurons is induced pluripotent stem cells (iPSCs) (Sundberg et al. 2013; Doi et al. 2014). The iPSCs are generated from reprogramming the adult fibroblast and they have the same proliferative and self-renewal potency of ESCs (Takahashi & Yamanaka 2006; Takahashi et al. 2007). They can be used for autologous transplantation providing the advantage of minimum immunological complications, however it also has disadvantage of the intrinsic vulnerability to the patients' main pathology. The other reprogrammed stem cells used for developing dopaminergic neurons is the induced neurons (iNs) (Kim et al. 2011). These cells directly converted from fibroblast to other differentiated cells without passing the pluripotent stage (Vierbuchen et al. 2010). Unlike pluripotent stem cells, they lack the limitless self-renewal capacity but can still produce an expandable source of neurons (Grealish et al. 2016). Foetal brain neural stem cells NSCs are a multipotent stem cell isolated from the brain of embryos and can be differentiated into neurons, astrocyte and oligodendrocyte. The isolated NSCs from the midbrain of embryos have been successfully converted to dopaminergic neurons. However, these cells need longer time of expansion in culture with less supply of cells and lower differentiation capacity (Yasuhara et al. 2006; Madhavan et al. 2012).

In principle, dopaminergic neurons derived from stem cells can solve many of the practical and ethical challenges of primary foetal tissues, they can be prepared in the lab, scaled up to high levels of production, and provided ready for the patients at time of surgery with fewer ethical hurdles. However, stem cell derived dopaminergic neurons have yet to demonstrate their ability to compete with primary cells in terms of efficacy and that they can be safely hosted by the PD brain. As yet there have only been studies in animal models and there is some way to go to demonstrate long term survival and efficacy, before going into clinical trials.

The main requirements of the transplanted cells are to be safe and able to survive, innervate a significant area of the striatum, form appropriate synapses and function adequately to produce sufficient dopamine to elicit recovery of motor deficits. Initially problems emerged in the technology and understanding of differentiation protocols to guide the cells towards a dopaminergic phenotype and in the timing of transplantation to create viable grafts. Various protocols have been designed and improved to generate reliable populations of dopaminergic neuron that have a midbrain phenotype of in term of transcriptional profile, protein expression, electrophysiological activity, and dopamine release. Early experiments succeeded in generating dopaminergic neurons from hESCs. However, the generated dopaminergic neurons didn't carry the essential protein expression of the midbrain dopaminergic neurons like LIM homeobox transcription factor 1, alpha **(**LMX1A) and forkhead box protein A2 (FOXA2) (Yang et al. 2008; Park et al. 2005) (Emborg et al. 2013). This was followed by protocols directing the fate of the embryonic stem cells to floor plate using dual SMAD inhibition followed by the induction of midbrain regional specification of dopaminergic neurons. Now, there are well described protocols to get authentic dopaminergic neurons which are indistinguishable from midbrain equivalent primary cells (Kriks et al. 2011) (Kirkeby et al. 2012). These cells can achieve behavioural recovery in different animal models of PD. For instance, Kriks and colleagues have described protocols based on directing the hESC to be floor plate derived dopaminergic neurons precursors using SHH and WNT signalling to yield dopaminergic neurons over a period of 25 days. These neurons showed a robust survival and behavioural functioning in three different animal models of PD (6-OHDA lesioned rat, 6-OHDA lesioned -mice and MPTP-treated non-human primates) (Kriks et al. 2011). Another successful protocol described by Kirkeby and colleagues is based on embryoid body formation with dual

SMAD inhibition followed by dose-dependent activation of WNT signalling and SHH activation to generate dopaminergic neurons. Similarly, these have been shown to be capable of reversing motor deficit in the 6-OHDA lesioned rat (Kirkeby et al. 2012). In comparing the efficiency of dopaminergic neurons derived from stem cells and human primary foetal cells, both grafts have the same apparent efficacy in reversing motor deficits (Grealish, Diguet, et al. 2014). Similar success with alternative 'starter cells' have also been successful in generating dopaminergic neurons and producing behavioural efficacy in animal models. This includes reprogrammed cells including induced pluripotent stem cells (iPSCs), direct conversion into induced neurons iNS and induced neural progenitor cells iNPCs (reviewed in (Grealish et al. 2016). Currently there is a global consortium under the name of "G-force PD" which shares the challenges and the solutions to pave the way for clinical trials to transplanting dopaminergic neurons directed from stem cells (Barker et al. 2015). Recently a company called "International Stem Cell Corporation (ISCO)" declared that they were conducting a clinical trial in the Royal Melbourne Hospital in Melbourne, Australia in which they are transplanting neural stem cells differentiated from a pluripotent parthenogenetic cell (hpNSC) in moderate to severe PD patients (International Stem Cell Coporation, 2015). This trial has created a lot of conflict in the field, especially as there is no clear evidence from pre-clinical studies about the safety and efficiency of these cells. One concern is that these cells express PAX6, while midbrain dopaminergic neurons are negative for this particular marker, raising the issue of what phenotype of the cells transplanted and the anticipated mechanism of action of these cells (reviewed in (Barker et al. 2016). The translation of stem cells as a source to transplantation in PD to clinical trial should be considered with caution and takes all the lessons from the previous clinical trials of primary cells transplantation to avoid possible inconsistency results that would have negative impact on the future of cell therapy. Several important features of stem cell transplantation have not yet been fully explored in the basic animal models deployed thus far.

1.6.6 Real world animal model of PD

Most of the animal models used to evaluate cell therapy have ignored the fact that PD patients are exposed to long term anti-parkinsonian medications, especially L-dopa treatment. These therapies continue for a prolonged period of time after transplantation, until reductions in medication can be introduced once the transplanted cells have matured enough to have independent therapeutic efficacy. As described above L-dopa elicits many

changes in striatal neurochemistry interfering with the molecular and synaptic plasticity of the host brain. It also causes changes in blood brain barrier permeability and inducing angiogenesis (Westin et al. 2006), all of which have the potential to influence graft outcomes. For instance the developed dyskinesia due to L-dopa treatment suggested to be precipitating factor for the development of GID (García et al. 2011b; Lane, Vercammen, et al. 2009). Moreover, the change in BBB permeability increase possibility of changing the inflammatory figure around the graft. In addition, L-dopa treatment has been suggested to increase the oxidative stress load in the brain because it is increase dopamine level which may lead to increase its oxidative metabolite (Felten DL, Felten SY, Steece-Collier K, Date I 1992). On the other hand, there is speculation that L-dopa could have a positive effect on the graft via modulation the inflammatory pattern from pro-inflammatory to protective phenotype (Carr et al. 2003; Nakano et al. 2009).

1.6.7 Peptides have neuroprotective properties for the midbrain dopaminergic neurons:

Recently some peptides illustrated that they can protect the nigrostriatal dopaminergic neurons in different animal models. Ghrelin, a stomach peptide, is one of these peptides that protected the nigral dopaminergic neurons in cell culture and animal model when challenged with different toxins (Moon et al. 2009a; Andrews et al. 2009). Its neuroprotection effect was accompanied with anti-apoptotic, anti-inflammatory ant anti-oxidant properties (Dong et al. 2009b; Jiang et al. 2008). The other peptides are Glucagon like peptide-1 GLP-1 receptor agonists (exendin-4 and liraglutide). These peptides are used to treat type 2 diabetes mellites T2DM. They also showed a neuroprotection property, anti-inflammatory and anti-apoptotic criteria (Perry 2002; Sharma et al. 2014; McClean et al. 2011). Exendin-4 illustrated ability to protect the nigral dopaminergic neurons in different PD animal models and cell culture models (Kim et al. 2009a). These peptides can cross blood brain barrier and some of the them has approved for clinical use (exendin-4 and liraglutide) which encouraged to use exendin-4 for clinical trial to treat parkinsonian patients (Aviles-Olmos et al. 2014). These neuroprotective agents (ghrelin and GLP-1R agonists) haven't tested yet if they have ability to protect transplanted dopaminergic neurons. Since the inflammation, oxidative stress and apoptotic cell death are the main causes of cell death of the transplanted cells in the host striatum (see section 1.6.1), these agents could have a potential to protect the transplanted cells and improve graft function. Especially, the transplanted cells (VM cells or stem cells

derived dopaminergic neurons) are phenotypes of the midbrain dopaminergic neurons which they protected by these peptides.

1.7 Thesis aims

Although many studies have tried to optimise cell therapy in animal models of PD, low cell survival rates and insufficient efficacy are still the major challenges that need to be overcome. In this thesis, I have tried to identify neuroprotective agents that may be able to support transplanted cell survival and efficacy, targeting the transplanted cells in the host brain through peripheral administration. Two key themes then follow, the first to apply the principles of an animal model more closely mimicking the reality of a PD patient thus far never considered, by involving anti-parkinsonian mediations such as L-dopa. Stem cell-derived dopaminergic neurons as an alternative source for cell therapy, these cells have not yet been tested in animal models that consider the clinical issue of exposing the cells to the ongoing anti-parkinsonian medications before and after transplantation that is the reality of cell transplantation. The key concern is how could these medications affect graft survival, function and safety. So, the second theme of this thesis is to understand survival and function of two different stem cells having different started cell source and different histological graft features in "real world animal model" using L-dopa treatment. A hitherto unaddressed issue is also the ability of hESC derived grafts to reduced LID.

The underlying hypothesis for this thesis is that using neuroprotective agents can improve of transplanted foetal and hESC derived dopaminergic neurons survival and efficacy, and L-dopa administration may compromise the survival of the transplanted foetal and hESC derived dopaminergic neurons.

The main aims of this thesis therefore were to:

1. Optimise cell transplantation therapy using neuroprotective agents to support cells survival and efficacy (addressed in chapters 3, 4, 5 and 6)
2. Understand the efficacy and histological features of human embryonic stem cell derived dopaminergic grafts (addressed in chapters 5 and 6).

3. Determine the effect of implementing a “real world animal model” (ie including the administration of L-dopa), on the efficacy of cell transplants and the impact of neuroprotective agents (addressed in chapters 4, 5 and 6).
4. Evaluate the ability of hESC derived dopaminergic neuronal grafts to improve LID (addressed in chapters 5 and 6)

2 Chapter 2: Methodology

2.1 Animal husbandry

Adult female Sprague Dawley and time mated pregnant Wistar rats, obtained from Envigo, were used in the experiments. At the start of each experiment, they had a weight range between 190-250 g and placed in an animal house at room temperature (20-22 ^{0}C). A humidity of 45-65% with a light cycle of 12 hours on and 12 hours off were maintained at the holding room. Four rats were housed per cage (L: 54 cm, W: 37 cm, D: 21 cm) and supplied with hygienic animal bedding with *ad libitum* access to food (14% protein, Harlan) and water. The Sprague Dawley rats were acclimatised to one week before starting the experiments. All the experiments were performed in agreement with the Animals Scientific Procedure Act 1996 and Home Office regulations (PPL: 30/3316; PIL: ISS4A938B).

2.2 Surgical Procedures:

All the surgical procedures were carried out in aseptic and equipped surgery room. Rats were anaesthetised with isoflurane at a concentration of 4-5% plus 8% oxygen for the induction then maintained at 2-3% isoflurane plus 8% oxygen and 4% nitrous oxide. All the surgeries were performed on a Kopf stereotactic frame which holds the rat's head in a fixed position and ensured precise moving of a drill or a cannula to a targeted point. A Harvard micro-drive infusion pump was used to infuse the 6-OHDA (details 2.2.1) solution or the transplanted cells suspension at a suitable infusion rate (details in 2.2.2). During the surgery, rats were covered by sterilised plastic sheets to keep them warm. At the end of the intervention, all rats were sutured with vicryl 4-0 sutures and injected with 3 µl Metacam (5 mg/ml) and 5 ml normal saline subcutaneously. Finally, they were transferred to a recovery chamber at 30 ^{0}C for few hours before returning them to their cages. The health and the weight of the animals were checked daily for 3 days following the surgery and then once weekly.

2.2.1 6-hydroxydopamine lesion

A fresh 5 mg vial of 6-HODA containing 0.2 mg of ascorbic acid was reconstituted for the lesion the nigrostriatal dopaminergic neurons. It was stored at -20 ^{0}C and reconstituted with 800 µl of a cooled normal saline to obtain a concentration of 25 mM. For long term storage, it was stored in aluminium foiled aliquots at -20 ^{0}C while during the surgery it was kept in an ice box. A 30-gauge stainless steel cannula was used to deliver the 6-OHDA solution. The

cannula was inserted into the medial forebrain bundle (MFB) of the right hemisphere on the following coordinates from the bregma: - 4 mm of the Anterior-Posterior axis (AP); - 1.3 mm of the Medial-Lateral axis (ML); - 7 mm under the Dura of the Dorso-Ventral line (DV) with the nose bar set at -4.5 (Ungerstedt 1968; Torres et al. 2011). After that, 3 µl of the 6-OHDA solution was infused over 3 min and then the cannula was left for 2 min before withdrawing to allow a complete diffusion.

2.2.2 Cells transplantation

Primary foetal dopaminergic neurons and stem cells derived dopaminergic neurons were used for transplantation in hemi-parkinsonian rats through different experiments of this thesis. They were grafted at one site of two deposits inside the dopamine depleted striatum of the right hemisphere according to the following coordinators from the bregma: AP: - 0.5 mm; ML: - 3 mm; DV - 5 mm and - 4 mm, while the nose set bar was at - 4.5 mm. The following is details of cells obtaining, preparation and transplantation details for each type of these cells.

2.2.2.1 Ventral Mesencephalon dopaminergic neurons:

E14 Wistar time-mated pregnant rats were ordered to obtain their embryos. They were anesthetised by administration a 500mg/kg of pentobarbitone (Euthatal®) i.p. followed by embryo collection and mother's termination via neck dislocation. The crown-rump-length (CRL) was checked to confirm the embryo's age (10.5-11 mm for E14). Then, embryo decapitation was carried out in a petri dish filled with Hanks' balanced salt solution (HBSS) solution. In a similar media, the ventral mesencephalon (VM) pieces were dissected under microscope using fine cutting edge scissors (2.5-3mm) and forceps with fine tips (0.1 x 0.06 mm) (see Figure 3). After that, the VM sections were transferred into a medium Dulbecco's minimum Eagle medium (DMEM) in 1.5 ml Eppendorf tube then a standard protocol was followed to make a single cell or cluster of cells suspension (Björklund et al. 1983; Torres et al. 2007)**.** The VM sections were washed 3 times with DMEM solution followed by incubation with 1.5 ml of TryplE™ express solution and 30 µl of DNase solution at 37 ^{0}C for 20 min (tube agitated gently after 10 min). This was followed by three washes of DMEM/DNase solution (14ml DMEM + 280µl DNase). After that, a 500 µl of DMEM/DNase solution was added to the dissected VM tissues and a mechanical dissociation process was applied to break them down using a few gentle trituration by 1000 µl Gilson pipette set at 200 µl then by 200 µl

Gilson pipette set at 200ul. The viability and count of the cells were checked under a microscope by staining a 2 µl sample of the suspension with a 0.04% trypan blue on a haemocytometer slide. After that, the suspension was centrifuged at a rate of 380 G for 3 min at room temperature. After removing the supernatant, the pellet was re-suspended with enough quantity of (DMEM/DNase) solution to achieve a concentration of two thirds of each VM section per two µl of the suspension (for instance, if 6 VMs sections were dissected, the pellet would be suspended with 18 µl of DMEM/DNase solution). On the same day, the suspension transferred to the surgical room and kept at room temperature during the surgical procedure. A 2 µl of the suspension was infused into the striatum (1 µl at each deposit) at a rate of 1 µl/ 90 sec. A 23-gauge stainless steel cannula connected to a Polyethylene tube which in turn joined to a 10 µl Hamilton syringe was used to deliver the cells. After the infusion, the cannula was left inside the striatum for 3 min before retraction to ensure a complete infusion.

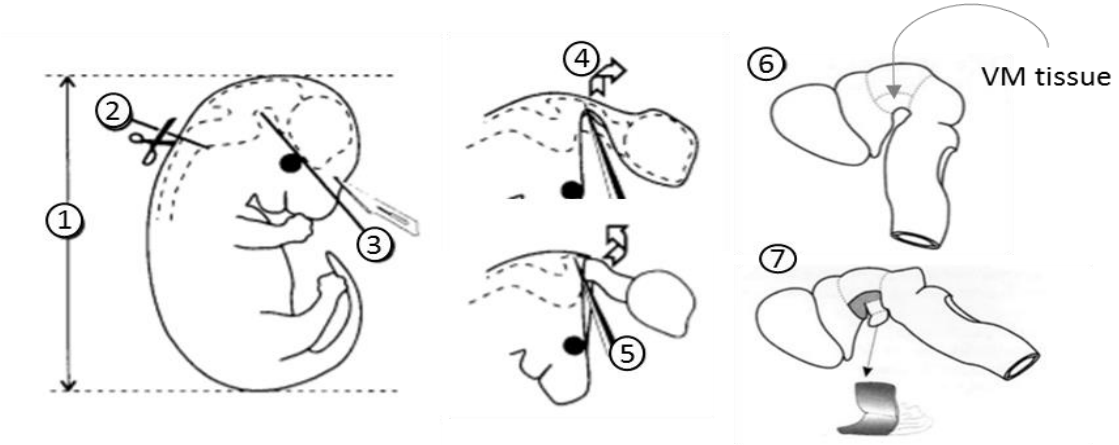


Figure 3 steps of VM dissection: 1. CRL measurement which is the distance between the highest and lowest point of the embryo and it is about 10.5-11 mm for E14 rat's embryo; 2. Neck dislocation; 3. Cutting above the eye and under the base of the brain using fine cutting edge scissor 4. Separating the brain, moving away skin and skull; 5. Peeling away all covering layers and meninges; 6. The drawing lines around the VM section is showing the cutting angles; 7. Releasing the VM tissue which have a butterfly shape (modified from (Dunnett & Bjourklund 2000)).

2.2.2.2 Stem cells derived dopaminergic neurons

two different types of stem cell-derived dopaminergic neurons were used in this thesis. Each type was developed at a different lab and followed a different protocol and this thesis is not involved in their generation or developing and the cells were received prior to transplantation procedure. The following is a brief description of the concept of generation with reference to

the lab or the group who created them in addition to details of transferring, and procedures of cells preparation for transplantation.

2.2.2.2.1 Stem cell derived dopaminergic neurons according to (Kirkeby et al. 2012) protocol:

Midbrain dopaminergic neurons generated from H9 hESCs was used as a cell source for transplantation in chapter 5. These cells were generated in Lund University in Kirkeby and colleagues' labs, and this thesis is not involved in the cells generation. In brief, a published protocol described the method of generation of these cells which involved formation of embroyed body with a dual SMAD inhibition to get neural progenitors. Then the cells regional specification was controlled by adding chemical inhibitors to glycogen synthase kinase 3 GSK3(CT99021) in a dose dependent pattern which activate canonical WNT signalling. A higher dose (1-4µM) was patterned the cells toward hindbrain characteristics while lower dose (less than 1 µM) gave more rostral criteria to the cells. Midbrain cell identities were obtained at a narrow range of CT99021 concentration between 0.7µM to 0.8µM while their fate to get more ventral properties controlled by adding the pattering factor SHH-C2411 (Kirkeby et al. 2012). Cell verification before sending for transplantation was performed at Lund University which involved plated cells on day 14 of differentiation to be stained with FOXA2, LMX1a and Otx2 markers (verification data is not available in this thesis).

Fresh cells were used for transplantation. On day 16 of differentiation, plated cells were transferred from Lund-Sweden using transport incubator to Cardiff university-UK, where transplantation surgery was performed, and they were kept overnight in the lab incubator (37 ^{0}C, 5% CO_2). On day 17 the cells underwent a procedure of dissociation and making cells ready for transplantation. They were washed twice with PBS solution before adding 200µl of accutase solution per well and left for 10 min in the incubator at 37 ^{0}C. Then the cells were dissociated thoroughly with a 1ml pipette and transferred to 10 ml tube containing 7-10 ml of DMEM: F12+B27 medium followed by centrifugation at 400 G for 5 min. The supernatant was removed and 1 ml of HBSS + dornase alfa (6:1) plus 10 µl of DMEM: F12+B27 were added and transferred to 1.5ml tube. 10 µl aliquot were taken for cells counting and viability assessment. Then, the cells were spun down at 400 G for 5 min and re-suspended with HBSS + Dornase alfa solution to achieve a concentration of 75,000 cells/ µl. Three batches of cells suspensions were prepared for transplantation and kept on ice during the surgical procedure

for no longer than 3hrs. Transfer of the cells from Lund to Cardiff and re-suspension procedure were done by Sarah Nolbrant, PhD student at Lund University.

A 10 µl Hamilton syringe connected to 26-G stainless steel Hamilton needle (19132-u, Sigma) was used to deliver the cells inside the striatum. they were infused at rate of 1 µl/min with leaving the cannula for 3 min before retraction. 4 µl of the cells suspension (300,000 cells) were transplanted in each depleted striatum (2 µl per deposit).

2.2.2.2.2 Stem cell derived dopaminergic neurons according to non-published work done by Tilo Kunath group at University of Edinburgh:

hESCs (RC17)- derived dopaminergic neurons were used as cell source for transplantation in chapter 6. This thesis is not involved in generation these cells and they generated in Tilo Kunath and colleagues' labs, Edinburgh University. According to presented work of this group in Parkinson's' UK conference 2014, The method of generation involved using dual SMAD signalling inhibition with an early strong SHH activation and controlling to the FGF signalling. At the start of differentiation, FGF signalling was inhibited with PD0325901 followed by adding FGF8 and an appropriate WNT activation (Tilo Kunath, personal communication, Parkinsons' UK 2014).

On day 11 of differentiation, the cells were frozen in a stem cell banker with ROCK inhibitor. Before shipping the frozen cells for transplantation, sample of frozen cells was thawed and cultured until day 16 for counting and evaluating the cells quality and criteria by measuring TFF3 level using ELISA; CORIN level using Fluorescence-Activated Cells Sorting (FACS); and immunostaining of FOXA2/LMX1a/CORIN. After cells quality verification, they were shipped from Edinburgh to Cardiff. The cells were thawed and plated (day 11) followed by feeding on day 14 (thawing and feeding procedures were accomplished by Dr Nicola Drummond, Edinburgh University). On day 16, the wells were rinsed with Accutase before incubating them in 0.5 ml of Accutase solution at 37 ^{0}C for 10 min. then in the Accutase media, they were pipetted up and down 10 times to make a single cell suspension. After that, the suspension was transferred to 5-fold volume of DMEM media. Then, they were centrifuged and re-suspended with appropriate volume of DMEM to get a concentration of 250,000 cells/µl (cells preparation for transplantation was performed by Dr Ngoc-Nga Vinh, Cardiff University). Two µl of the cells suspension were grafted inside the striatum (1 µl at each deposit) on a rate of

1 µl/ min with leaving the cannula for 3 min before retraction. Hamilton syringe connected to 26-G stainless steel Hamilton needle was used to deliver the cells.

Table 2 summary for stem cells derived dopaminergic neurons details which used for transplantation.

Referring group	Kirkeby et al. 2012 cells	Tilo Kunath group cells
hES Cell line	H9	RC17
Differentiation Protocol	Dual SMAD inhibition+ dosed dependent activation of WNT signalling + SHH activation	Dual SMAD inhibition+ early SHH activation+ FGF modulation
Storage and transfer	Fresh cells	Frozen and thawed cells
Cannula size and type	26-G Hamilton needle	26-G Hamilton needle
Site of transplantation	One site of 2 deposits at coordinators AP: +0.5; ML: +3; DV +5 and +4 from bregma	One site of 2 deposits at coordinators AP: +0.5; ML: +3; DV +5 and +4 from bregma
Cells suspension concentration	75,000 cells/ µl	250,000 cells/µl
Infusion rate	1 µl/ min	1 µl/ min
Dose of transplantation per striatum	300,000 cells	500,000 cells

2.3 Behavioural assessments

2.3.1 Amphetamine induced rotation test

Meth- amphetamine powder was dissolved with 0.9% normal saline to make a concentration of 2.5mg/ ml. Each rat was received 2.5 mg/ kg i.p. of amphetamine solution and placed immediately in a Perspex cylinder. The rats were harnessed to an automated rotometer connected to software set to count the number of fractions of rotation fractions toward

either direction (clock-wise or anti-clock wise) (Ungerstedt & Arbuthnott 1970). The net number of rotations and direction was determined by counting the number of one-eighth rotations toward ipsilateral direction subtracted from the number of one-eighth rotations towards contralateral side during 90 or 180 min (test running time) divided by 8 (fractions of one full rotation). The total rotation per 1 min was determined by dividing the net rotation by the test running time. A successful 6-OHDA lesioned rat was assessed 2-3 weeks after lesion by amphetamine induced rotation test. Rats who rotated ipsilaterally more than 6 rotations per a minute were considered to have more than 90% lesioned nigral dopaminergic neurons (Torres et al. 2011).

2.3.2 Apomorphine rotation test

Apomorphine powder was dissolved with 0.9% of normal saline obtaining a concentration of 0.05 mg/ ml prior to use and kept in dark containers to avoid drug oxidation. The rats were injected with a dose of 0.05 mg/ kg subcutaneously (s.c.) and placed immediately in the Perspex cylinder connected to automated rotometer. The rotometer was set in the same way described in amphetamine induced rotation test (see 2.3.1) to count the number and direction of rats' rotations. This test was run for 1 hr post drug administration. This test used in chapter 5 and 6, and only at the last time point of behavioural assessments post-transplantation.

2.3.3 Motor tests

The stepping, vibrissae and cylinder tests were used to evaluate the asymmetrical motor behaviour of the rats at different time points before and after cells transplantation surgery. These tests were conducted during light phase. It is expected that the rats with right hemisphere nigral-striatal dopaminergic neurons lesion lose the ability to use the contralateral forelimb during these tests while a successful and efficient dopaminergic cells transplantation should be able to reverse this deficit.

2.3.3.1 Stepping test

Each rat was placed on the surface of a bench and hold in a manner that let one forelimb make a contact with the surface and support the body weight while the other forelimb restrained (see Figure 4). Then the researcher moved the rat over one meter of the surface during 10 sec forward. After that, the same process was repeated with the other forelimb. The percentage of left forelimb steps (contralateral side) from the right forelimb (ipsilateral

side) steps was determined and considered as the value for the rats' performances in stepping test.

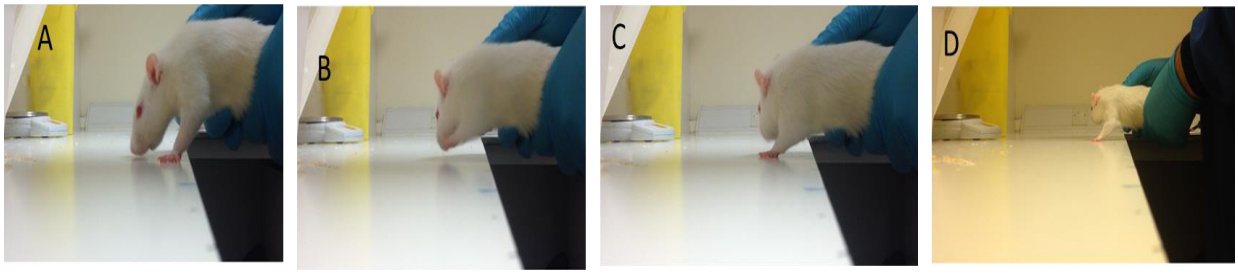


Figure 4 stepping test: images from A to D showing the way of constrain a rat and moving a contralateral forelimb over one meter of the surface bench.

2.3.4 Vibrissae (whisker) test

The researcher held the body and hind limbs of the rat to restrain one forelimb and let the other move freely. The rats were moved gently upward from a point under the bench and adjacent to the edge letting the rat's whisker make contact with the edge. The paw placing reflex on the bench surface, which stimulated from whisker contact, were counted (see Figure 5). This movement was repeated 10 times for each forelimb then the percentage of contralateral placing reflex of the ipsilateral side was calculated and considered as the value of whisker test.

Figure 5 Vibrissae (whisker) test: images from A to C showing the way of holding the rat and passing it from under the bench closer to its edge allowing a contact the whiskers and inducing the paw placing reflex over the bench surface.

2.3.5 Cylinder test

Each rat was placed inside a Perspex cylinder (high: 33.5 cm, diameter 19 cm) and surrounded by two connected mirrors by an angle of 90^{o} which allowed easily seeing the forelimb paws touches on the cylinder surface from all view directions (see Figure 6). A video camera was placed in front of the cylinder to record these touches and repeat recording if needed. The percentage of contralateral paw touches out of the first 20 total touches

(ipsilateral and contralateral) was calculated and considered as a value for rat's performance in the cylinder.

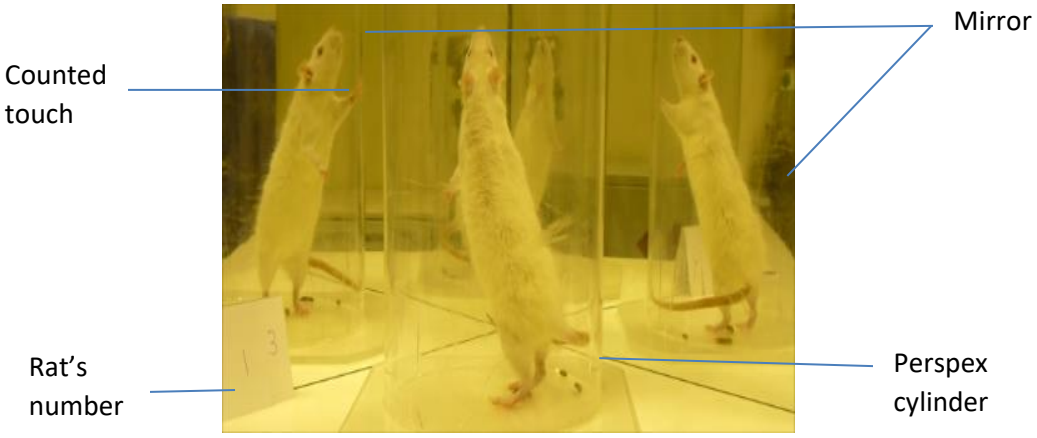


Figure 6 cylinder test

2.4 L-dopa induced abnormal behavioural and motors movements

2.4.1 L-dopa treatment:

A reconstituted powder of L-3,4-Dihydroxyphenylalainine Methylester (L-DOPA) was used for peripheral administration in combination with benserazide hydrochloride, which is a peripheral aromatic amino acid decarboxylase (AADC) enzyme inhibitor preventing conversion of L-dopa to dopamine in the periphery. The 2 drugs were mixed together and stored at -20 ^{0} C for no longer than 4 days before dissolving with 0.9% normal saline and injected s.c. in the rats. The dose of administration and proportions of each drug in the combination in addition to dose frequency and duration of treatment will be stated in the method of the chapters where it is appropriate.

2.4.2 L-DOPA induced rotation:

As with amphetamine, the L-dopa injected rats were putted immediately in perspex cylinder and joined to an automated rotometer. Number of rat's rotations with either clockwise or anti-clock wise direction were counted via a software program linked to the rotometer. The program was set to count number of one-eighth rotation at each direction during 180 minutes of injecting the rat. The total number of rotations per minute were determined by subtracting the total one-eight rotations toward clockwise (ipsilateral side) from the total one-eight rotations anti-clockwise (contralateral side) divided by 8 (fractions to complete one rotation) and by 180 (the duration time of running the experiment in minutes).

2.4.3 Abnormal involuntary movements (AIMs) rating scale:

The comparison between different AIMs scales which performed by Breger and colleagues (2013) was considered to choose an appropriate scale to assess the AIMs induced by L-dopa injection. They found that developing of the AIMs score described by Winkler and colleagues scale (Winkler, Kirik, Björklund, & Cenci, 2002) is more sensitive in response to doubling the dose of L-dopa or alleviation the dyskinesia by using amantadine (anti-dyskinesia agent) than other scales. They also showed that hind-limb movements described (Steece-Collier et al. 2003) is correlated with doubling L-dopa dose and increase the significance of amantadine effect (Breger et al. 2013). The AIMs scale used in this thesis involved evaluating the duration and intensity of dyskinesia movements of the forelimb, hind limb, trunk and mouth which is performed concurrent with L-dopa induced rotation test. The examiner observed each rat for one minute every 20 minutes during 180 minutes starting immediately after L-dopa injection. At each observation, the duration and the intensity of the abnormal movements were scored as described in Table 3 and some images in Figure 7. The total AIMs score is the summation of the multiplying of the intensity score with duration score at each observation point. AIMs scoring test were started 3 weeks post 6-OHDA lesion surgery and repeated several times before and after cell transplantation surgery, more details about frequency of performing the test and dose of L-dopa administration are stated in the next chapters where it is appropriate.

Table 3 Abnormal Involuntary Movements (AIMs) rating scale as described by Breger et al., 2013

Dyskinesia	Score = Intensity Description	Score = duration
Forelimb	1= Tiny oscillatory movements of the paw and the distal forelimb around a fixed position 2= Movements of low amplitude but causing visible translocation of both distal and proximal parts of the limb. 3= Translocation of the whole limb with visible contraction of shoulder muscles 4= Vigorous limb and shoulder movements of maximal amplitude.	0= none 1= occasional: less than 50% of the time. 2= frequent: more than 50 % of the time. 3= continuous but interrupted by strong sensor stimuli. 4= continues but uninterrupted by strong sensor stimuli.
Hind limb	1= abnormal posturing of limb 2= sustained posturing of the limb, mildly extended in abnormal posture. 3= severely hyperextended in abnormal position 4= hind limb fully extended, causing the rat to lose balance.	
Axial	1= lateral deviation of head and neck <30 2= lateral deviation of head and neck 30-60 3= lateral deviation or torsion of head, neck and upper trunk 60-90 4= torsion of neck and trunk at >90 causing the rat to lose balance.	
Oro-lingual	1=jaw movements and facial grimacing 2= tongue protrusion	
locomotion	No intensity score	

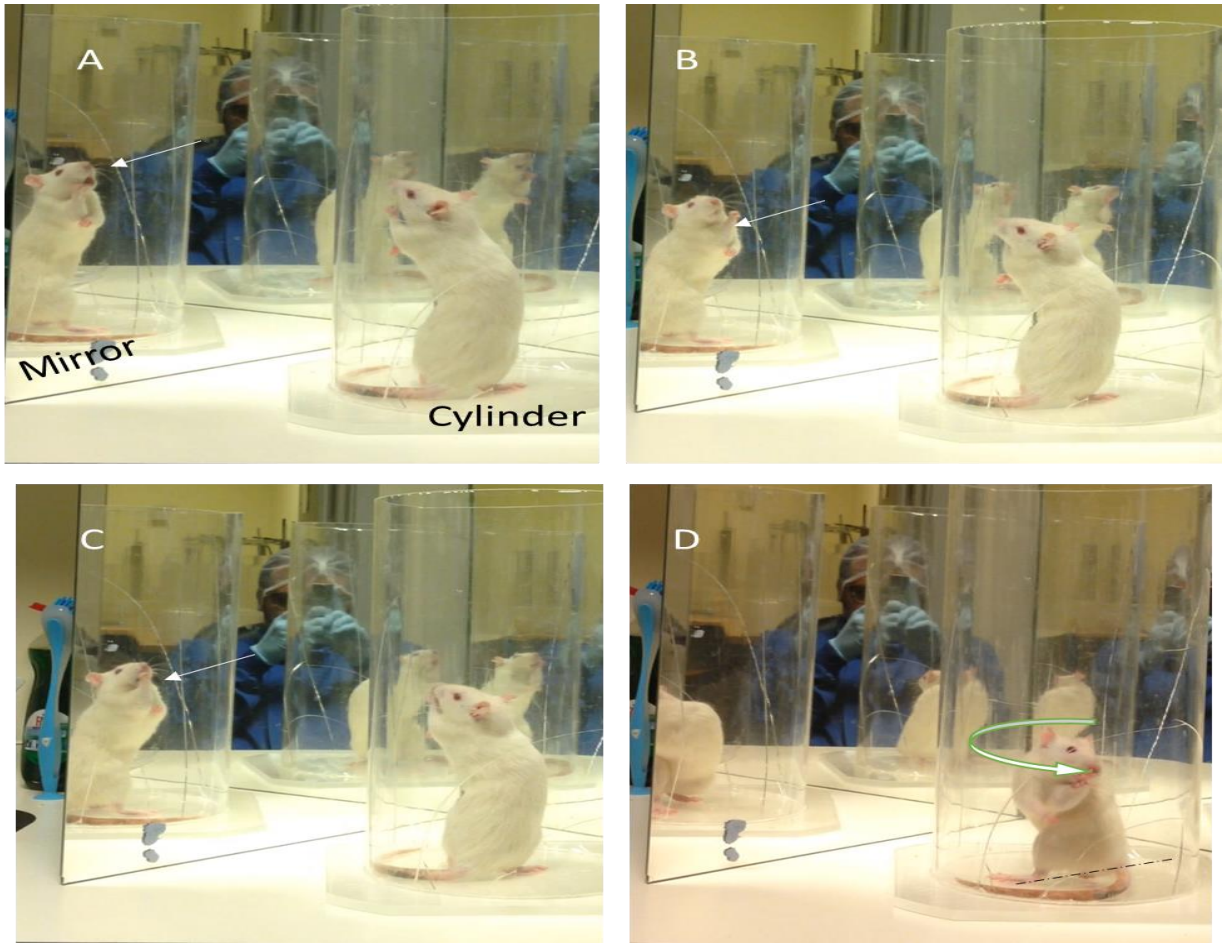


Figure 7 images of Abnormal Involuntary Movements observations during AIMs scoring: image (A) shows jaw movement; following the paw position between the images (B and C) figured out the way of paw oscillation; image (D) showing axial dystonia where the head and the neck deviated about 90 degree of the normal posture.

2.5 Rats perfusion and Fixed Brain extraction

Rats were anesthetized prior to perfusion by injecting a 0.7 ml of 200mg/ml Euthatal solution (i.p.). They were perfused transcardially with a pre-wash of phosphate-buffered saline PBS solution (90 g disodium phosphate, 45 g sodium chloride, 5 L DW, and adjusted with ortho-phosphoric acid at PH 7.4) at a rate of 25 ml per minute for 2 min using an infusion pump. At the same rate, the pump was switched to infuse 4% para-formaldehyde PFA solution (200 g of PFA in 5 L of pre-wash solution) for 4 min. Following perfusion, the heads were cut off and the brains were extracted and post-fixed in 4% PFA solution for 4 hours. After that, the PFA solution were replaced by 25% sucrose solution (250 g of sucrose dissolved in the pre-wash solution) till they saturated and sunk followed by cutting the brain or storing at 4 ^{0}C. A freeze microtome was used to slice the brains to coronal sections with a thickness of 30µm. The

brain sections in 1:12 series were kept in a 1 ml tube filled with an anti-freeze solution (sodium monophosphate 8.72g, sodium diphosphate 2.52g, D.W. 640 ml, ethylene glycol 480 ml, glycerol 480 ml) and stored at 4 ^{0}C till use for histological analysis.

2.6 Fresh brain dissection and other organ tissues collection

Fresh samples of striatum, substantia nigra, hippocampus and frontal cortex were dissected from 3 normal rats. The rats were anaesthetised by injecting 0.8 ml of Euthatal i.p. (200 mg/ml) before decapitation using a guillotine. The rat brains were extracted and put inside rat's brain mould on ice and dissected by inserting blades through the brain on the following distances from the front of the brain: 3 mm, 7 mm, 11 mm and 14 mm. The striatum was dissected from the sections between 3 mm and 11 mm while the frontal cortex was dissected from 0-3 mm. The section between 11 mm and 14 mm was used to dissect the hippocampus and to pinch out a piece of tissue including substantia nigra by using 4 mm pinch pen. The samples were immediately placed in 1.5 ml tubes and kept on dry ice then stored at – 80 ^{0} C till use for protein extraction and western blotting. Other fresh tissue samples were snipped from liver, pancreas, muscles and adipose tissue from normal rats or rats used in the experiments of chapters 4, 5 and 6 and they were yielded prior to perfusion of the rats. They were kept immediately in dry ice and then stored at – 80 ^{0} C for western blotting and histological analysis.

2.7 VM Primary cells plating down and fixation

VM cell culturing and fixation was operated in sterilised cell culture cabinet. The process was started by preparing a sterile 24 wells plate covered with sterile 13 mm glass coverslips and coated with 500 µl 0.01% Poly-lysine PLL to improve adherence. Then VM sections obtained from E14 Wistar rat embryos was dissociated to a single cell suspension by following the same protocol described in section (2.2.2.1). The cells were suspended with a culture media (50 ml DMEM, 1 ml B27 (2%) and 500 µl Foetal Bovine Serum (1%)) to yield a concentration of 50,000 cells per 30 µl. Before seeding the cells, each well was washed 3 times with a sterile distilled water and left under UV light for 30 min to dry. Then 30 µl of the cell suspension was dropped in the centre of the coverslip of each well and left in an incubator at 37 ^{0} C for 3 hrs. The wells were then washed with culture media 3 times before adding a 500 µl of 4% PFA solution in

each well for and left for 10 min. After that, the cells were washed with BPS solution and stored at 4 ^{0}C.

2.8 Plasma collection and analysis:

2.8.1 Blood samples collection

Blood samples were collected immediately before trans-cardial perfusion from the left ventricles of the rats in the experiments of chapters 4, 5, and 6. All rats were food restricted for 9-11 hrs and they were received their regular treatments 80 min before gathering the samples (except in chapter 6, there was no treatment before collecting the blood). 2-3 ml of the blood samples were collected using 23 Gx1'' needle and putted immediately in EDTA tubes (VACUET lavender, greiner bio-one) with 200 µl of Aprotinin solution and mixed thoroughly. They were centrifuged at 2000 G for 15 min using cooling centrifuge system, Eppendorf – 5841. Then plasma supernatant was taken and stored in -80 ^{0}C.

2.8.2 Glucose analysis:

Plasma glucose level measured by using YSI 2300 STAT PLUS™ analyser. The analyser automatically withdrew 25 µl of plasma samples and gave two readings of glucose level (mmol/ L) for each sample then the average value was considered.

2.8.3 Luminex assay:

Luminex xMAP technology were used to determine plasma levels of insulin, endogenous GLP1, IL-6 and TNF-a. Rat Metabolic Magnetic Bead Panel from Millipore Company (RMHMAG-84k) and Bio-Plex 200 system powered by luminex x-map TM technology were used analyse the plasma samples. The panel includes magnetic beads of anti-insulin, anti-GLP-1, anti- IL-6 and anti-TNFα antibodies in addition to other reagents including standard, quality control, 96-well plate, assay buffer, serum matrix, bead diluent, 10 times wash buffer, detection antibodies, streptavidin-phycoerythrin and mixing bottle. The well plate was divided to include double wells for each sample and double wells for 7 standards, 2 quality controls and one background. The antibody beads were mixed together in a bottle then the reagent solutions which include standard, quality control and blank solutions were prepared according to Milliplex map kit guidelines (Milliplex map Kit 2015). Then the Milliplex immunoassay procedure was followed which included adding 200 µl of the Assay Buffer into each well of the plate with shaking for 10 min at room temp. This was then decanted and 25 µl of serum matrix solution added to the background, standard and control wells. A 25 µl of

Assay Buffer was added to the background and sample wells then a 25 µl of the standard, control or sample was putted in the appropriate wells. After that, the mixed antibody beads were vortexed and 25 µl were put in each well followed by sealing the plated and incubating it at 4 ^{0}C overnight. Next day all the well contents were washed 3 times with wash buffer then 50 µl of detection antibodies were added and incubated on plate shaker for 30 min at room temp. 50 µl of streptavidin- Phycoerythrin was then added to each well without decanting the detection antibodies and incubated for 30 min at room temp followed by removing all well contents and washing 3 times with the washing buffer. After that, a 100 µl of Luminex Sheath Fluid (which used to drive the samples to the optic component of the Bio-plex machine) was added to each well before putting the plate inside the machine. Bio-Plex machine set up with all the required information before running which involved: beads region and name of each analyte; concentration of the standards of each analyte; location of the standards, controls, backgrounds and samples in the well map; concentration of the quality controls; sample information (like number of sample for the appropriate well). After running, reports of the results were obtained including standards, controls, background and samples concentrations for each target.

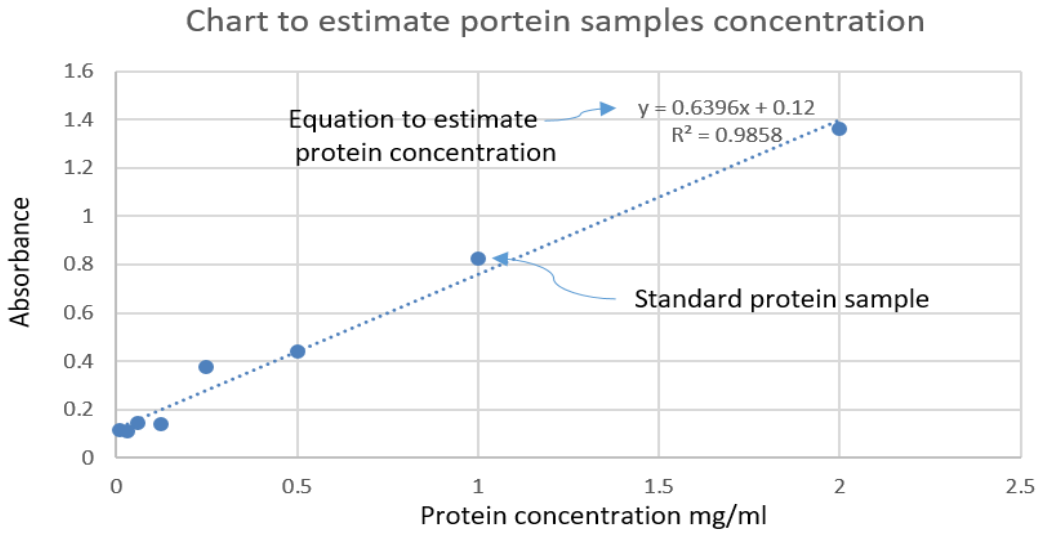
2.9 Protein lysis and Western blots:

2.9.1 Protein lysis:

Frozen tissue samples obtained from fresh organs (described in section 2.6) were mixed with a Lysis buffer (see Table 4) in different proportions (see Table 5) in 2 ml tube containing ceramic beads. Then a protease inhibitor 30 µg/ml and phosphatase inhibitor 60 µg/ml were added to the mixture which kept in ice box during all the procedure time. After that, the tubes were homogenised in a Precellys® 24 homogenizer which was ran 3 cycles at a rate of 5500 rpm for 20 second at each cycle to yield a grinded homogenate of the tissue sample. Then the suspension transferred to 1 ml tube without ceramic beads and centrifuged with a pre-cooled centrifuge (Eppendorf 5810R) at a rate of 17000 G for 60 min. then the supernatant was kept for protein denaturation and protein concentration validation before storing at –20 ^{0} C or using in western blotting. Protein solution were mixed with an equal volume of Laemmli Lysis-buffer (see Table 4) and heated at 90 ^{0}C for 5 min for denaturation.

2.9.2 Protein concentration validation:

Bicinchoninc acid (BCA) assay kit was used to detect the protein concentration of each sample. Protein samples were diluted with deionized water in 1:10 ratio then 10 µl of each sample were loaded in double wells of 96 wells plate. 10 µl of 7 standard concentrations of BSA protein [2, 1, 0.5, 0.125, 0.0625, 0.03, 0.015] mg/ml was also added in double wells of the plate and 10 µl of deionized water was loaded in 2 wells to background. After that, 200 µl of BCA reagents which was prepared in ration of 50 of reagent A to 1 of reagent B (see Table 4) were loaded on all the wells of the samples, standards and blanks followed by incubating the plate for 30 min at 37 ^{0}C. Labtech Microplate Reader LT-5000 ® NS set up on wave length 560 was used to measure the wells absorbance values. Then the samples concentrations were estimated from a protein concentration vs absorbance chart. The chart was created by applying standard samples absorbance (recorded by the machine) with their knowing concentration then form the chart the equation to estimate sample concentration from it absorbance can be obtained (as described in this chart).



2.9.3 Western Blot:

10 % SDS-PAGE running gel (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophorese) (see Table 4) and stacking gel (composition: see Table 4) were used for separating the protein samples. After defrosting, the samples were denatured by heating to 70 ^{0}C prior to loading in the gel and placed in electrophoresis chamber (Bio-Rad Laboratories) containing a proper volume of running buffer (composition, see Table 4) then run at 200 v for 1-3 hrs. Separated proteins were transferred from the gel to a Nitrocellulose blotting membrane (0.2 µm) using

a semi-dry membrane transfer at 39 A for 1 hr which involve scaffolding the gel and the blotting paper with filter papers soaked with semi-dry blotting buffer (composition, see Table 4) inside an electric field. The blotting membrane was then blocked with either 5% non-fat milk (NFM) or 5% BSA in TBST solution (composition: see Table 4) for 1 hr at room temperature. Then the membrane was incubated with a primary antibody at proper dilution in 5% NFM blocking solution and kept overnight at 4 ^{0}C. Next day, the blotting membrane was washed 5 times, 5 min at each wash, with TBST solution followed by incubating with a proper horseradish peroxidase linked secondary antibody solution for 2 hrs at room temperature. Then the blot was washed 5 times with TBST solution followed by bands developing using SuperSignal® West Dura kit. Bands were visualized using was the gel imaging Syngene® G BOX linked to an automatic control software (GeneSys).

Table 4 component of solutions and kits used in protein extraction, protein concertation validation and western blotting.

Reagents or Buffers	Composition
Lysis Buffer	1.46 g NaCl, 0.91 g KCl, 0.15 g MgCl and 0.93 EDTA were dissolved firstly with 230 ml distilled water DW followed by adding 20 ml TBS solution (pH 7.6) plus 625 µl Triton X-100.
BCA kit	Reagent A: 1 gm sodium bicinchoninate (BCA), 2 g sodium carbonate, 0.16 g sodium tartrate, 0.4 g NaOH, and 0.95 g sodium bicarbonate, brought to 100 ml with distilled water (pH to 11.25). Reagent B: 0.4 gm cupric sulfate in 10 ml distilled water
Laemmli Lysis-buffer	bromophenol blue, 0.004%, DTT, 400 mM, glycerol, 20%, SDS, 4%, TRIS, 0.125 M
10 % SDS gel	5.27 ml DW, 100 µl of 10% SDS (sodium dodecyl sulphate) and 1.25 ml (3 M tris Hcl, pH 8.8), 3.33 ml 30% Acrylamide, 50 µl of 10% APS (Ammonium pre sulfate) and 5µl TEMED (tetra methyl etheylen diamine
Running Buffer	Tris base 3 g, Glycine 14.25 g, SDS 0.5 g, DW 1 L, pH 8.3.
Stacking Gel	5.77 ml DW, 1.67 ml 30% Acrylamide, 2.5 ml SDS/Tris (0.4% SDS/ 0.5 M Tris.Hcl pH 6.8), 50 µl of 10% APS, and 5 µl TEMED.
TBS solution	2.4 g Tris base, 8.7 g NaCl, 1 L DW, pH 7.4
TBS-tween 20	1 L TBS and 1 ml of 0.1% tween 20
Semi-dry blotting buffer	5.8 g Tis base, 2.92 g Glycine, 0.38 g SDS, 800 ml DW, 200 ml Methanol.

Table 5 the optimised proportions of tissue samples mixed with the lysis buffer

Tissue sample	Tissue weight/ Lysis buffer volume	Loaded volume in the gel
Striatum tissue	80 mg/ 160 µl	15 µl
Substantia Nigra	14 mg/ 100 µl	30 µl
Frontal Cortex	40 mg/ 250 µl	10 µl
Hippocampus	90 mg/ 90 µl	10 µl
Ventral Mesencephalon	(6 VM sections) 25 mg/ 160 µl	50 µl

2.10 Cells and Histological determinations

2.10.1 DAB Immunohistochemistry (DAB-IHC)

Free floating Immunohistochemistry IHC was used to identify and quantify different protein targets of fixed coronal sections of the brains. One or more of a 1:12 series of brain sections were washed 3 times with TBS solution (Tris base 12 g, sodium chloride 9 g, D.W. 1L, pH 7.4) each wash 5 min. Then the sections were quenched (methanol 10 %, hydrogen peroxide 10 %, D.W. 80 %) for 15 minutes followed by 3 washes with TBS solution, 10 min each wash. After that, they were blocked with an appropriate serum in a concentration of 3% in TBST solution (TBS 250 ml, triton 500 µl) solution for 2 hrs. Without washing, the brain sections were incubated overnight at room temperature with a primary antibody prepared in an optimised concentration in 1% of the block solution. Next day, the tissues were washed 3 times with TBS solution before incubating with a proper biotinylated secondary antibody in 1% serum of TBS solution at concentration of 1:200 for 2 hrs at room temperature. Afterwards the sections were washed 3 times with TBS solution followed by adding Vectastain Elite ABC solution (Biotin/ Avidin system linked to the horse peroxidase) in a concentration of 1:200 of 1% serum TBS solution and incubated for 2 hrs at room temp. The tissue sections were then washed 3 times with TBS solution and 2 times with TNS solution (Tris base 9 gm, D.W. 1L, pH at 7.4) followed by staining with DAB solution (2 ml of 0.1 % of 3,3'- Diaminobenzidine tetra-hydrochloride DAB, 40 ml TNS solution, 12 µl of 0.03% hydrogen peroxide) then washed twice with TNS solution. Finally, they were mounted on gelatinised slides and left to dry at room

temperature. They were then dehydrated by passing the slides through alcohol baths in series of increasing concentrations 70%, 95%, 100% and cleared with double xylene solutions (5 mins in each solution) before cover-slipping with DPX solution (distyrene plasticizer and xylene). (see Appendix A for list of antibody details)

2.10.2 Single or double Fluorescence IHC (F-IHC)

Similar to DAB-IHC, a free floating IHC process was used. The F-IHC started with washing tissue sections with TBS solution 3 times but without quenching step followed by blocking with 3% of a serum in TBST solution for 2 hrs. Then without washing, the blocking solution were replaced by an optimised concentration of primary antibody in 1% serum TBST solution and incubated at room temp overnight. Next, the sections were washed 3 times with TBS solution followed by adding a fluorescent secondary antibody using a concentration of 1:500 in 1% serum TBS solution (all the process after this step involved covering the tissues with an Aluminium foil or placed in a dark area to avoid fluorescence bleaching) then incubated for 2 hrs at room temp. Afterward the tissue sections were washed 3 times with TBS solution then either mounted on slides or processed to add another primary antibody. The process of adding a second primary antibody involved repeating the same steps of blocking, adding primary antibody, washing and adding secondary antibody but with consideration of choosing a secondary antibody with a different florescent dye and not interact with the first antibody. Finally, tissue sections were mounted on gelatinised slides followed by dehydration and coverslip similar to DAB-IHC (see section 2.10.1) but in a dark place. (see Appendix A&B for list of antibody details)

2.10.3 Immunocytochemistry ICC

Fixed cells seeded in PLL coverslips of 24-well plate (see section 2.7) were underwent immunocytochemistry ICC. Starting with 3 washes using 0.1M PBS solution (80 g NaCl, 2.0 g KCl, 21.7 g Na2HPO4, 2.59 g KH2PO4, prepared in 1 L of distilled water, pH 7.4) it was then followed by adding 500 µl of a blocking solution (3% serum, 1% BSA, 0.1% triton X-100, 0.1M PBS,) for 30 min. After that, without washing, the blocking solution containing an appropriate concentration of a primary antibody was added and incubated overnight at 4 ^{0}C. Next day, the cells were washed 3 times with PBS-T (0.1M PBS and 0.1% triton X-100) solution followed by adding a fluorescent secondary antibody in a concentration 1:400 with the blocking solution and incubated for 2 hrs at room temp (all the processes after adding the secondary

antibody was performed with covering the plate with Aluminium foil or in a dark area). Afterwards, the cells were washed with PBS 3 times and the processes of adding a second primary antibody was started. Similarly, the process involved blocking the cells using similar blocking solution but with a suitable serum followed by adding the primary antibody, washing and adding the secondary antibody followed by washing. The species of the used second primary and secondary antibodies were chosen to be not interact with the first primary and secondary antibodies. In addition, the second secondary antibody had fluorescence dye different from the first one. At the end, the coverslips were removed from the plate and mounted on slides using anti-fade mounting media (VECTASHIELD) with DAPI to stain the nuclei. (see Appendix A&B for list of antibody details)

2.10.4 Optimisation of immunoassays

IHC, F-IHC and ICC procedures optimised to detect the target and/ or to get the best image. The process of optimisation involved using a series of antibody concentrations and comparing it with a negative antibody control and/ or incubating the primary antibody for longer period. The target signal, for some fluorescence immunoassays, was measured by using biotinylated secondary antibodies before adding the fluorescent label or by increasing blocking serum concentration or incubation time. In the double immunoassay, each antibody target was optimised separately before detecting them together. (Appendix A pointed to the procedures that involved an adjustment and optimisation)

2.10.5 Oil red O staining

Oil red O staining is a method used to stain lipids in the tissues which depend on the physical property of Oil Red O, a diazole dye, being more soluble in lipids than the vehicle solvent. It was used to quantify lipids in liver samples used in different experiments in this thesis (see section 2.6 for details of liver sample collection). A frozen liver sample was sliced to a 20 µm thickness using a cryostat machine set on -32 ^{0}C and then mounted on negatively charged slides. The sections were then immediately fixed with 4% PFA solution for 10 min followed by washing with a distilled water for 5 min. The slides were dipped in 60% isopropanol solution for 5 sec followed by immersing them in a staining jar containing a filtered ORO dyes diluted with a distilled water in a ratio of 50:50 for 15 min. After that, the slides were dipped again in 60 isopropanol solution for 5 sec then passed in a double washing with distilled water for 10 min. Finally, A Glycerine jelly, an aqueous mounting media, was used to coverslip the slides.

This method was optimised after using a range of tissue section thicknesses, a series of ORO dilution ratios and different dipping time with isopropanol solution.

2.10.6 Haematoxylin and Eosin (H and E) staining

Fixed liver slices obtained from frozen liver samples (see 2.10.5) underwent Haematoxylin and Eosin staining. This method was used to examine the histological and morphological appearance of the liver sections. The slides were passed through a series of solutions according the following schedule: 2X xylene for 4 min; 2X 100% Alcohol for 4 min; 95% Alcohol for 2 min and 70% Alcohol for 2 min. After that, the slides were washed with tap water and dipped in Mayer's Haematoxylin solution for 10 min followed by washing with tap water and stain with 1% aqueous Eosin solution for 2 min. Then the slides rinsed with water and passed on the following series of alcohol solutions: 70% alcohol for 20 sec; 95% alcohol for 20 sec, double 100% alcohol for 3 min and xylene solution for 4 min. At the end, the slides were coverslip using DPX as mountant.

2.11 Microscopy and image analysis

2.11.1 Graft analysis

The number of dopaminergic cells in the graft was determined from counting TH labelled cells. This were determined by direct counting the cells on 20X or 40X magnification on a bright field using Leica light® or Feltiz dilux 22 ® microscope except in chapter 5 where the cells were counted using the stereology microscope (Olympus B 50 linked to Visopharm integrator system software). The total number of TH cells in the graft was estimated by applying Abercrombie equation: $N=\sum \{n \times F \times T/(T+H)\}$ where N = Total corrected number, n= number of the counted cells, F= frequency of the sections, T= thickness of the sections (30um), and H= mean diameter of the cells (Hedreen 1998)**.** The total number of cells in the graft of human Embryonic Stem Cells hESC-derived dopaminergic neurons transplantation, in chapter 5 and 6, was determined by counting HuNu (human nuclei) labelled cells. This was accomplished by obtaining several images at 10X magnification for all graft using Leica light® microscope followed by image analysis for counting using ImageJ 1.51e software. First, to get a clearer image, the images pixel values were inverted followed by background subtraction by 50 pixel-value before re-invertion. Then, the images converted to 8-bit scale followed by an auto threshold determination. After that, a particle analysis function was commanded set up on particle size (5 pixel - infinity) and circularity ratio (0.1-1) to count all the particles (HuNu

labelled cells). Finally, the images were zoomed and screened to count the undetectable particles and adding them to the total number (see Figure 8). The Abercrombie equation was then applied to estimate the total HuNu labelled cells in the graft.

The volume of dopaminergic neurons in the graft was calculated by summation of all the areas containing TH^+ cells in all the striatal sections in the series multiplied by section thickness (30 mm) and by series frequency (12). The surface area was measured either by a software supplied with the microscope (Olympus B 50 microscope linked to Visopharm integrator system software) or by ImageJ software using images captured at 4X magnification. Similarly, the volume of all hESC-derived dopaminergic neurons graft was measured by determining the surface area of STEM121 stained sections (specifically staining human cells cytoplasm) in one 1:12 series.

Dopaminergic graft fibre innervation was evaluated by obtaining images for TH^+ labelled grafts on using Olympus B 50 or Leica light® microscopes. The image analysis for fibre innervation was evaluated by ImageJ 1.51e using two different methods. Both methods depended on measuring images threshold surface area limited to the stained TH markers. However, in the first method, TH density inside the graft border was evaluated (i.e TH fibres and cell bodies, used to evaluate TH density in chapter 4). The TH density was measured by dividing the mean of TH innervation area (threshold surface area) over the graft surface area (see images A and B of Figure 9). while, in the second method, fibre innervation out of the graft border was measured (used to evaluate fibres outgrowth in chapter 5). The section located in the middle of the graft was chosen to estimate fibre innervation toward the medial or lateral sides at distances of 200, 400 and 600 µm from the graft border. 3 vertical lines of squares (200*200 μm^2) extended medially and laterally from the graft then the summation of threshold surface area (fibres surface area) in the squares of each line was calculated (see images C and D of Figure 9).

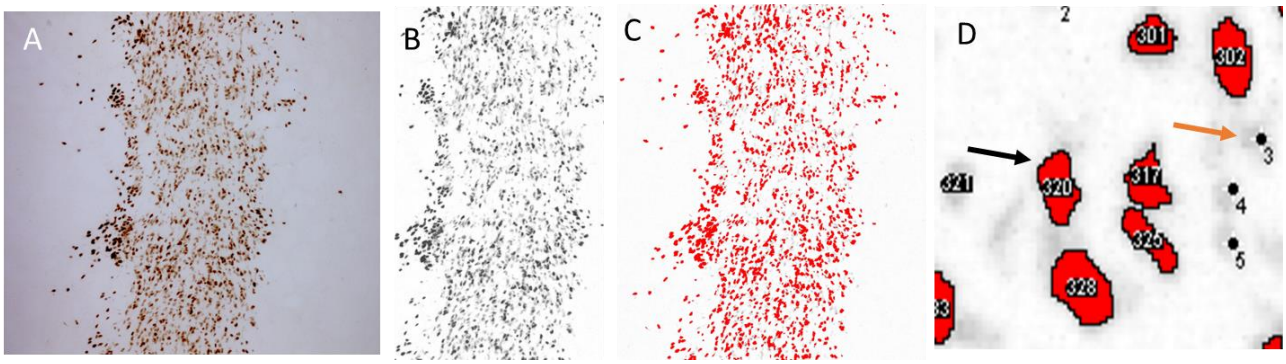


Figure 8 ImageJ software process to count Hunu labelled cell: A, captured image; B, converted image to 8-bit scale; C, auto-threshold to determine the HuNu particles; D, zoomed image showing detected and counted particles by the software (black arrow) and the undetected manually count particles (orange arrow).

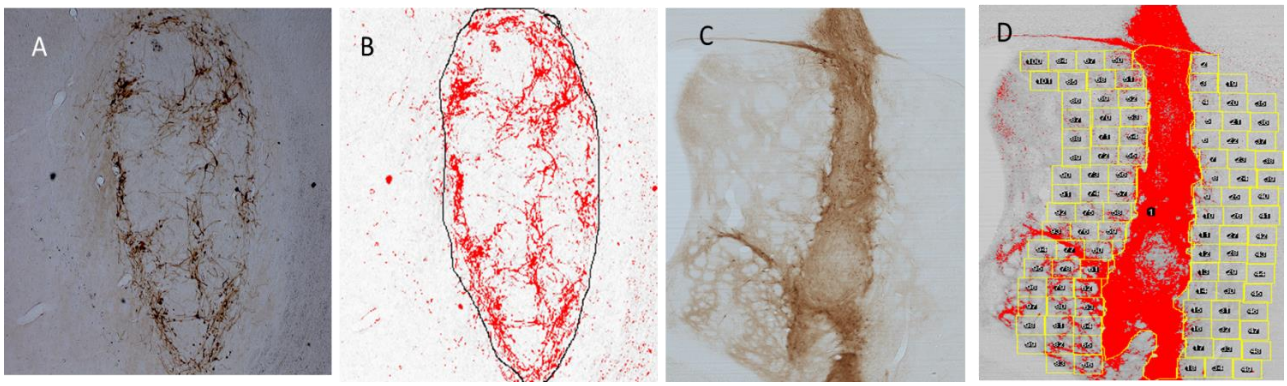


Figure 9 fibres innervation estimations: images A and B shows first method of evaluation; image C and D shows method 2 of evaluation

2.11.2 SN TH^+ lesion analysis

The percentage of the lesion TH^+ labelled cells in the SN were estimated at the level of the medial terminal accessory nucleus of the optic tract where the SN is delineated from the VTA. This located on the following coordination from the bregma: AP = -5.8; ML = +1; DV = -8. The extent of the lesion was measured by expressing the number of the remaining TH^+ cells in the right hemisphere (lesioned) as a percentage of the left side (intact).

2.11.3 Striatal inflammation analysis

Microglial density and leukocyte infiltration was investigated around the transplanted graft in the striatum using CD11b and CD45 markers respectively. CD45 labelled cells were counted directly from under Feltiz dilux 22® microscope then the total number corrected using the Abercrombie equation. Microglial quantification was evaluated by measuring optical density (OD) of CD11b labelled cells in the middle section of the graft. Images were obtained by

Olympus B 50 at 4X magnification and analysed by ImageJ 1.51e. Firstly, the ImageJ was calibrated for measuring the OD according to ImageJ optical density instructions (ImageJ n.d.). The OD was measured around the graft within 40 µm distance of the graft border. Similarly, the OD was measured in corresponding places of the intact striatal side in addition to different points in the cortex to act as a background control. The OD values of the graft and intact striatal sides were subtracted from OD values of the background control. Then evaluation of OD around the graft was expressed as a relative percentage to OD of the intact striatum.

2.11.4 Striatal blood vessels image analysis

Striatal blood vessels were visualised by Leica light® microscope on 10X magnification after labelling with tomato-lectin marker (*Lycopersicon esculentum)*. Then images were obtained and analyzed with ImageJ to quantify the blood vessel surface area. The images were converted to 8-bit scale followed by determining an image threshold (limited to the labelled tomato lectin target) followed by measuring the threshold surface area which was considered a measure of the blood vessel surface area per image. The average reading from 3 different images obtained from the middle graft section was considered.

2.11.5 Analysis of co-localised targets

Co-localisation of two or more targets was examined either under the LEICA DMIRE2® Microscope on 63X objective or using Leica microsystem confocal microscope on 40 objectives. With LEICA DMIRE2® Microscope, the slide position was fixed prior to capturing an image of a certain target followed by switching the laser beam to a proper wave length to detect another marker. The localisation of the targets in the two-dimensional image was then detected using arrow detecting tools of ImageJ. While with confocal microscope, prior to running, the laser bands were adjusted to be within the targets wave length and the Z-width was determined from the bottom and upper limit of existence of the focused section. Following, the optical sectioning was run to get a 3-dimensional structure of the image then the co-localisation of targets was tested at each depth.

2.11.6 Lipids accumulation in the liver

The quantity of fat droplets in the liver sections was estimated by capturing images of Oil Red O stained sections on 10X objectives of Leica light® microscope and analysed with ImageJ. Similar to the method of blood vessels image analysis, 5 images were captured of each liver section and converted to 8-bit image scale followed by determination the image threshold

surface area which reflecting the fat droplets surface area per image. After that, the liver steatosis state was evaluated as a percentage fat surface area per section.

2.11.7 Stereological cells counting

Olympus B 50 Stereology microscope linked to Visopharm software was applied to estimate the total number of TH^+ cell in the graft of chapter 5 using optical fractionator method. This method depends on selecting the area of the interest (graft area) then counting the object (TH^+) located in systematically random samples within the selected area. The sampling method depends on counting spaces covering the entire area of interest then unbiasedly the sample regions are selected in uniform distances in directions of X and Y within the counted spaces. The dimension of the sample box (counting frame) is determined to include an average of 2 to 3 objects per frame. To count the object in the samples criteria rules were followed including: the object should be either inside the frame or intersect with the top or the right vertical line of the frame; while the objects outside the frame or intersect with the bottom or the left vertical line of the frame was excluded. In chapter 5, the area of interest (graft area) was encircled under 4X object of the microscope while the counting the cells in the frames was accomplished under 40X object. The selected dimensions for the frame were 58.7 µm on X- axis and 73.46 µm On the Y-axis. Number of samples was determined by a step length between samples equal to 283.54 µm. Then the total number of cells was counted using the following equation:

$$N = n * A / (a * S) * F * (T / (T+ D);$$

N = total estimated number; n = counted number inside sampling boxes; A = region of interest area (graft surface area); a = frame surface area; S = number of samples; F = series frequency; T = section thickness; D = cell diameter.

2.12 Statistical analysis

The data were analysed using either Prism 5 in chapter 3 or IBM SPSS in the followed chapters (chapter 4, 5 and 6). The statistical analysis method explained in details at each experimental chapter, in short, the behavioural data was analysed using repeated measure analysis of variance ANOVA, except apomorphine test where either one-way or two ANOVA used. In histological data either one-way or two-way ANOVA analysis was used unless T- test was used when it is appropriate. The analysis test followed by post- hoc test to compare the significance

of the difference between the groups and data was considered significant if *p <0.05 and highly significant if **p <0.01 or ***p <0.001.

3 Chapter 3: Impact of ghrelin and JMV-2894 on the survival and efficacy of transplanted allogenic Ventral Mesencephalon cells in 6-OHDA rat model of Parkinson Disease

3.1 Introduction

Ghrelin is described as an acylated hormone consisting of 28 amino acids, isolated for the first time from the rat stomach in 1999 (Kojima et al. 1999). In humans, it is mainly produced by X/A like cells of the gastric mucosa, in addition to other sites including the intestine, lung, heart, pancreas, kidney, testis, pituitary and hypothalamus (Delporte 2013). In human serum, it is present in two main molecular forms: the acyl form, which is responsible for the activation of the ghrelin receptors at physiological concentrations; and the des-acyl form which represents more than 90% of the total serum ghrelin but cannot interact with the receptors at a physiological concentration (Delporte 2013). Pharmacokinetic studies have illustrated that it has a short half-life in the human circulation ranging between 9-11 min in the case of the acyl form while the total, for the acyl and des-acyl form, is about 35 min (Tong et al. 2013)**.** Moreover, it has been established that its serum level is affected by many factors including the circadian cycle, fasting state and exercise (Bertani et al. 2010; Toshinai et al. 2001; Shiiya et al. 2011)**.** Another important pharmacokinetic aspect is its ability to cross the blood brain barrier and interact with the target receptors after a systemic administration (Jerlhag et al. 2012) (Kawahara et al. 2009). Physiologically, ghrelin has shown a wide range of activities including: stimulation of growth hormone secretion, promotion of appetite and food intake, regulation of energy homeostasis , activation of gastric secretion and motility, in addition it interferes with many aspects of the reproductive system, the cardiovascular system, bone formation, pancreatic secretions and anti-inflammatory function (reviewed in (Delporte 2013)**.**

The role of ghrelin in the protection of neurons first emerged from evidence showing that treating cells with a rat serum of hypo-calories (in which ghrelin level increased) leads to improving their biogenic characters and decreases reactive oxygen species (ROS) production (López-Lluch et al. 2006). Evidence later confirmed this protective role of ghrelin and importantly in the dopaminergic neurons of PD models (Andrews et al. 2009). This protection for the dopaminergic neurons and possession anti-inflammatory properties (discussed below 3.1.23.1.3) would make ghrelin a good candidate for refining VM transplantation.

3.1.1 Ghrelin receptors:

Ghrelin receptors or growth hormone secretagogus receptor GHSR-1, which was considered as one of the orphan G protein coupled receptors until the discovery of ghrelin. It consists of

two types: GHSR1a is responsible for exerting the functional activity of acylated ghrelin or synthetic growth secretagogues hormone and consists of 366 amino acids and holds seven transmembrane helix domains (TMD1-7)(Kaiya et al. 2013); The second, GSHR1b, does not have a clear functional activity yet and consists of 289 amino acids and has five transmembrane domains (Kaiya et al. 2013). The molecular signalling of GHS-R1a involves the activation G protein subtypes and causes a subsequent activation to the phospholipase C, inositol triphosphate and intracellular Ca^{2+} pathways (Smith et al. 1999). The downstream signalling is usually affected by the dimerization state which appears between ghrelin receptors and other receptors like the somatostatin receptor (Park et al. 2012), melanocortin-3 receptor (Rediger et al. 2011) and (D1 and D2) dopaminergic receptors (Jiang et al. 2006; Kern et al. 2012). For instance, the heterodimerisation between the D2 receptor and GHS-R1a in hypothalamic neurons causes an allosteric modification to the D2 receptor intracellular signalling as using GHSR-1a antagonist leads to block D2 agonist effect (Kern et al. 2012). Ghrelin receptor distribution in the brain was detected in different regions including hypothalamus, substantia nigra and ventral tegmental area (Howard et al. 1996; Bennett et al. 1997; Zigman et al. 2006). Importantly, the localisation of ghrelin receptor on the dopaminergic neurons of the SNpc (Zigman et al. 2006; Andrews et al. 2009; Jiang et al. 2008) led to discerning a protective effect of ghrelin on dopaminergic neurons in PD models (Moon et al. 2009b; Andrews et al. 2009; Dong et al. 2009a; Jiang et al. 2008).

3.1.2 Anti-inflammatory effect of ghrelin:

It has been suggested that striatal inflammation has repercussions on the survival of dopaminergic neurons transplanted into the dopamine denervated striatum. Consequently, this impairs the motor response achievable by the transplant and in addition may contribute to the GID side effect (discussed previously 1.6.1.4 &1.6.4.2). The potential for ghrelin to have a positive impact on graft transplantation was illuminated from a series of studies coming from both animal and cell line experiments. These have pointed to its capability to suppress harmful inflammatory responses. First, on microglia, ghrelin can inhibit microglial activation and prevent pro-inflammatory cytokine expression when stimulated by different types of microglial activators such as threo-hydroxy-aspartate (THA) and fibrillar β-amyloid protein (fAβ), (Lee et al. 2012; Bulgarelli et al. 2009). Similarly**,** in a mouse Parkinsonian model, Moon and colleagues confirmed that ghrelin has been shown to have an inhibitory

effect on the microglia of the SNpc and striatum when they are activated by MPTP and this effect is mediated indirectly through the inhibition of matrix metalloproteinase-3 (MMP-3) expression of the affected DA neurons. They noticed that ghrelin causes a decrease in the activation of the inducible nitric oxide synthase and the expression of the pro-inflammatory cytokines (TNF-alpha and IL1B) (Moon et al. 2009b). Secondly, on dopaminergic neurons, ghrelin can inhibit release of IL6, a pro-inflammatory cytokine, from dopaminergic neurons when they are stimulated by lipopolysaccharide (LPS) and mediate this effect through their receptors on the DA neurons (Beynon et al. 2013). Thirdly, on leukocytes in the peripheral immune system, ghrelin has demonstrated the capability to prevent proliferation of splenic T cells when they are stimulated by anti-CD3 and it can diminish Th1 and Th2 cytokine (IL2, IFN-γ, IL4, and IL10) mRNA expressions (Xia et al. 2004). Finally, by its anti-apoptotic properties, ghrelin may attenuate microglial activation which has been reported to have originated from apoptotic caspase 8 and 3/7 signalling (reviewed by (Bayliss & Andrews 2013). However, the effect of ghrelin on the inflammatory response in the striatum as a recipient of an ectopic, allogeneic graft has not been investigated yet. This makes the prediction of the beneficial effect of ghrelin on the graft induced inflammation uncertain.

3.1.3 Protection of dopaminergic neurons:

Different studies have illustrated that Ghrelin exerts its neuroprotective effect on the dopaminergic neurons by different mechanisms including anti-apoptotic, anti-oxidant and/or boosting the mitochondrial function. Andrews and colleagues reported that ghrelin can protect the nigral dopaminergic neuron when exposed to MPTP toxin in mice model. They demonstrated that ghrelin mediates the protective effect through uncoupling molecule 2 (UCP2), which is a mitochondrial protein responsible for regulating mitochondrial biogenesis. They also showed that ghrelin increases the number of mitochondria in the nigral dopaminergic neurons and promote mitochondrial respiration and proliferation. This protection occurred only when the exogenous ghrelin was given during the ghrelin physiological window (i.e. at fasting state and dark phase condition) while the osmotic infusion of ghrelin showed no effect. They suggested that ghrelin activates the AMPK-SIRT1-UCP2 pathway similar to its role in the hypothalamus (Andrews et al. 2008) and they attributed the absence of the protection in the normal fed animals to the role of glucose which inhibits this pathway (Andrews et al. 2009).

Other studies have illustrated the anti-apoptotic effect of ghrelin in neuronal and non-neuronal cells (Kui et al. 2009; Miao et al. 2007) and importantly on nigro-striatal dopaminergic neurons. Dong and colleagues have reported that ghrelin can restore mitochondrial membrane potential, reduce reactive oxygen species (ROS) production and abolish caspase 3 levels (the key protein in the apoptotic process) which is induced in MES23.5 cells by the MPTP neurotoxin (Dong et al. 2009a; Jiang et al. 2008). Jiang and colleagues confirmed the anti-apoptotic effect of ghrelin on protecting the dopaminergic neurons in MPTP mice model as ghrelin promoted mRNA expression of Bcl-2 with attenuating Bax expression and reducing caspase-3 activity in the SN (Jiang et al. 2008). Moreover, ghrelin showed an anti-oxidant effect on the dopaminergic neurons. Liu and colleagues used MES23.5 dopaminergic cells challenged with MPTP toxin and they illustrated that ghrelin inhibit the elevation of the malonaldehyde (MDA) level, which was used as an indicator of the extent of the oxidative stress, after exposure to the MPTP. In addition, it reversed the reduction in the concentration of anti-oxidant enzymes, Cu–Zn SOD and catalase (CAT) enzymes(Liu et al. 2010) which are responsible on converting the ROS to hydrogen peroxide then to water (Wang et al. 2007).

3.1.4 Increasing dopamine secretion

The other effect for ghrelin may apply on the transplanted dopaminergic neurons is increasing the dopaminergic neurons firing rate. A previous study has demonstrated that ghrelin propagates the electrical activity of the nigral dopaminergic neurons causing an elevation in the tyrosin hydroxylase enzyme expression and an increase in synaptic dopamine concentration (Andrews et al., 2009). This study was confirmed later when Shi and colleagues showed that ghrelin causes this effect via inhibition of the Kv7/KCNQ/M, voltage-gated potassium –channels which is mediated by binding to GHSR1a receptor and stimulation of the PLC-PKC pathway (Shi et al. 2013). Moreover, apart from its direct action, ghrelin can also increase DA secretion in the striatum via the amplification of the nicotine inducing dopamine released from the dopaminergic neurons. New evidence has shown that ghrelin can exert this role by stimulating the striatum nicotinic acetylcholine receptors (nAchR) in the cholinergic neurons which probably express the GHS-R1a receptors (Palotai et al. 2013). Such an effect may increase the efficiency of the transplanted dopaminergic neurons by providing sufficient synaptic dopamine to stimulate the striatal DA receptors.

3.1.5 Long acting ghrelin receptor agonist JMV-2894

Since ghrelin has short half-life, new GHSR-1a receptor agonist compounds have better pharmacokinetic property and lower cost selectivity was produced. JMV-2894 is one of these compounds that illustrated high affinity and selectivity for GHSR-1a receptor in vitro (Moulin et al. 2007). It also tested in vivo to validate its effectiveness on inducing behavioural food intake and releasing growth hormone. The data clarified it has a potent effect on increasing growth hormone release with no effect on food intake and this effect was abolished in presence of GHSR-1a antagonist (Moulin et al. 2007). There is no data available about the neuroprotection effect of this compound or about its ability to cross blood brain barrier.

3.1.6 Validation of JMV-2894 permeability through the blood brain barrier

Previously ghrelin showed it can induce neurogenesis in the dentate gyrus of hippocampus (Li et al. 2013). In addition, the GHSR-1a receptor has been detected in the dentate gyrus (Zigman et al. 2006). Since there is no enough data about the ability of JMV-2894 to cross blood brain barrier, this effect of JMV-2894 in the hippocampus was tested in the current experiment to validate that JMV-2894 has ability to cross the BBB. In addition, it was used to ensure that the used dose of ghrelin in the current experiment has central action.

3.1.7 Hypothesis, aim and objectives:

It was clarified in the general introduction (section 1.6.1) that one of the challenges facing cell therapy is the low survival rate of the transplanted cells and there are several factors that contribute to the death of the transplanted cells like low trophic factor tone, inflammatory reaction and oxidative stress. From above, ghrelin showed that it can protect the nigral dopaminergic neurons via different mechanisms including anti-apoptotic, anti-inflammatory and anti-oxidative effects. These observations suggest that ghrelin has the potential to support survival of the transplanted dopaminergic neurons. However, the effect of ghrelin on the protecting transplanted dopaminergic neurons in the striatum has not been explored yet and needs to be investigated.

I hypothesise that

- The above neuroprotective and anti-inflammatory properties of ghrelin may support allogenic ventral mesencephalon VM graft survival and efficacy.

The aim of this study was

- Determine whether ghrelin and ghrelin long acting analogue (JMV-2894) administration can improve survival and functionality of VM cells transplanted into the 6-OHDA lesioned rat model of Parkinson Disease.

The specific objectives were

1) confirmation of ghrelin receptor, GSH-R1a, and Ghrelin-O-acyl-transferase (GOAT) enzyme expressions in E14 VM and graft
2) confirmation of GSH-R1a receptor expression in adult rat brain regions
3) evaluation of therapeutic benefit of administration of acyl ghrelin or JMV-2894 with VM graft via
    a. assessment of the motor and behavioural recovery tests and
    b. histological determinations.
4) Validation of the blood brain barrier permeability of JMV-2894 by determining its effect on the hippocampal neurogenesis.

3.2 Methodology and Experimental design

The unilateral nigrostriatal DA neurons of 50 Sprague Dawley rats were lesioned by an infusion of 6OHDA into the medial forebrain bundle using stereotaxic surgery (section 2.2.1). A successful lesion was evaluated 3 weeks after the surgery using amphetamine-induced rotation (rats who rotated 6 or more ipsilateral rotations per minute were expected to have more than 90% DA lesion). In the same week, the deficit in motor activity was measured using the cylinder, stepping and vibrissae tests. Rats were then allocated into 5 groups (n=10), each group has a similar or closer average level of the rotations and the motor tests. A week later, four groups underwent allogenic E14 VM cells transplantation and one group remained as a lesion control (section 2.2.2). Each group received one of the following treatments: saline; acyl ghrelin (10 µg/kg); acyl ghrelin (50 µg/kg) or the ghrelin agonist JMV-2894 (160 µg/kg). The treatments were started immediately after the transplantation and continued for 8 weeks. The recovery of motor tests and amphetamine induced rotations were re-evaluated at 3 timepoints after transplantation (4, 6 and 8 weeks). Then, all the rats were perfused and the brains were extracted and cut for histological analysis (see Figure 10).

DAB-IHC were run to label the dopaminergic neurons in the striatum and the SN using TH as a marker (section 2.10.1). The survival of the dopaminergic neurons in the graft was estimated from direct counting of the TH labelled cells in the transplanted striatum. While percentage of the unilateral nigrostriatal dopaminergic lesion was confirmed from counting TH+ cells in the right and left side of the SN as defined in sections 2.11.1 and 2.11.2.

Ghrelin receptor (GSH-R1a) and Ghrelin-O-acyl transferase enzyme (GOAT) expression in E14 embryonic VM cells of Wistar rats and their co-localisation with markers for ‘stemness’ (SOX2), neuronal progenitors (BIII tubulin) and mature dopaminergic neurons (TH) were determined using the F-ICC assay and Western blot (sections 2.10.2 and 2.9.3). In addition, the presence of GHSR1a in different brain regions of the adult SD rat (striatum, SN, hippocampus and frontal cortex) was investigated using Western blot (section 2.9.3). While expression of the GHSR1a receptor on the grafted dopaminergic neurons was investigated from their co-localisation with TH labelled cells in the graft using double F-IHC (section 2.10.2).

The effect of the peripheral administration of all treatments on hippocampal neurogenesis was investigated by labelling the neuronal progenitor with doublecortin (DCX) antibody using DAB-IHC. The labelled cells were counted in the sub Granular Zone (SGZ) of the Dentate Gyrus (DG) via Olympus B 50 stereology microscope. The granular cell layer (GCL) and (SGZ) area in one section of (1:12) series sections were encircled and measured at 4X objective then all the labelled DCX cells within this area were counted on 20X objectives. The cell density within the counted area was considered to compare between the treatments effect on the neurogenesis.

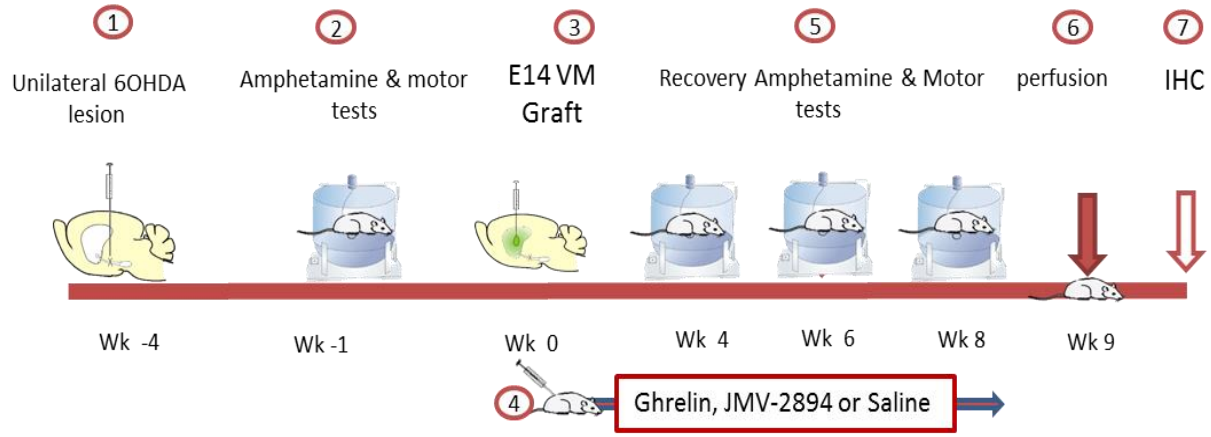


Figure 10 the time line for the in vivo part of the experiment

3.2.1 Treatments

Acyl Ghrelin (rat) was obtained commercially from TOCRIS bioscience (Cat number 1465) in the form of 1 mg reconstituted powder vial and stored at -20 ^{0}C. Each vial was reconstituted with 1000 µl of distilled water then diluted with a normal saline to reach the desired concentrations and stored in the refrigerator at 4 ^{0} C for a maximum of 4 days. JMV-2894 was obtained from Aeterna Zentaris company in the form of 10.25 mg powder which was stored at 4 ^{0}C then reconstituted with distilled water and diluted with normal saline to the desired concentration and then stored at 4 ^{0}c for 14 days.

Doses and schedules were determined by previous work reported in the literature. The effect of ghrelin at a low dose 10 µg/kg and enhanced dose 50 µg/kg once daily was tested in this experiment. Both doses were given intraperitoneally informed by previous work showing that ghrelin can protect the nigrostriatal dopaminergic neurons in an MPTP mice model via peripheral administration (Moon et al. 2009a). While administration of JMV-2894, a long

acting ghrelin agonist (Leyris et al. 2011), at a dose of 160 µg/kg (i.p.) once daily was shown to stimulate growth hormone secretion in rodents (Moulin et al. 2007). Treatments were started at 16:00 every day. All the rats had restricted access to food for the 3 hours after drug administration, aiming to decrease the serum glucose levels which may potentiate the protective effect of the ghrelin (Andrews et al. 2009).

3.2.2 Statistical analysis

The data from the rats who had unsuccessful cell transplantation were excluded (number of excluded rats = 15) therefore the final number of rats in each transplanted group that underwent statistical analysis was between 5 to 7 as the following: graft plus saline (n = 5); graft plus ghrelin 50 (n=6); graft plus ghrelin 10 (n = 7); graft plus JMV-2894 (n = 7). Behavioural data was analysed using IBM SPSS while histological data was analysed using Prism 5. Repeated measure analysis ANOVA was performed to analyse behavioural data followed by Bonferroni post hoc test; time considered as within subject factor while the groups (lesion; graft + saline; graft +ghrelin 10; graft+ ghrelin 50; graft+JMV-2894) considered as between subject factor. The histological data was analysed using one-way ANOVA followed with Dunnett post-hoc test.

3.3 Results

3.3.1 Ghrelin receptor GHS-R1a and related enzymes investigations

As expected, F-ICC for the E14 VM cells illustrated the presence of SOX2, BIII tubulin and TH labelled cells in the mix of cells used for transplantation. GHSR1a co-localised with SOX2 and BIII tubulin demonstrating that it is present in stem cells and differentiated neurons at the time of transplantation (Figure 11). GHSR1a was also co-expressed with TH showing that transplanted dopaminergic neurons are expressing the receptor (Figure 11). Similarly, GOAT was expressed in the differentiated and undifferentiated neurons in addition to co-existence with dopaminergic neurons (Figure 11). Western blot results identified the GHSR1a in samples extracted from 7 different pieces of E14 VM obtained from 2 different mothers. GHSR1a was detected in the SN, striatum, hippocampus and frontal cortex of two adult SD rats (Figure 12). Post mortem double F-IHC analysis illustrated the presence of GHSR1a on the TH labelled cells in the grafted striatum demonstrating that the target was present throughout the transplantation to graft maturity (Figure 13).

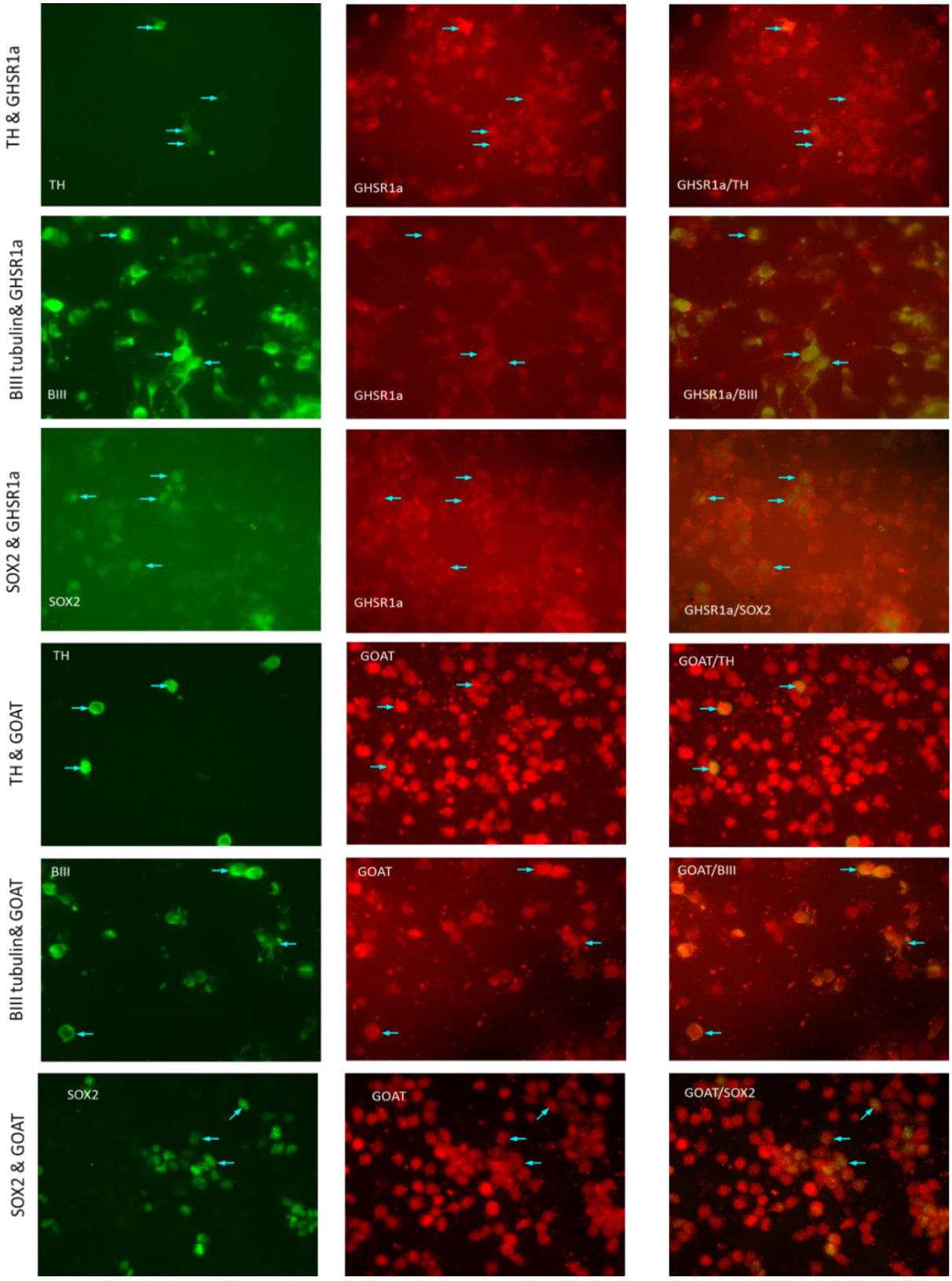


Figure 11 GHSR1a and GOAT expression on VM cells: F-ICC on E14 VM cells showed expression of GHSR (red) with TH, sox2 and BIII tubulin labelled cells (green). Similarly, Ghrelin O Acyl transferase enzyme GOAT (red) expressed on TH, SOX2 and BIII tubulin labelled cells (green).

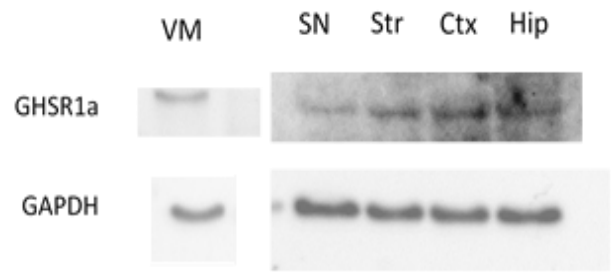


Figure 12 Western Blot analysis: WB showed detection of GHSR1a protein (size 41Kda) in tissue samples dissected VM sections and from the following brain regions of adults female SD rats: SN; hippocampus; striatum; and frontal cortex. The GAPDH protein was used as a house-keeping and detected at size 37 Kda.

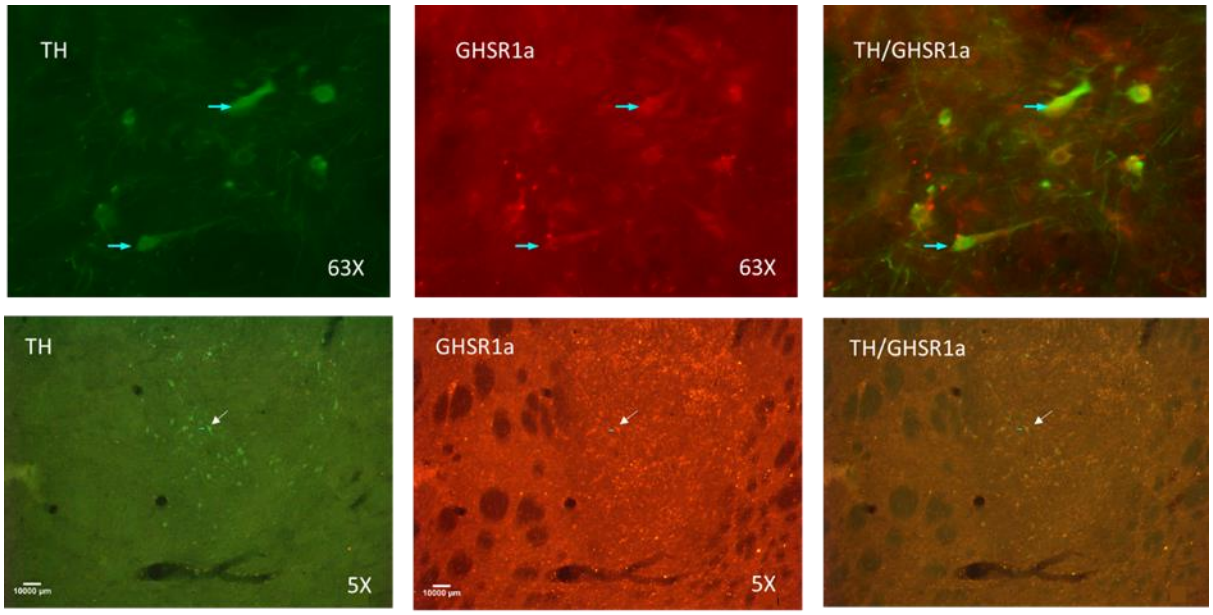


Figure 13 GHSR1a expression in the graft: double F-IHC illustrating co-localisation of the TH and GHSR1a markers on the graft. The images were captured using 5x and 63X microscope objectives.

3.3.2 Motor and behavioural results

In amphetamine-induced rotation task, lesion only control rats showed a sustained rotational bias to the ipsilateral side after amphetamine administration. Data analysis showed a significant difference between the groups ($F_{(4,29)} = 15.4$, $p<0.001$) and significant interaction with the time ($F(4,29) = 7.8$, $p< 0.001$) (repeated measure analysis, groups = between subject factor; time = within subject factor). The group * time analysis showed significant difference between the groups on week 4, 6 and 12 (min: week 6: $F_{(4,29)} = 16.4$, $p<0.001$). The pairwise comparison adjusted with Bonferroni post hoc test at each time point showed that the graft plus saline group has a high significant a reduction in number of ipsilateral rotations compared to lesion control group ($p<0.001$); and there was no difference between the transplanted groups treated with ghrelin (both doses) or JMV-2894 compared to graft plus saline group

(Figure 14). *In vibrissae and stepping motor tasks,* the data analysis showed there is no significant difference between the groups (max: vibrissae test: $F_{(4,29)} = 1.8$, n.s). *In cylinder test,* the data analysis showed significant difference between the groups ($F_{(4,29)} = 3.2$, $p<0.05$), However, the pairwise comparison adjusted with Bonferroni post hoc test showed that there was no significant difference between lesion group and graft plus saline group; and there was no difference between the transplanted groups treated with ghrelin (both doses) or JMV-2894 compared to graft plus saline group. The only difference was between the lesion group and graft plus ghrelin 50 µg group ($p<0.05$), which is not relevant comparison in this experiment.

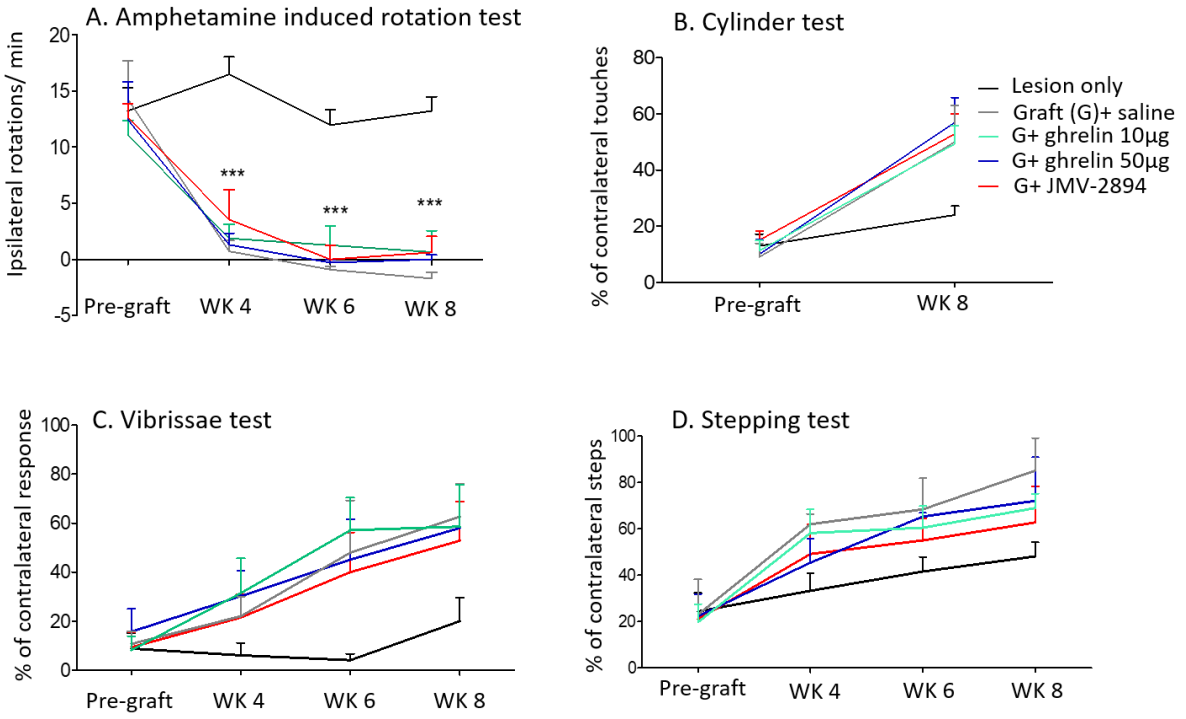


Figure 14 Motor and behavioural outcomes: (figure A) Amphetamine induced rotation test showed that the graft plus saline group ameliorated ipsilateral rotations significantly compared to lesion control (repeated measure analysis one-way ANOVA, ***P< 0.001). (figures B, C & D) cylinder, vibrissae and stepping test illustrated on effect for the graft plus saline to improve rats' performances compared to lesion group and no effect for the ghrelin either dose and JMV-2894 on these tests compared to the graft plus saline control group (repeated measure analysis).

3.3.3 Dopaminergic neuronal survival in the graft and lesion in the SN:

The percentage loss of TH+ cells in the right side of the SN compared to the intact side exceeded 97% in all groups suggesting nearly a complete degeneration of the endogenous nigrostriatal dopaminergic neurons of the right hemisphere. Counting the TH labelled cells in the grafted striatum revealed that TH positive graft were present with average size: graft with

saline group = 891.4±398; graft with JMV= 597±226; graft with ghrelin 50ug/kg= 375±153; graft with ghrelin 10ug/kg= 434±164. There was no effect of drug treatment on graft size, reflecting the lack of difference in behavioural outcomes.

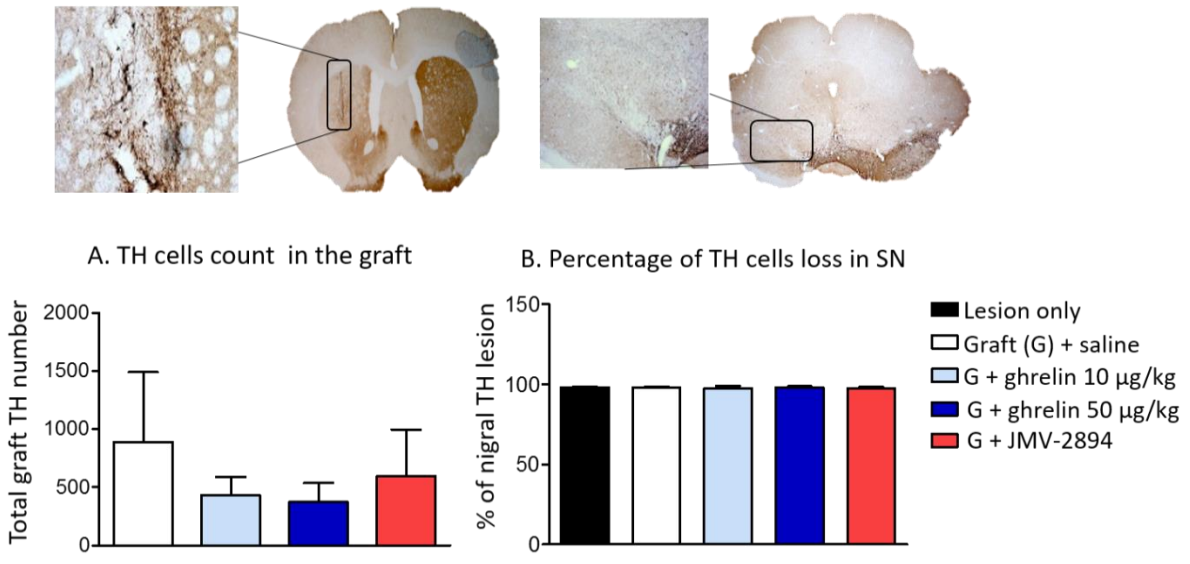


Figure 15 TH cell counting in the graft and SN: (A) mean number of the TH in graft showed there was no significant difference between number of cells that survived in groups treated with ghrelin or JMV 2894 comparing to saline control group (one-way ANOVA analysis). (B) percentage of the degenerated TH labelled cells in the lesioned side compared to the intact side was more than 97% in all groups.

3.3.4 Effects of ghrelin and JMV 2894 on the hippocampal neurogenesis

The DCX labelled cells in the dentate gyrus of the right hemisphere (lesioned or transplanted) and the left side (intact) was compared separately in all groups. On the left hemisphere, ghrelin showed a tendency to increase DCX density at a dose of 50 µg/kg while the lower dose 10 µg/kg exerted a significant increase (one-way ANOVA $p < 0.05$) compared to saline and lesion controls. While, JMV-2894 showed it had no effect on the DCX cells in the dentate gyrus of the intact side (left side). On the right hemisphere, ghrelin showed a tendency to increase the neurogenesis only at the lower dose but without significance. While neither ghrelin 50 µg/kg nor JMV-2894 showed any effect on the DCX cell density of dentate gyrus (Figure 16).

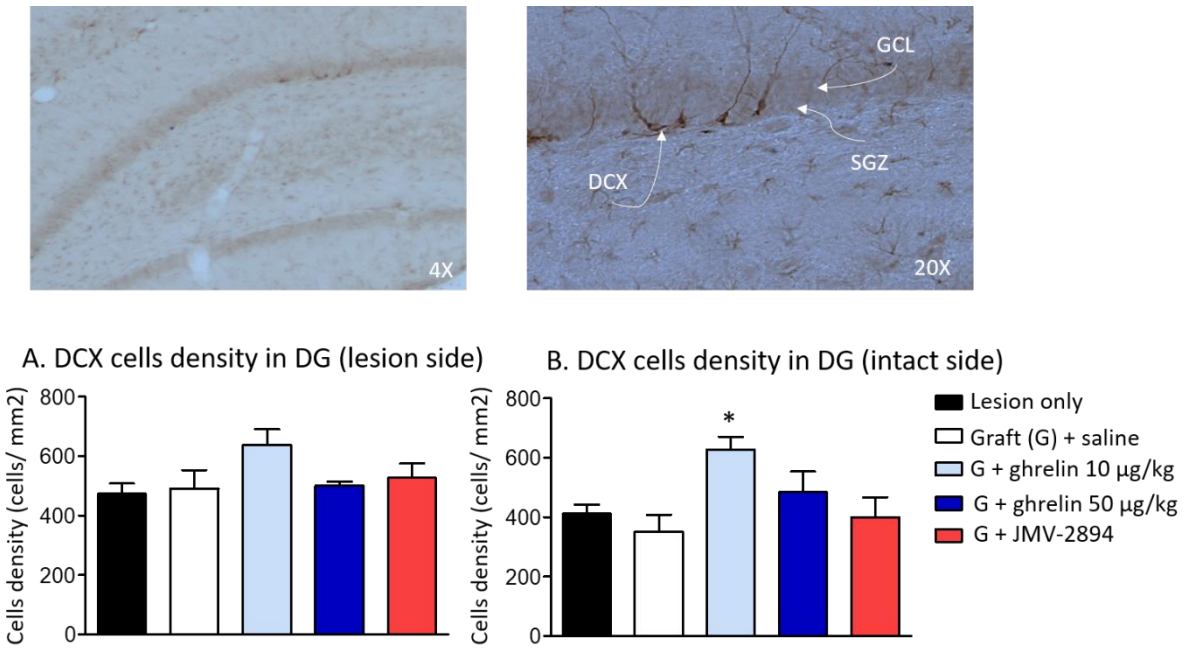


Figure 16: effect of treatments on hippocampal neurogenesis: DCX labelled cell density in the DG of the left (intact) and right (lesion & transplanted) hemisphere was compared between the different groups. In the intact side, the enhanced dose of ghrelin showed a tendency to increase DCX density while the lower does made a significant increase comparing to the control groups (one-way ANOVA, $P< 0.05$) while JMV-2894 didn't show a difference. In the lesion side, only the lower dose-ghrelin group showed a tendency to increase DCX density without significant difference.

3.4 Discussion

This data confirmed the presence of GHSR1a and GOAT on the mature and immature E14 VM cells and importantly on the dopaminergic precursors. This displayed availability of the basic component for transduction of the ghrelin signal into the neurons. GHSR1a is the receptor responsible for the biological function of ghrelin and GOAT is the enzyme responsible for converting the ghrelin to its active form (acylation of the the third serine residue) (Gutierrez et al. 2008). Furthermore, co-expression of GHSR1a with TH in the graft confirmed maintaining the receptor after 8 weeks of transplantation in the host striatum. This would suggest presence of appropriate machinery that ghrelin maintained functional activity on the transplanted cells throughout the experiment. The current data consolidate previous reports that showed expression of GHSR1a in different brain regions SN, hippocampus, cortex (Zigman et al. 2006) and striatum (Kern et al. 2012).

Amelioration of rotation deficit in the amphetamine test implied that the transplanted allogeneic VM cells were functional in all transplanted groups. Amphetamine administration induces release of dopamine into the synapse leading to a difference in dopamine levels between the intact striatum and lesion side and creating asymmetrical rotations (Hefti et al. 1980; Torres & Dunnett 2007). The grafts were capable of compensating for the amphetamine-induced ipsilateral dopamine drive indicating release of dopamine in the lesion side. However, these grafts were inadequate to improve motor performances in stepping and whisker tests and only showed a tendency to improve the response in the cylinder test. This is likely to be due to the graft size being relatively small. Previous studies illustrated that there may be a threshold level required for the size of the graft needed to improve the motor deficits (Hagell & Brundin 2001). Nevertheless, this small graft would give ground for better evaluation of whether ghrelin and JMV-2894 have the potential to increase graft size.

Despite the presence of the appropriate machinery, the behavioural results showed no additional improvement in functional benefit of the graft plus treatments with ghrelin at either dose or with JMV-2894. The behavioural data reflected the histological analysis of the grafts which illustrated no significant difference in the number of surviving TH+ cells in all groups. These observations lead to the conclusion that peripheral administration of ghrelin and JMV-2894 at the doses used does not have a supportive effect on the survival and efficacy of transplanted VM cells. VM dopaminergic neurons are precursors for the developed endogenous nigrostriatal dopaminergic neurons. Ghrelin in previous experiments was protective for these neurons and exerted its protective effect through GHSR1a receptors which was confirmed by either knocking out the receptor (Andrews et al. 2009) or blocking it with D-Lys-3- GHRP-6 (Moon et al. 2009a). This experiment is the first to explore the potential protective effect of ghrelin on E14 VM cells transplanted ectopically in the striatum which could involve factors which halt the protective effect. Moreover, ghrelin has a short half-life, 9-11 min, and was administered as a single daily dose. This was consistent with previous work in a mouse model of PD showing that single daily dosing was effective in protecting dopaminergic neurons from MPTP toxin challenge (Moon et al. 2009). It is reasonable to think that this dose frequency, even if it was protective for the nigral dopaminergic neurons, may not be capable of supporting the survival of dopaminergic neurons in an ectopic environment.

Alternatively, it could be that this would be sufficient in the mouse but it may not sufficient for rat model to achieve the require action.

JMV-2894 is a long acting GHSR1a agonist and didn't show any preferential effect on transplanted cell survival or efficacy. JMV-2894 is a ghrelin receptor agonist product and there is no information about its ability to cross the BBB. However, Moullin and colleagues (Moulin et al. 2007) demonstrated that JMV-2894 (compound number 41 in the paper) was not capable of increasing food intake in rats through peripheral administration, although it was able to increase growth hormone levels. One possible explanation is that it may have poor bioavailability at receptor sites in the pituitary gland to stimulate food intake while it provoked growth hormone levels peripherally via vagus nerve by increasing GHRH and neuropeptide Y release. It has been confirmed that ghrelin can stimulate GHRH release peripherally via the vagus nerve and centrally at the hypothalamus level (Wagner et al. 2009).

To determine whether there was a functional effect of either, and to confirm that they can passed the BBB, I explored the effect of the ghrelin and ghrelin mimetic on inducing the hippocampal neurogenesis. Previous work indicated that ghrelin has a significant effect on increasing the number of DCX labelled neuroblast cells in dentate gyrus from peripheral administration of 80 µg/kg once daily for 8 days in SD rats (Li et al. 2013). My data confirmed this positive effect of ghrelin in both left and right hippocampi while JMV-2894 had no effect on the DCX cells compared to the control groups in both sides. This support to the idea that JMV-2894 was not capable to cross the BBB while ghrelin was available at the hippocampus and induced the neurogenic effect in both low and high doses over 8 weeks.

3.5 Conclusion

Despite the accumulating evidence that supports the protective effect of ghrelin on the dopaminergic neurons in different PD models, this study clarified that peripheral administration of ghrelin and JMV-2894 were not able to support the survival or the efficacy of VM dopaminergic neuron transplantation. The results confirmed expression of the ghrelin receptor GHSR1a and GOAT enzyme on the VM cells at the point of transplantation in addition to maintaining receptors expression on grafted dopaminergic neurons through maturation and innervation of the striatum. However, inadequate dose or administration frequency of ghrelin and the possibility of poor penetration of JMV-2894 through the BBB were suggested to be the reason for halting their effect in supporting the graft. To this end, the next chapter will explore another potential neuro-protective agent that may support graft survival and efficacy from peripheral administration.

4 Chapter 4: Impact of glucagon Like peptide-1 agonists on support survival and efficacy of allogenic ventral mesencephalon transplantation in rats' model of Parkinson Disease

4.1 Introduction

Effect of glucagon like peptide-1 receptor GLP-1R agonists on the neurodegenerative disease started from evidence showed that Type 2 diabetes mellitus T2DM is one of many etiological factors in Alzheimer Disease (AD) (Luchsinger et al. 2007; Ristow 2004; Strachan 2005). So, some hypotheses suggest that brain insulin resistance contributes to the pathology of the disease (Talbot & Wang 2014). For this reason, some trials have investigated normalisation of insulin signalling in the brain using anti-diabetic drugs, including GLP-1 agonists, in an AD model. GLP-1 can cross the blood brain barrier and its receptors are expressed in many regions of the brain, however it has a short half-life and is rapidly degraded by the DDP-4 enzyme which limits its exploration in AD models. Instead exendin-4 and liraglutide, GLP-1 analogues, dominated the studies in the AD and other neurodegenerative diseases because they mediate the same GLP-1 physiological effect when bound to the GLP-1 receptor and they are relatively stable in blood circulation with the capability of crossing the blood brain barrier (Kastin & Akerstrom 2003; Hunter & Hölscher 2012). In an APP/PS1 mouse model of AD, peripheral administration of liraglutide reverses the main neurodegenerative signs of the AD model including preventing synapse loss in the hippocampus, reducing β-amyloid plaque count (pathological mark of the disease) in the cortex, reducing microglia inflammation, increasing neurogenesis in the dentate gyrus and preventing memory impairment in object recognition and water maze tasks (McClean et al. 2011). The neuroprotection effect of GLP-1 agonists in AD models motivated exploration of their effects on other neurodegenerative disease including multiple sclerosis, stroke, amyotrophic lateral sclerosis, peripheral neuropathy (reviewed in (Hölscher 2014)) and importantly in models of PD (next section).

4.1.1 Exendin-4 effects on models of Parkinson's disease

Several studies have tried to characterise the protective effect of exenidin-4 on the dopaminergic neurons and models of PD. Li and colleagues elucidated that exendin-4 has a protective effect on VM dopaminergic neurons in cell culture. The cells were exposed to 6-OHDA which caused a significant reduction in number of TH^+ cells by 30%, while the addition of exendin-4 completely preserved the cells. Furthermore, it caused an elevation in the yield of TH^+ cells by 60% in the absence of 6-OHDA. The same group explored the mechanisms by which exendin-4 protected the cells in the SH-SY5Y cell line and they found that exendin-4

inhibits levels of pro-apoptotic proteins, caspase-3 and BAX and elevates levels of anti-apoptotic proteins Bcl2 after exposure to 6-OHDA (Li et al. 2009).

In the MPTP treated mouse model of PD, peripheral administration of exendin-4 caused a significant reduction in the loss of SN neurons and striatal fibres (Kim et al. 2009b; Li et al. 2009). The same study showed a parallel reversal of motor deficits in rotarod, pole test, beam walk, and open-field activity tests (Li et al. 2009). Exendin-4 is also involved in reduction of microgliosis in the SN and the striatum of the mice after MPTP challenge. In addition, it caused a reduction of the matrix metalloproteinase-3 (MMP-3) expression on the SN dopaminergic neurons, which is involved in microglial activation, and reduced levels of microglial derived pro-inflammatory mediators (TNF-α and IL-1β) after MPTP exposure (Kim et al. 2009b). Similarly in 6-OHDA and LPS rat models of PD, exendin-4 protected nigral dopaminergic neurons, increased levels of striatal dopamine and reversed the deficit in the behavioural test (apomorphine-induced rotation test) (Harkavyi et al. 2008).

Exendin-4 efficacy on the progression PD was assessed in a single blind clinical trial of 45 patients. Twice daily Exenatide (exendin-4) for 12 months improved scores in the Movement Disorders Society Unified Parkinson's Rating Scale (MDS-UPDRS) by 2.7 points in the treated group while the control group declined by 2.2 points (Iciar Aviles-Olmos et al. 2013). Those patients had been followed up in an open labelled randomised control trial after 12 months of cessation of the exenatide treatment. The results showed that the patients who were previously treated with exenatide maintained improved MDS-UPDRS scores compared to the control group by 5.6 points (Aviles-Olmos et al. 2014). These data illustrate a potential advantage of using exenatide in modulating PD progression which may be mediated through neuroprotective effects on the nigral dopaminergic neurons.

4.1.2 GLP-1R agonists effects on other neurodegenerative disease models

From cell culture models, cumulative evidence has demonstrated that GLP-1 agonists have neuroprotective properties on different types of neurons. Perry and colleagues explored the effect of GLP-1 and exendin-4 on pheochromocytoma (PC12) cell line (mostly used for studying neuronal differentiation) which expressed GLP1 receptors. Both treatments induced neurite outgrowth similar to the effect of nerve growth factor NGF. Exendin-4 potentiated the effect of NGF on inducing neuronal differentiation, in addition it inhibited neuronal degeneration in NGF deprived media (Perry et al. 2002). Exendin-4 may also be able to protect

cultured hippocampal neurons against cell death when exposed to beta amyloid peptide (neurotoxic molecule played a role in pathogenesis of AD) and oxidative insults (Perry et al. 2003). In human neuroblastoma cell line SH-SY5Y, Sharma and colleagues found that liraglutide can improve cell viability and reduce cytotoxicity and apoptosis when stressed with methyl glyoxal (stressor attenuated energy metabolism in the cells). They also reported an increase in the expression of anti-apoptotic protein Mcl-1 and reduce the pro-apoptotic proteins Bax and Bik (Sharma et al. 2014).

Other studies suggested that GLP-1 agonists could have modulatory effects on neuroinflammation. In cell culture, Iwai *et al*. identified the presence of GLP-1 receptor on microglia and astrocytes and they reported that GLP-1 prevented release of IL-1β (pro-inflammatory cytokines) from the microglia after lipopolysaccharide LPS (endotoxin) induction (Iwai et al. 2006). In APP/PS mice (AD model), liraglutide reduced levels of activated microglia associated with amyloid-β plaque pathology of the disease (McClean et al. 2011; Long-Smith et al. 2013). Other studies exposed mouse brain to x- ray irradiation to induce chronic inflammation characterised by increasing levels of activated microglia and concentration of pro-inflammatory cytokines IL-6 and IL-2 in the dentate gyrus and cortex while pre-treatment with liraglutide illustrated high significance on reducing the inflammation (Parthsarathy & Hölscher 2013).

4.1.3 Glucagon like peptide-1

Glucagon like peptide-1 (GLP-1) is an incretin hormone secreted from L-cells in the distal ileum (Eissele et al. 1992). It is responsible for the augmentation of insulin secretion under high glucose conditions (Kreymann et al. 1987; Gromada et al. 1998). It also induces B cell proliferation and increase resistance to apoptosis (Drucker & Nauck 2006; Li et al. 2003). It has a role in maintaining a variety of haemostatic functions like feeding behaviour, gastric motility, glucose regulation and cardio-vascular function via its receptors in the peripheral and central nervous system (Baggio & Drucker 2007). Human plasma levels of GLP-1 are 5 to 10 pmol/L in fasting conditions which increase 2 to 3 fold after meals (Orskov et al. 1994). This level is affected by food intake which causes an increase in GLP-1 level after 10-15 min of the meal followed by a second increase after 30-60 min (Herrmann et al. 1995). GLP-1 levels are increased by leptin release (Anini & Brubaker 2003) while its release is decreased by insulin, insulin resistance, somatostatin and neuropeptide galanin (Baggio & Drucker 2007).

Exercise is another factor which has been reported to have effects on GLP-1 levels as acute to moderate exercise boosts GLP-1 secretion after food intake (Martins et al. 2007; Ueda et al. 2009; Martins et al. 2010). GLP-1 is rapidly deactivated in the circulation by ubiquitous proteolytic enzyme dipeptidyl peptidase-4 (DDP-4) to its N- terminal metabolite(Deacon et al. 1995) with a short half-life about 5-6 min (Ørskov et al. 1993) while the intact GLP-1 is cleared via kidney with half-life of 2 minutes (Ruiz-Grande et al. 1993).

The GLP-1 receptor is related to the B-family of 7- transmembrane spanning, heterotrimeric G- protein coupled receptors GPCR (Mayo et al. 2003). Binding of the ligand to the GPCR causes dissociation the heterotrimeric G-protein to G_{α} and $G_{\beta\lambda}$ subunits which each may link to effector molecules (Wess 1997). GLP-1 receptor is similar to other GPCR, the binding of the ligand to the receptor leads to activate the Gα subunit. The GLP-1R ligands mostly activate Gαs which is associated with activation of adenylate cyclase, however it is also able to activate Gαq, associated with activation phospholipase C, and Gαi, associated with inhibition to the cAMP (Fletcher et al. 2016). These causes that GLP-1 agonists have more than one downstream signalling pathways. Different agents of GLP-1R agonists have different bias in selecting the signalling pathway which results in some differences in clinical effects of the GLP-1 agonists (Pabreja et al. 2014). Extended exposure of the GLP-1 receptor to the ligand is terminated by a regulatory mechanism via two methods: 1. Desensitisation, which involves direct cessation of the signalling cascade by uncoupling with G protein or indirectly via phosphorylation of one type of the receptor and desensitise other type; 2. Internalisation, which involves endocytosis of the receptor-ligand complex, followed by recycling the receptor to the cell surface (Fletcher et al. 2016). GLP-1 receptor is located at several tissues including pancreatic cells, lung, heart, kidney, stomach, intestine, pituitary, skin and ganglion neurons of the vagus nerve (reviewed in (Baggio & Drucker 2007). It is also located in different CNS sites like the hippocampus, cerebellum, neocortex (Hamilton & Hölscher 2009) and hypothalamus (Acuna-Goycolea & van den Pol 2004). Moreover, Yazhou and colleagues identified expression and functionality of GLP1 receptors on the embryonic ventral mesencephalon and cerebrocortical cells (Li et al. 2009).

4.1.4 Exendin-4 (exenatide):

Exendin-4 is a GLP-1 receptor agonist consisting of 39 amino acids terminated with an amino group histidine residue, isolated for the first time from Glia lizard venom *Heloderma*

suspectum in 1992 (Eng et al. 1992). The amino acid sequence of exendin-4 is identical to 53% of the amino acid sequence of endogenous GLP1 (Blonde & Montanya 2012). The presence of a glycine at position 2 makes it resistant to degradation by the DDP-4 enzyme and circulates in blood vessels for a longer time compared to the endogenous GLP1 with a half-life range between 60-90 minutes (Kolterman et al. 2005) and its biological action on lowering glucose levels extends for 4 hrs after a single dose (Young et al. 1999). The downstream signalling of exendin-4 results from activation of Gαi and Gαs protein subtype in the GLP-1 receptor with bias toward the inhibitory subtype Gαi (Weston et al. 2014), while the receptor-ligand complex termination is regulated via internalisation (Gao & Jusko 2012; Gao & Jusko 2011). Clearance of exendin-4 from the body is achieved by degradation to peptide fragments in kidney membranes and eliminated either intact or as fragmented peptide via glomerular filtration and tubular secretion (Copley et al. 2006). Some of these fragmented peptides have been reported to have low to moderate antagonism potency on GLP1 receptors like exenatide (15-39) and exenatide (16-39) (Copley et al. 2006).

Exendin-4 was registered as a treatment for T2DM patients by the Food and Drug administration in the United States in 2005. Clinical trials revealed that exendin-4 is effective in the treatment of T2DM patients whose glucose levels are not controlled by metformin and/or sulfonylurea treatments. Twice daily sub-cutaneous injection of exendin-4 is effective in controlling fast and postprandial glucose levels, reducing HbA1c levels and improving B cells function markers (Fineman et al. 2003; DeFronzo et al. 2005). The most common side effects reported in clinical trials were nausea, usually happening in the first week of the therapy, and mild to moderate hypoglycaemia when its used at therapeutic doses higher than 10 µg twice daily (Kendall et al. 2005; Buse et al. 2004).

4.1.5 Liraglutide:

Liraglutide is another GLP1 agonist anti-diabetic drug that has been approved for clinical use. It has a longer half-life (12.53hr) than exendin-4 and longer control on glycaemia which can be maintained by a single daily dose (Campbell 2011; Han et al. 2013). Liraglutide has 97% amino acid sequence homology with GLP-1 but it differs in that it includes a palmitic acid moiety linked to the lysine at position 26 via glutamic acid spacer (Madsen et al. 2007) (Figure 17). It has a delayed absorption after subcutaneous injection and lower affinity for degradation by PPD4 enzyme which leads to a prolonged half-life and efficacy (Malm-erjefält

et al. 2010; Blonde & Montanya 2012). Dissimilar to exendin-4, the downstream signalling of liraglutide results from activation of both Gαi and Gαs subtypes without bias (Weston et al. 2014) and it has 10 times higher affinity to undergo receptor complex internalisation (Roed et al. 2014) which may be the reasons for some differences between their effects.

Liraglutide has the same action as exendin-4 on alleviating hyperglycaemia via insulintropic effects and suppression of glucagon release (Blonde & Montanya 2012). However, liraglutide showed it has a more significant effect on reducing fast plasma glucose levels and HbA_{1c} than exendin-4 while exendin-4 it is more effective in reducing postprandial glucose level (Buse et al. 2009). The main side effects of liraglutide are mild nausea, vomiting and diarrhoea with a lower incidence of hypoglycaemia compared to exendin-4 (Campbell 2011).

GLP-1 (7-36)NH2 : HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH2

Exendin-4: HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS

Liraglutide: HAEGTFTSDVSSYLEGQAAKEFIAWLVRGR-NH2

E

Figure 17 amino acid sequences of GLP-1, exendin-4 and liraglutide. The highlighted amino acid block represents the change from GLP-1 structure (modified from (Pabreja et al. 2014)).

4.1.6 Aims and objectives of this chapter

Exendin-4 has shown that it can protect the dopaminergic neurons in cell culture and animal models. In addition, another GLP-1 agonist, liraglutide demonstrated advantages on protecting the neurons and reducing brain inflammation in different models. This raised a question about the capablity of GLP-1 agonists on supporting survival and efficacy of cell therapy in PD which illustrated incosistencey in efficacy and low rate of cell survival in animal models and clinical trials. Specifically, exendin-4 and liraglutide hold the advantages of crossing the blood brain barrier and their safety is already approved for clinical use which would make them ideal agents for supporting cell transplantation. It was hypothesised that both exendin-4 and liraglutide can support survival and efficacy of allogenic VM cell transplantation in a 6-OHDA rat model. This needs to be tested in a clinically relevant animal model by exposure to a chronic anti-PD medication, like L-dopa, before and after cell

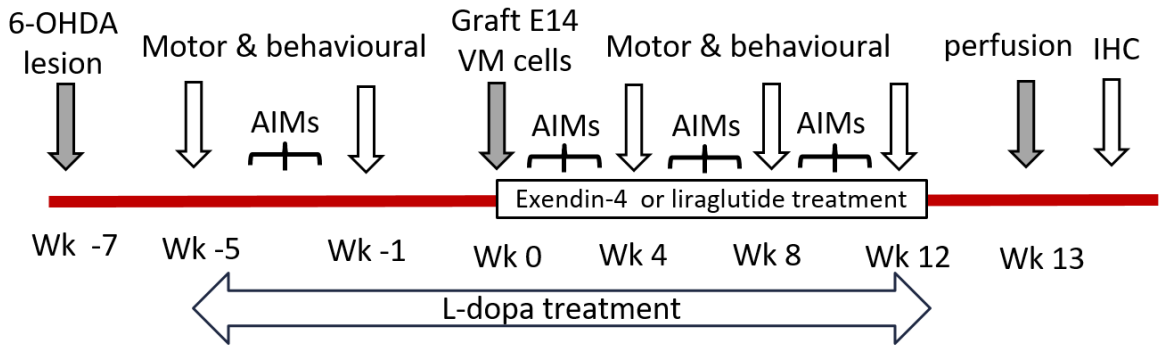
transplantation and using allograft transplantation to add a level of immunolgical reaction. So the aims of this chapter are to determine whether:

1. GLP-1 agonists (exendin-4 and liaglutide) can be effective in improving the survival and efficacy of transplanted VM dopaminergic neurons in a 6-OHDA parkinsonian rat model.
2. L-dopa has additional impact on the neuroprotective effect of the GLP-1 agonists (exendin-4 and liraglutide) on the graft.

The following objectives were followed to test the hypothesis of that exendin-4 and liraglutide are able to support survival and efficacy of allogneic VM graft in presence and abscnese of L-dopa:

1. Detection expression of GLP-1 receptors on the transplanted cells at the transplantation time and on the graft during the post-mortem analysis.
2. Assessment of exendin-4 and liraglutide influence on the motor and behavioural recovery in the presence and absence of L-dopa treatment in 6-OHDA lesioned rats.
3. Determination the effect exendin-4 and liraglutide on post-mortem histological measurments of the graft in presence of absence of L-dopa.

4.2 Methodology and Experimental design:



Rats divided into the following groups:

A. Lesion control (n=8)
B. Graft+ saline (n=8)
C. Graft+ exendin-4 (n=9)
D. Graft+ liraglutide (n=6)
E. Graft+ L-dopa (n=8)
F. Graft+ L-dopa+ exendin-4 (n=8)
G. Graft+ L-dopa+ liraglutide (n=7)

Figure 18 the experiment groups and time line

Nigrostriatal dopaminergic neurons were degenerated unilaterally by infusion of 6OHDA into the right medial forebrain bundle of 70 female Sprague Dawley rats (section 2.2.1). The lesions were assessed 2 weeks later by performance in the amphetamine-induced rotation (ipsilateral rotations ≥ 6 times/ min, was considered to be successful, ie a loss of more than 90% of the dopaminergic neurons). 9 rats were excluded due to incomplete lesion and 5 rats excluded due to unsuccessful transplantation because of technical issue (number of rat per group in figure 18). The rats were then allocated into 7 groups; one group was the lesion control and 6 groups were transplanted with Wister E14 VM cells into the striatum of the right hemisphere (section 2.2.2). The grafted groups were subdivided into two sets each containing 3 groups: the first set received L-dopa and pair of them treated with either exendin-4 or liraglutide; the second set was without L-dopa and received either exendin-4, liraglutide or saline (see Figure 18). L-dopa treatment was started on week 3 after the lesion surgery and continued for 5 weeks before transplantation surgery. The amphetamine-induced rotations and simple hand motor tests were carried out before starting the L-dopa treatment, one week before cell transplantation and three times after the transplantation. While the Abnormal Involuntary Movements (AIMs) and L-dopa-induced rotations tests were recorded once

weekly before and after cell transplantation (section 2.4). L-dopa and GLP-1 agonists were stopped 2 days before each behavioural and motor test to wash out any acute effect. Blood sampling and perfusion were carried out at week 13 post transplantation. The rats were on food restriction for 9 hrs before blood sample collections and they received their appropriate treatment 80 min before blood sampling. This was followed by perfusion of the rats and collection of the brains for histological analysis. The cellular and histological determinations included: IHC to detect TH, CD11b, CD45, 5HT and IRS-1 pS^{1011} markers (section 2.10.1); F-IHC to detect striatal blood vessels using the marker tomato lectin (section 2.10.2); double F-IHC to detect co-localisation of TH with Girk2 and TH with GLP1R (section 2.10.2); double F-ICC to detect co-localisation of GLP1R with TH, GLP1R with SOX2 and GLP1R with BIII tubulin (section 2.10.3); Oil Red-O staining to stain fat in the liver (section 2.10.5); Eosin and Haematoxylin to stain liver sections (section 2.10.6). Western blot analysis was used to determine GLP1R in the substantia nigra, striatum, hippocampus, frontal cortex (section 2.9.3). Plasma analysis was used to determine insulin, glucose and GLP1 levels (section 2.8).

4.2.1 Treatments

L-dopa was combined with benserazide (section 2.4.1) at a dose of 12 mg/kg (s.c.). The L-dopa/ benseraside treatment commenced 3 weeks after lesion surgery for 5 weeks before transplantation surgery with a single daily dose for the first week then with a single dose each alternative day. L-dopa treatment continued after transplantation surgery until the end of the experiment on week 12 using the same dose each alternate day. This dose combination was used and obtimised previously in our lab (Breger 2013).

Exendin-4 was given (i.p.) at a dose of 0.5 µg/ kg twice daily. This was consistent with a previous study illustrating that 0.5 µg/kg tiwce daily of exendin-4 was effective on protecting the dopaminergic neurons in a 6-OHDA rat model (Harkavyi et al. 2008). The treatments started immediately after transplatation of the VM cells in the depleted striatum and continued until week 12 post transplantation. Exendin-4 (1 mg vial, Tocris Bioscience) was dissolved in 1 ml of 0.9% sterlized normal saline then aliquoted into 30 aliquots and stored on -20 ^{0}C for a maximum of 30 days.

Liraglutide was administered to the rats (i.p.) at a dose of 100ug/kg. This dose was selected based on a previous study in an AD rat model (McClean et al. 2010). Pre-filled pen liraglutide

(Victoza$^{(R)}$) at concentration 6 mg/ml then 83 µl of the drug solution withdrawn from the pen and diluted with 5 ml of 0.9% saline to get a concentration 100ug/ml prior to injection.

4.2.2 Statistical analysis

The data analysis was performed using IBM SPSS while Prism 5 software was used to create the figures. For the assessment of graft effect compare to lesion control in behavioural tests, the graft plus saline group and lesion only group were analysed separately.

Repeated measure ANOVA (time was the within subject factor; treatment group was the between subject factor) was performed for the behavioural data. Two-way ANOVA was applied for the histological and blood analysis data (between subject factor include L-dopa, exendin-4 and liraglutide). Bonferroni's post hoc test was used for pairwise comparisons and statistical adjustment. Two tailed t-test was used to compare between lesion control and graft plus exendin-4 group in oil liver test.

All the data sets have been checked for the normality distribution test. In amphetamine rotation test, (graft+ exendin-4) and (graft+ L-dopa+ liraglutide) groups had significant abnormality distribution (Shapiro-Wilk test, $p<0.001$). Two outliers, one in each group, were detected and excluded from the analysis. The outlier was considered if it has a value lower or higher than the double standard deviation distance from the data mean.

4.3 Results

4.3.1 GLP1 receptor expression in the VM cells and the graft:

Investigation of the GLP1 receptor on E14 VM cell populations identified its co-expression on the neurons marked with BIII tubulin and importantly on the dopaminergic neurons (TH+). The GLP-1R was not present on immature stem cells marked as SOX2 expressing cells (Figure 19). This demonstrates that it appears in the relevant grafted population of cells at maturity. Western blot analysis detected the GLP1 receptor proteins in the E14 VM sections obtained from 7 embryos collected from two Wistar rats which is representative of the tissue that was transplanted. The post-mortem analysis of the brains of transplanted rats, showed co-expression of the GLP-1 receptor on the grafted dopaminergic neurons TH^+ after 13 weeks of the transplantation (Figure 21). In adult SD rats, the western blot analysis identified the GLP-1 receptor in different brain regions including striatum, substantia nigra, hippocampus and frontal cortex which are representative of the host brain (Figure 20).

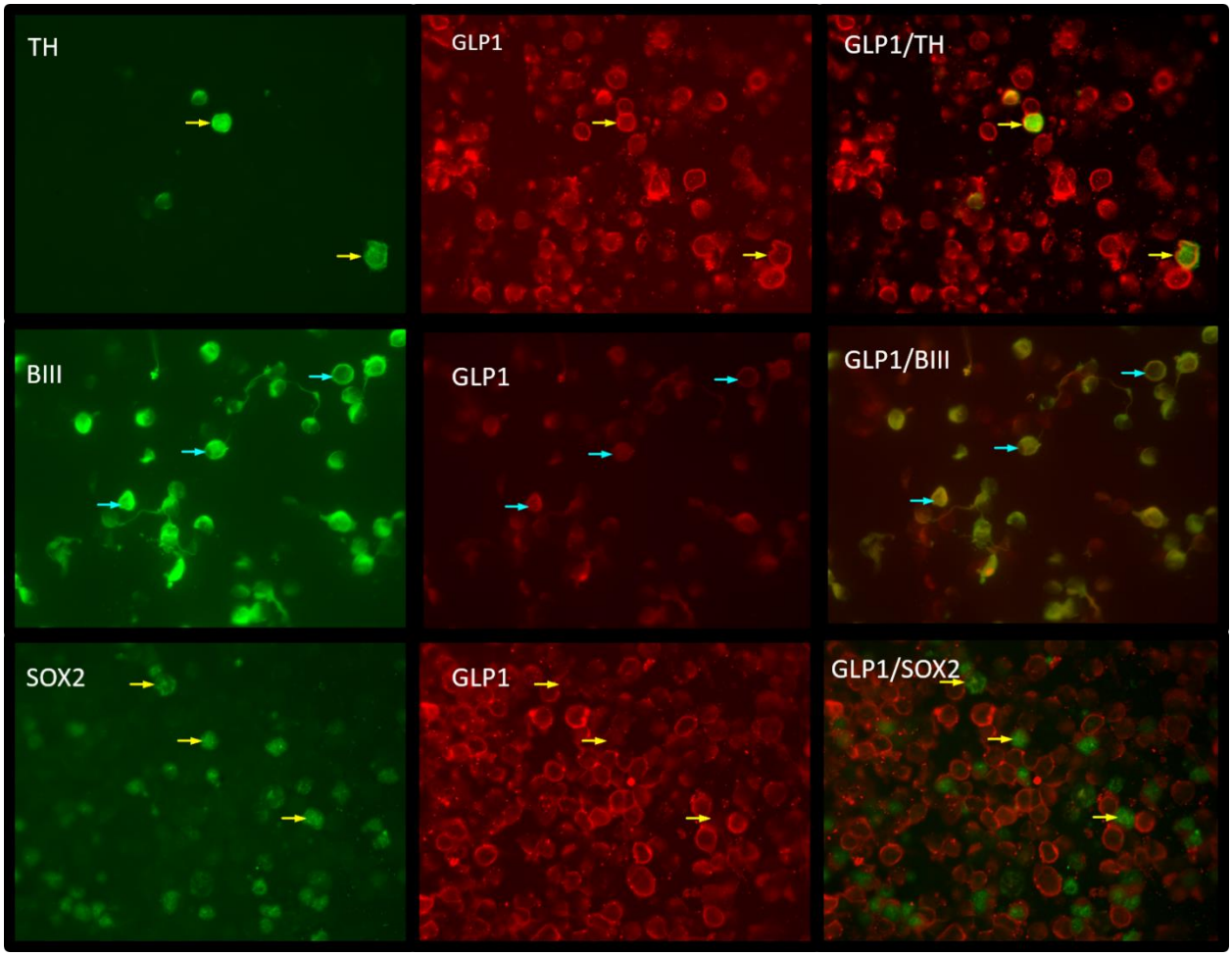


Figure 19 GLP1R expression in E14 VM cells: F-ICC showed expression of GLP1R (red labelled cells) with BIII tubulin and TH cells but it is not present with SOX2 (green labelled cells).

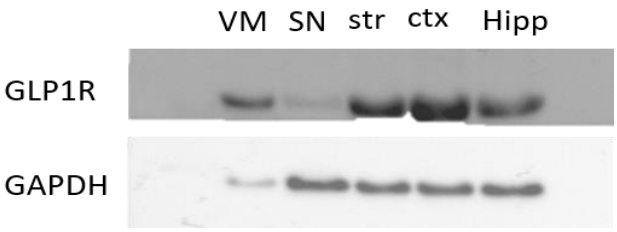


Figure 20 Western Blot of GLP1R protein (size 53 kDa) in E14 VM sections of Wistar rats. It was also detected in different brain regions Substantia nigra (SN), striatum (str), frontal cortex (ctx) and hippocampus (Hipp). GAPDH was used as a house keeper, size 37 kDa.

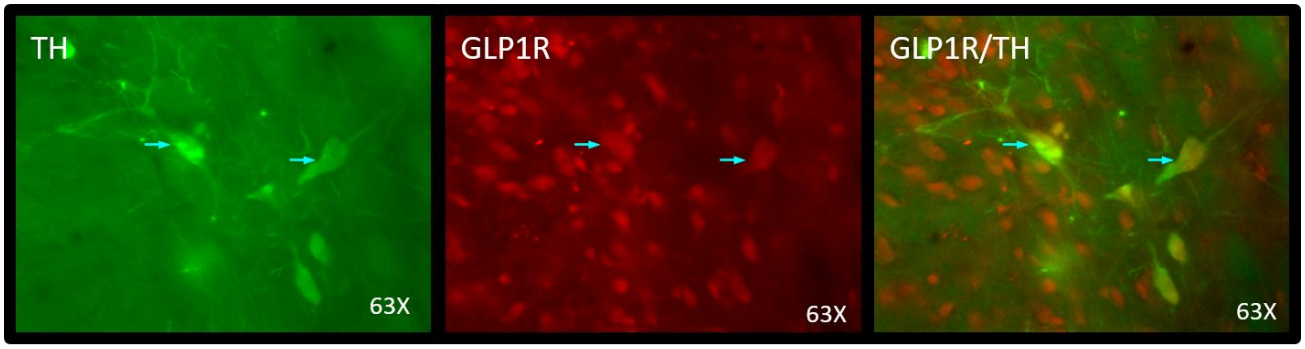


Figure 21 GLP1R expression in the grafted neurons: double F-IHC showed co-localisation of the TH cells (green) and GLP1R (red) in the striatum 13 weeks of transplantation (The arrow points to the co-localised cells).

4.3.2 Behavioural assessment of the graft

The control graft (saline group) showed a highly significant overall effect on reducing the number of amphetamine rotations toward the ipsilateral side compared to the lesion control group (repeated measure ANOVA, group was between subject factor, time was within subject factor) ($F_{(1,16)}$ = 12.6, **p< 0.01). The interaction with time showed the significant amelioration was on week 4, 8, and 12 (Min: week4 $F_{(1,16)}$ = 10.5, P< 0.01). The graft also had an overall significant effect on amelioration of the motor deficit in the cylinder test (repeated measure ANOVA, group was between subject factor, time was within subject factor) ($F_{(1,16)}$ = 12.4, **p< 0.01). The interaction with the time showed the graft group has significant effect on week 12 ($F_{(1,16)}$ = 19, P<0.001). It had no overall effect in the other sensorimotor tests, stepping and vibrissae (max: vibrissae: $F_{(1,16)}$ = 3, n.s).

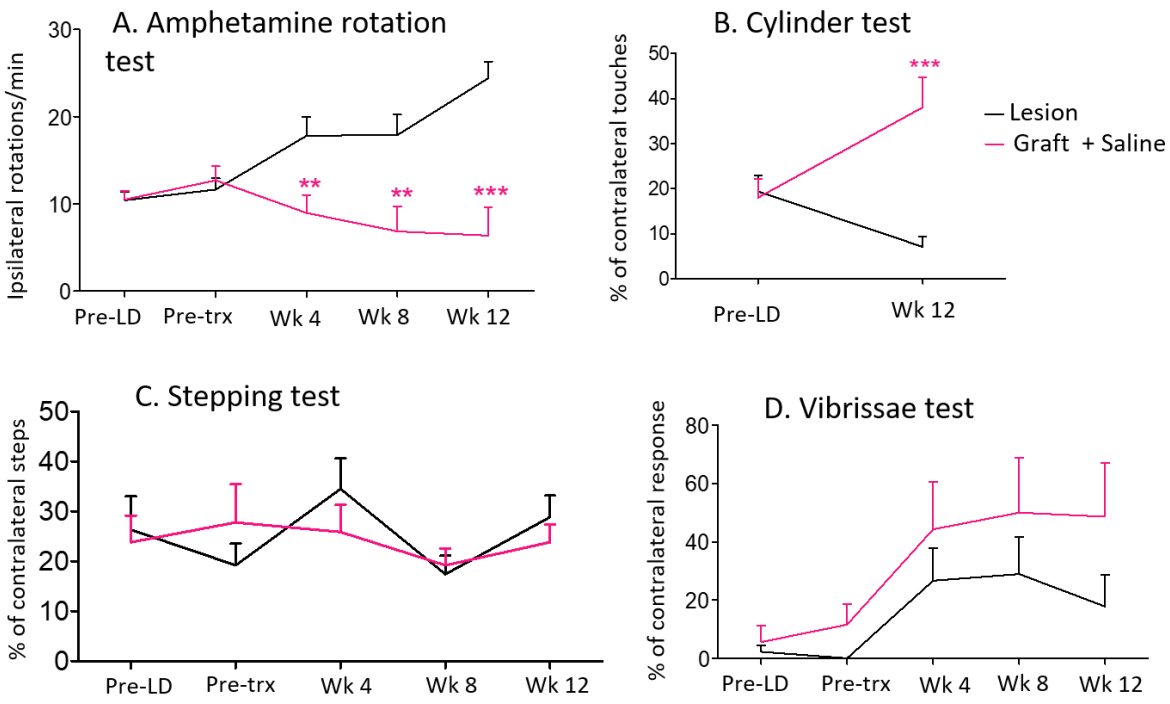


Figure 22 graft effect on the motor and behavioural tests: (A) the graft plus saline had overall significant effect on ameliorating behavioural deficits in amphetamine rotation test compared to lesion group (***P< 0.001), (B) the graft had overall significant effect on cylinder test (***p<0.001) but it had no effect on the stepping (C) and vibrissae (D) tests.

4.3.3 GLP-1 agonist Effect on behavioural results of the grafted groups in presence and absence of L-dopa treatment

Amphetamine rotation test: over all there is significant effect of Exendin-4 and liraglutide on amelioration of amphetamine rotations while L-dopa has no significant effect ($F_{(1,37)\ min}$ exendin-4 = 4.1, *P<0.05) (repeated measure ANOVA, between subject factors include: exendin-4, liraglutide and L-dopa; within subject factor include the time). There is a significant interaction between L-dopa treatment and liraglutide (L-dopa x liraglutide, $F_{(1,37)}$ = 6, *p<0.05); liraglutide illustrated significant effect on reducing amphetamine rotations in presence of L-dopa treatment ($F_{(1,37)}$ =14.3, ***p< 0.001) but it has no effect in saline treated group ($F_{(1,37)}$ = 1.3, n.s). There is also a significant interaction between exendin-4, L-dopa and the time (L-dopa x exendin-4 x time, F =5, p<0.01); exendin-4 ameliorate rotations significantly in saline groups on weeks 4, 8 and 12 (min: week 12; $F_{(1,37)}$ = 8, **p<0.01) but it has no effect in presence of L-dopa (max: $F_{(1, 37)}$ = 1.3, n,s).

Vibrissae test: only liraglutide showed an overall significant effect on improving rats' performance in vibrissae test ($F_{(1,37)}$ = 6.4, p<0.05) and there is no effect for exendin-4 and L-

dopa (max: exendin-4: $F_{(1,37)} = 2$, n.s) (repeated measure ANOVA, between subject factors include: exendin-4, liraglutide and L-dopa; within subject factor include the time). The interaction with the time shows that liraglutide produced the significant effect on week 4 in presence of L-dopa ($F_{(1,37)} = 6.3$, $p<0.05$) and on week 8 in saline group ($F_{(1,37)} = 4.3$ $p<0.05$).

Stepping test: overall only liraglutide significantly improved rats' performance in stepping test ($F_{(1,37)} = 8.4$, $p<0.01$). exendin-4 illustrated tendency to improve but didn't reach significant difference and L-dopa has no effect (exendin-4: $F_{(1,37)} = 3.7$, $p = 0.06$) (repeated measure ANOVA, between subject factors include: exendin-4, liraglutide and L-dopa; within subject factor include the time). The interaction between liraglutide, L-dopa and time shows that liraglutide has the significance effect on week 12 in L-dopa group.

Cylinder test: overall, exendin-4 has significant effect on improving rats' performance in cylinder test ($F_{(1,35)} = 4.7$, $p<0.05$); L-dopa illustrated overall significant effect on reducing rats' performance (L-dopa, $F_{(1,35)} = 10$, $P< 0.01$); liraglutide has no effect ($F_{(1,35)} = 1.7$, n.s) (repeated measure ANOVA, between subject factors include: exendin-4, liraglutide and L-dopa; within subject factor include the time). There is a significant interaction between L-dopa and exendin-4 ($F_{(1,35)} = 4.2$, $p<0.05$); exendin-4 has significant effect in saline treated group on week 12 ($F_{(1,35)} = 7.7$, $p<0.01$) but it has no effect in L-dopa treated group ($F_{(1,35)} = 0.6$, n.s); L-dopa has significant effect on reducing rats' performance only in exendin-4 treated group ($F_{(1,36)} =12.5$, $p<0.001$) and no effect in non-exendin-4 group ($F_{(1, 36)} = 2.6$, n.s).

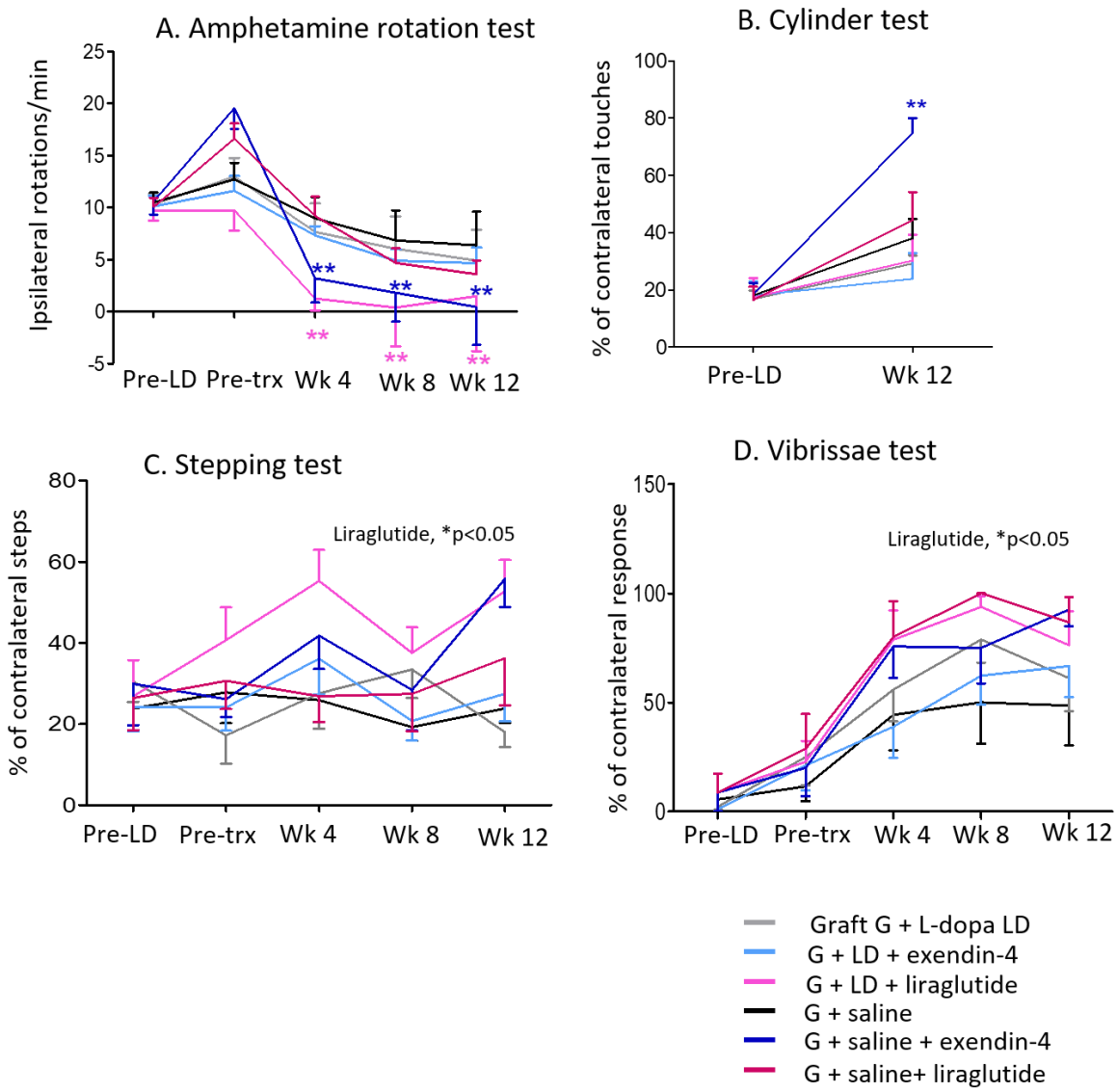


Figure 23 behavioural results: (A) exendin-4 ameliorate rotations in saline treated group only on week 4, 8 and 12 (**P<0.01), liraglutide ameliorate rotations in L-dopa treated group only (**P<0.01). (B) exendin-4 improved rats' performance in stepping test only in saline treated group (**p<0.01). (C) liraglutide has overall effect on improving rats' performance in stepping test (C) and vibrissae test (D) (*p< 0.05, **p<0.01)

4.3.4 L-dopa induced abnormal involuntary movements (AIMs) and rotations:

Total AIMs score, pre-transplantation, increased gradually over 5 weeks to achieve the higher point prior to transplantation. Then, post-transplantation, AIMs declined over 10 weeks to reach the lower point on week 10 followed by a brief increase on week 11. The time factor has significant effect on the AIMs score ($F(12,240) = 22.6$, $p<0.001$). The pairwise comparison adjusted with Bonferroni post hoc test showed that the reduction in AIMs score was significant since week 4 compared to base line time point week 1 pre-transplantation

(**p<0.01, ***p<0.001). No significant difference was recorded between GLP-1 agonist treated groups and graft control group (repeated measure ANOVA, time = within subject factor; groups= between subject factor).

L-dopa induced rotations towards the contralateral side, pre-transplantation increased gradually from the initiation of L-dopa treatment to reach a higher level prior to transplantation in all groups. Then, post-transplantation, the level of the contralateral rotations was ameliorated throughout the experimental time to reach the lower level at week 11. Data analysis showed a significant effect of the time of number of rotations ($F_{(12,240)} = 8.6$, p<0.001). Pairwise comparison adjusted with Bonferroni post hoc test showed that the slope of the reduction reached a significant difference on weeks 10 and 11 compared to the baseline time point week 1 pre-transplantation (*p<0.05). The data demonstrated that there was no significant difference between the groups treated with either exendin-4 or liraglutide compared with the graft control group (repeated measure ANOVA, n.s.).

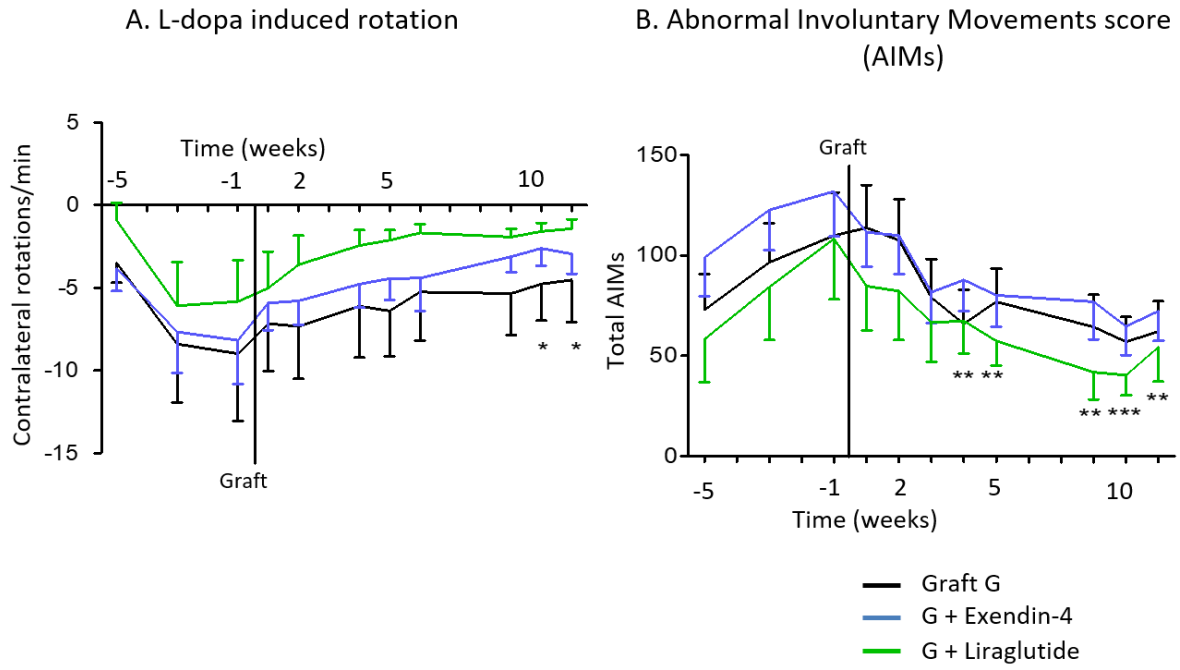


Figure 24 L-dopa induced rotation and Abnormal Involuntary Movements AIMs: (A) L-dopa induced contralateral rotations reduced gradually post-grafting reaching a significant difference on weeks 10 and 11 compared to week -1 (repeated measure analysis, *p<0.05). (B) total AIMs score reduced gradually post grafting with significant difference since week 4 compared to week -1 (pre-graft) (repeated measure analysis, **p<0.01, ***p<0.001). There were no differences between the graft control and treated groups with either exendin-4 or liraglutide in both L-dopa induced rotations (A) and AIMs score (B) (repeated measure ANOVA).

4.3.5 Dopaminergic neurons loss in the substantia nigra

The TH+ cells counted in the right (lesioned) and left (intact) sides of the substantia nigra demonstrated that the percentage of TH+ loss in the lesioned side compared to the intact side was higher than 97% in all groups. This confirmed that the recovery in behavioural tests resulted from the transplanted cells and not from endogenous nigral dopaminergic neurons.

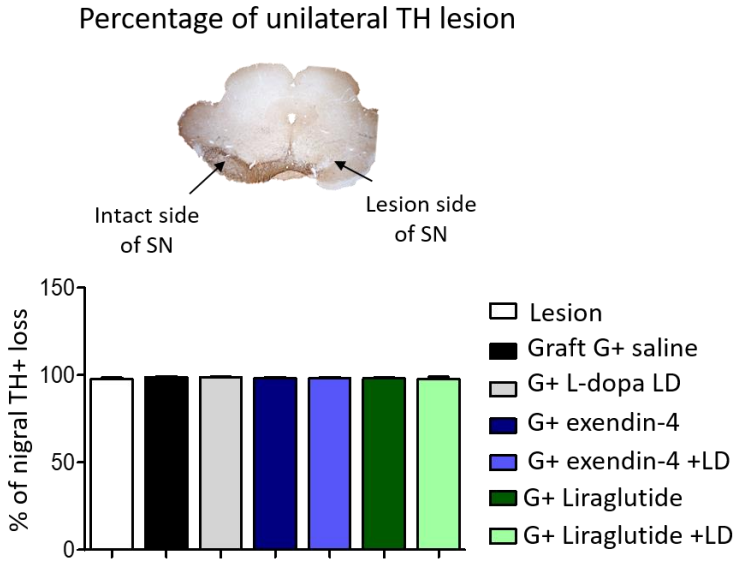


Figure 25 TH+ count in the substantia nigral: the percentage of the TH loss in the lesioned side (right side) of the SN was more than 97% compared to the intact side in all groups.

4.3.6 Graft TH+ count, volume and density

TH^+ count: overall, there is no significant effect of L-dopa, exendin-4 and liraglutide on increase total TH+ count with a tendency of exendin-4 to increase the total number ($F_{(1,39)}$ max exendin-4 = 3.55, p = 0.06, n.s) (two-way ANOVA, between subject factor include: L-dopa, exendin-4 and liraglutide). There is a significant interaction between L-dopa and exendin-4 (L-dopa x exendin-4, F (1,39) = 6.64, $p<0.05$). Exendin-4 increase TH+ count significantly in graft plus saline group (F (1,39) = 11.7, $p<0.001$) but it has no effect in the presence of L-dopa ($F_{(1,39)}$ = 2.8, n.s).

TH+ volume: overall there is no effect no effect for L-dopa, exendin-4, and liraglutide ($F_{(1,39)}$ $_{max}$ liraglutide = 3.5, n.s) (two-way ANOVA, between subject factor include: L-dopa, exendin-4 and liraglutide). However, there is tendency to improve the total volume by liraglutide and

there is a tendency for interaction between L-dopa and liraglutide (liraglutide x L-dopa, $F_{(1, 39)} = 3$, n.s).

TH+ fibres density: liraglutide has a significant overall effect on increasing TH+ fibres density in the graft core ($F(1,39) = 8.3$, $p<0.01$), exendin-4 and L-dopa has no effect on the TH+ density (max: exendin-4: $F_{(1,39)} = 0.39$) (two-way ANOVA, between subject factor include: L-dopa, exendin-4 and liraglutide). There is a significant interaction between L-dopa and exendin-4 ($F_{(1,39)} = 10$, $p< 0.01$); exendin-4 increase fibres density significantly in saline treated group ($F_{(1,39)} = 4.4$, $p<0.05$) while it reduced fibres density significantly in L-dopa treated group ($F_{(1,39)} = 12.7$, $p<0.001$).

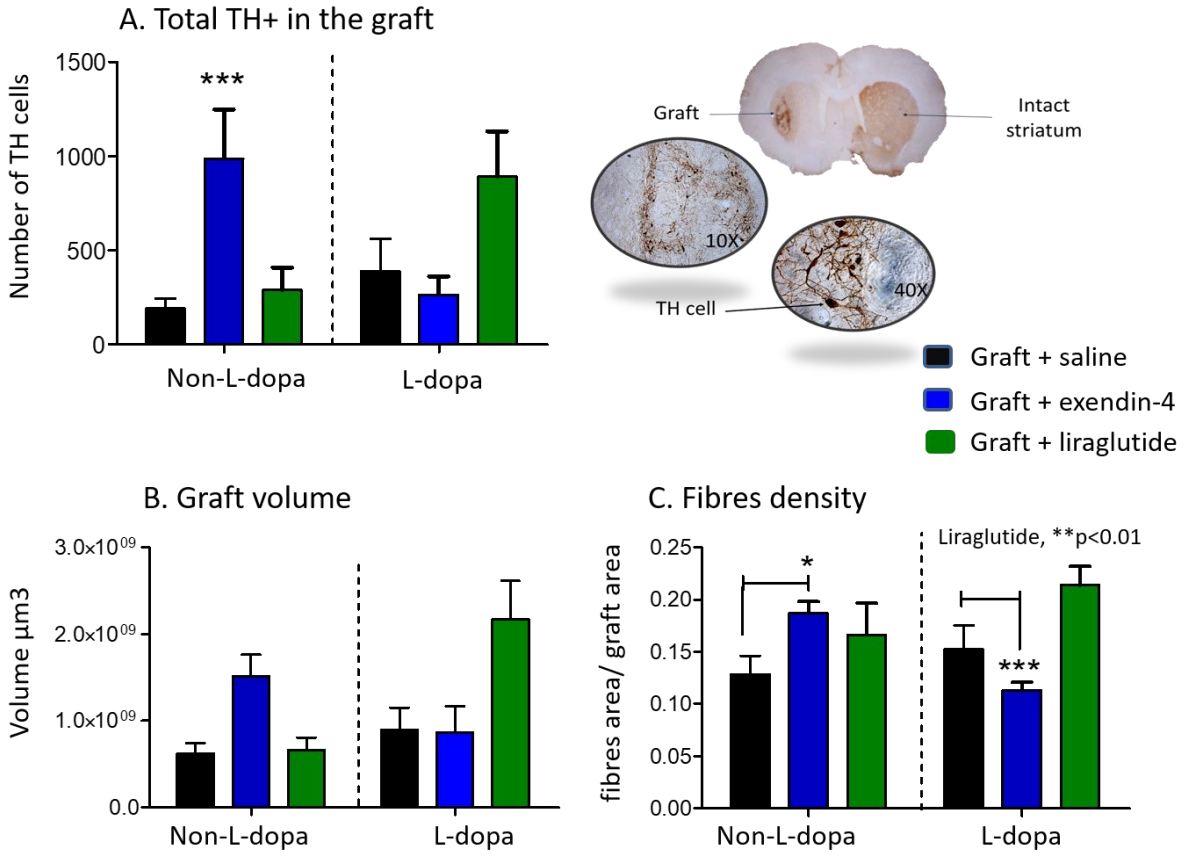


Figure 26 histological characterisation of the graft: (A) exendin-4 had a significant effect on increasing TH+ count in saline group but no effect in L-dopa group (***p< 0.001), (B) no effect for exendin-4 and liraglutide on graft volume, (C) liraglutide has significant overall effect on increasing TH+ fibres density while exendin-4 increase fibres density in saline group and reduced the density in L-dopa group (*p<0.05, **p<0.01, ***p<0.001) (two-way ANOVA).

4.3.7 Effect of GLP1 agonists on dopaminergic cells subtypes and serotonergic neurons in the graft

The double labelling of the TH and Girk2 in the graft illustrated that there was no effect of exendin-4, liraglutide and L-dopa on the ratio of A9 (Girk2+ cells) dopaminergic neuron subtype to the total dopaminergic neurons (TH+ cells) (two-way ANOVA, n.s.). The data also demonstrated that there was no effect of exendin-4, liraglutide and L-dopa on the number of serotonergic neurons labelled 5-HT in the graft (two-way ANOVA, $F_{(1, 39)\,max}$ L-dopa = 2.4, n.s.).

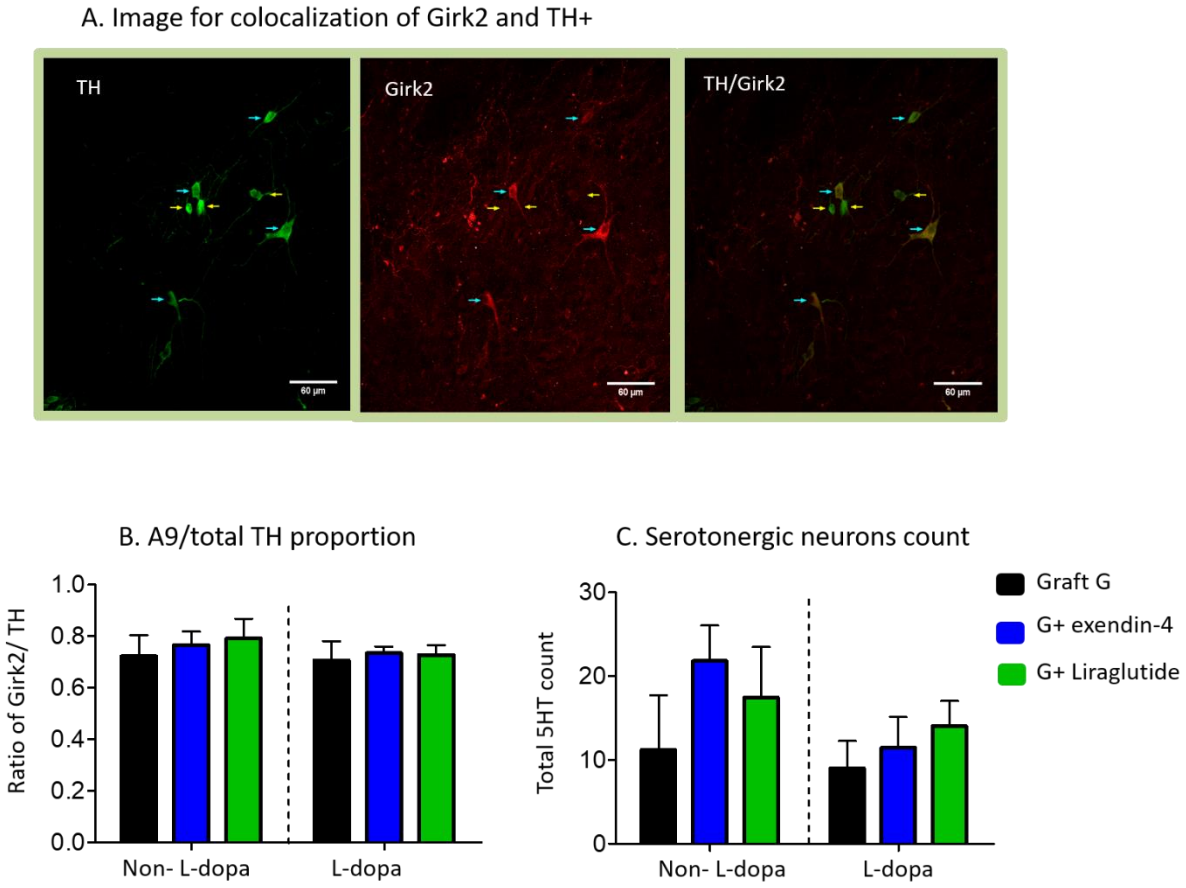


Figure 27 Subpopulations of dopaminergic neurons and serotonergic neurons in the graft: figure A shows co-localisation of TH (green) representing the dopaminergic neuron and Girk2 (red) representing the A9 subtype of the dopaminergic neuron in the graft (blue arrow pointed to some of co-localised targets while yellow arrow pointed to some of non-co-localised targets). (B) both exendn-4 and liraglutide had no effect on the A9/TH proportions (two-way ANOVA). (C) exendin-4 and liraglutide show no effect on the total number of the serotonergic neurons in the graft (two-way ANOVA).

4.3.8 Microglial stained CD11b analysis

The level of the microglial reaction around the graft was expressed as a ratio between the optical densities of the activated microglia around the graft to the optical density of the microglia at corresponding places in the intact side. The data showed that L-dopa has overall effect to increase the level of microglia labelled CD11b around the graft significantly ($F_{(1,39)}$ = 16.8, $p<0.001$), and there is no overall effect of exendin-4 or liraglutide on the level of CD11b (max: liraglutide: $F_{(1,39)}$ = 1.9, n.s) (two-way ANOVA, between subject factor include: L-dopa, exendin-4 and liraglutide). There is a significant interaction between L-dopa and exendin-4 ($F_{(1,39)}$ = 4.9, $p<0.05$); L-dopa increase level of CD11b significantly in in exendin-4 treated rats ($F_{(1,39)}$ = 14, $p<0.001$), but it has high tendency but not significant effect in non-exendin-4 treated rats ($F_{(1,39)}$ =3.9, n.s).

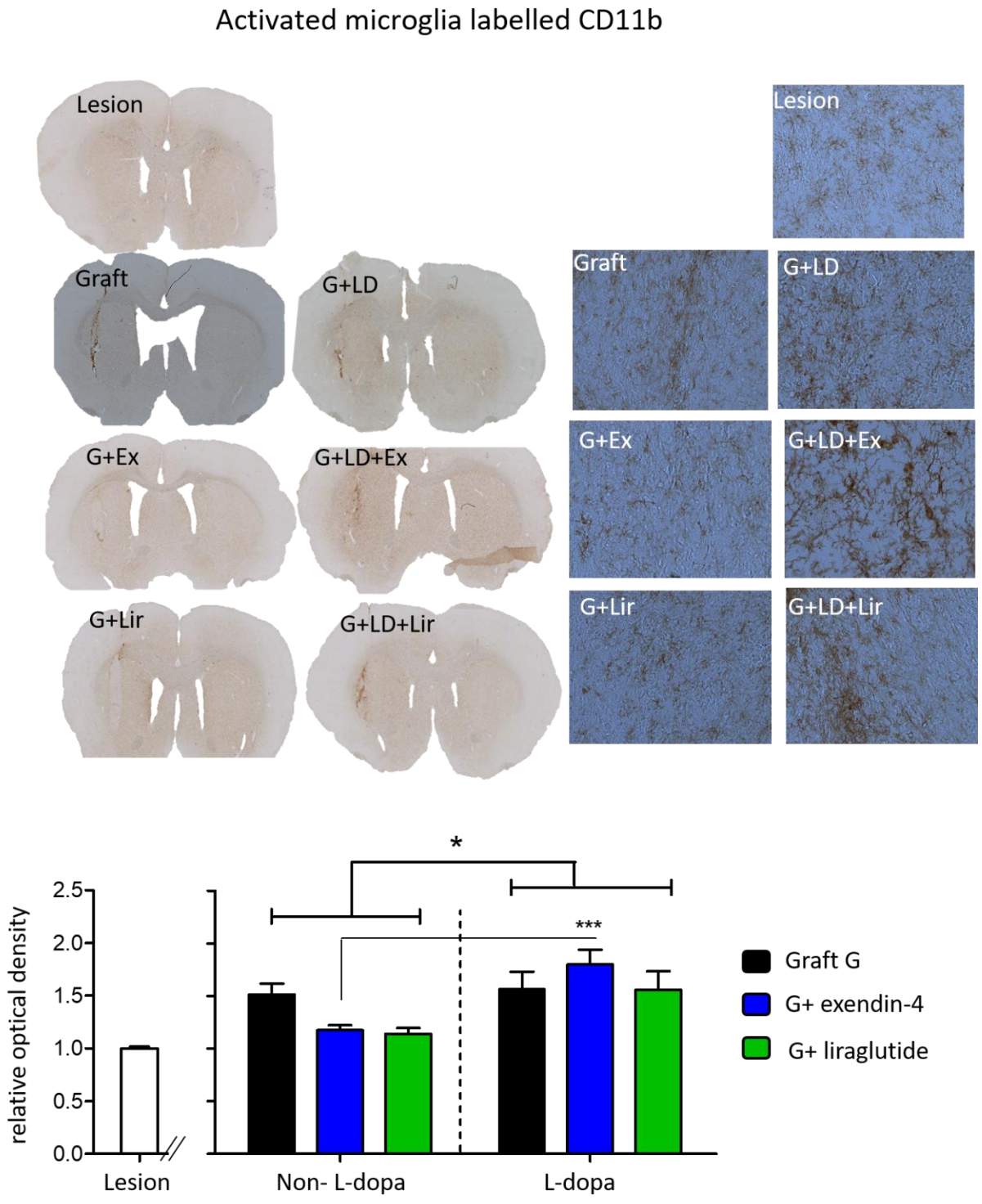


Figure 28 microglial stained (CD11b) density around the graft: L-dopa has overall significant effect on increase level of CD11b labelled cells around the graft and no effect for exendin-4 or liraglutide (*p<0.05), there is an interaction between L-dopa and exendin-4 shows that L-dopa increase CD11b cells in presence of exendin-4 (***p<0.001).

4.3.9 Leukocyte stained CD45 analysis

The data showed that there are only few infiltrated leucocytes labelled CD45 detected in the striatum of the lesion control group. In grafted groups, liraglutide has overall effect to increase number of infiltered CD45 cells ($F_{(1,39)} = 4.8$, $p<0.05$) and there is no effect of L-dopa and exendin-4 ($F_{(1,39)\,max}$ L-dopa = 3.3, n.s) (two-way ANOVA, between subject factor include: L-dopa, exendin-4 and liraglutide). There is a significant interaction between L-dopa and liraglutide ($F_{(1,39)} = 7$, $p<0.01$); liraglutide increase CD45+ cells in L-dopa treated group ($F_{(1,39)} = 13$, $p<0.001$) but it has no effect in saline treated rats ($F_{(1,39)} = 0.4$, n.s).

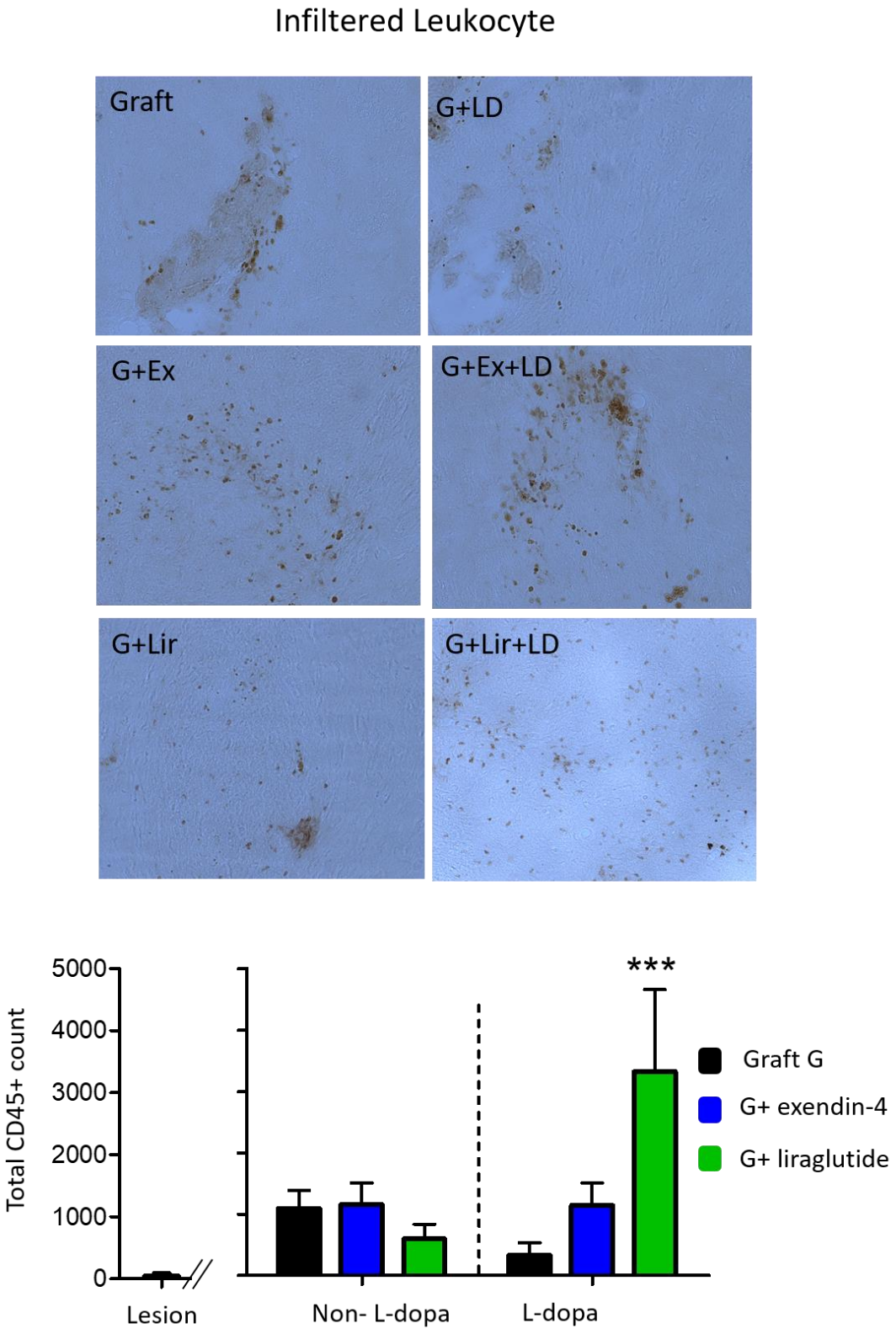


Figure 29 leucocyte staining (CD45) count in the grafted striatum: liraglutide has a significant overall effect on increasing the number of infiltrated leukocytes to the grafted striatum ($p<0.05$); liraglutide has significant interaction with L-dopa illustrated it significantly increase CD45 level in L-dopa group and no effect in saline group (***$p<0.001$).

4.3.10 Striatal blood vesseles length and diameter

Blood vessel surface area analysis, stained with tomato lectin, in the right hemisphere of the striatum (grafted side) illustrated there was no effect of L-dopa, exendin-4 and liraglutide in the average blood vessel surface area in grafted rats ($F_{(1,36)\ max}$ L-dopa = 2.25, n.s). The blood vessels diameter analysis showed that liraglutide has overall effect on increase diameter of the blood vessels in the grafted stratum ($F_{(1,36)} = 9.1$, $p < 0.01$) and there was no effect of L-dopa and exendin-4 on the blood vessels diameter ($F_{(1,36)\ max} = 0.5$, n.s) (two-way ANOVA, between subject factor include: L-dopa, exendin-4 and liraglutide).

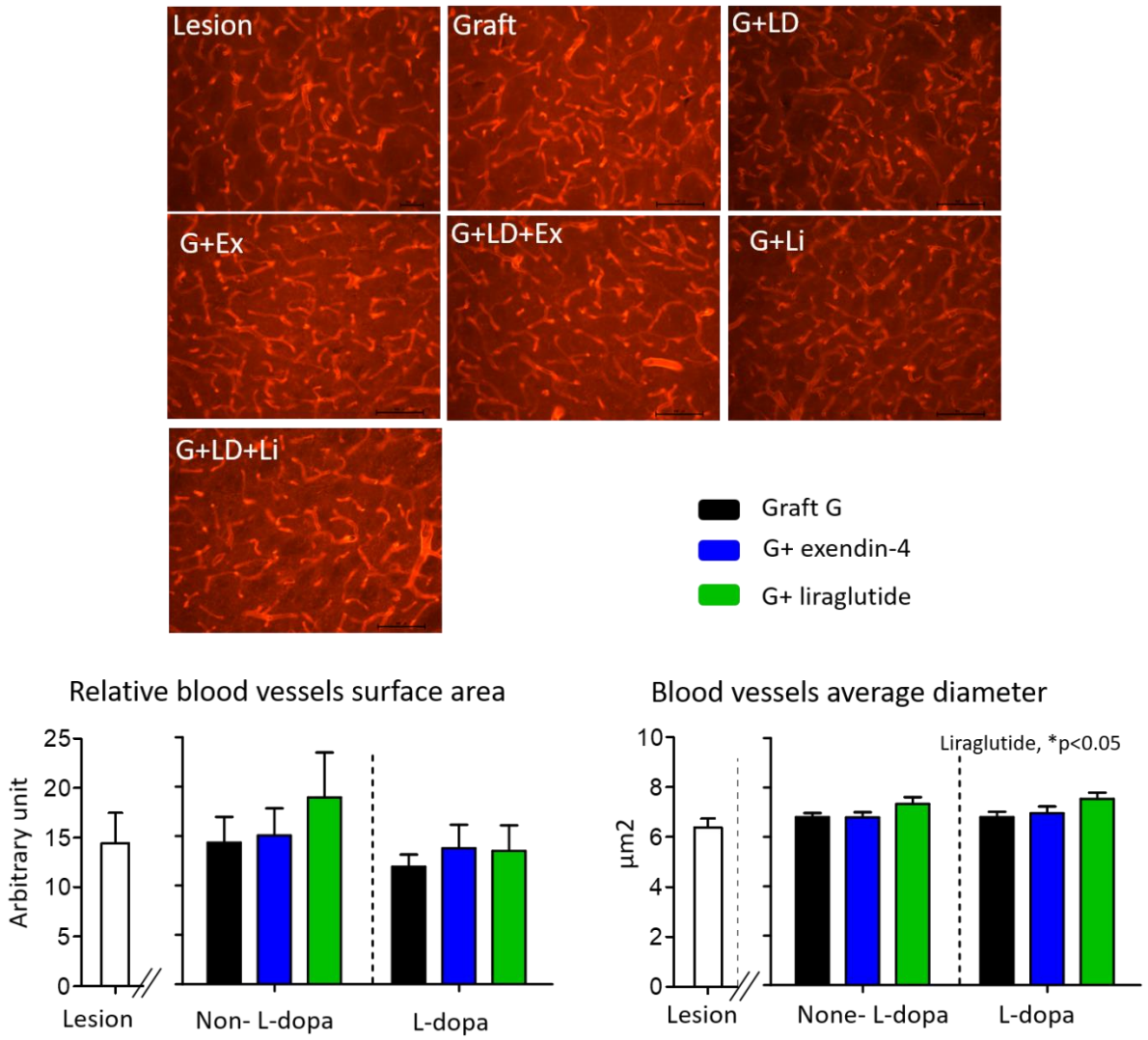


Figure 30 blood vessels staining (tomato lectin) in the grafted striatum: L-dopa, exendin-4 and liraglutide had no effect on the striatal blood vessels surface area; while liraglutide shows overall significant effect on increase the diameter of the blood vessels at the grafted side (two-way ANOVA, **p<0.01). The images were taken on 10x magnification from the striatum of the right hemisphere of each group (G= graft; LD= L-dopa; Ex= exendin-4; Li= Liraglutide).

4.3.11 The plasma level of insulin and glucose investigations

The GLP-1 agonists exendin-4 and liraglutide produced different actions on graft function and histological charachteristics in the presence and absence of L-dopa. To explore the reasons for this difference, the plasma level of insluin and glucose was investigated to understand whether these differences are related to changes in glucose and insulin hemostasis or not.

The analysis of insulin level in the plasma of the graft groups illustrated that exendin-4 has overall significant effect on increasing insluin level ($F_{(2, 45)} = 7.78$, $p<0.001$) while liraglutide and L-dopa have no effect ($F_{(1,45)\,max}$ L-dopa = 3, n.s) (two-way ANOVA, between subject factor

include: L-dopa, exendin-4 and liraglutide). There is a significant interaction between L-dopa and exendin-4 ($F = 6.1$, $p < 0.05$); exendin-4 increase insuline level significantly in L-dopa treated group ($F_{(1,45)} = 10.2$, $p < 0.001$) and it has no effect in the saline treated group ($F_{(1,45)} = 0.5$, n.s). The average of insulin level in group treated with exendin-4 and L-dopa equal to (718 ± 209 pg/ ml).

The analysis of glucose level in the plasma of the grafted groups illustrated that exendin-4 has high significant overall effect on increaing glucose level ($F_{(1,45)} = 24{,}7$, $p<0.001$), liraglutide also has significant overall effect on increasing glucose level ($F_{(1,45)} = 4.3$, $p<0.05$) (two-way ANOVA, between subject factor include: L-dopa, exendin-4 and liraglutide). The average of te glucose level in group treated with exendin-4 and L-dopa equal to (10 ± 0.68 mmol/ liter)

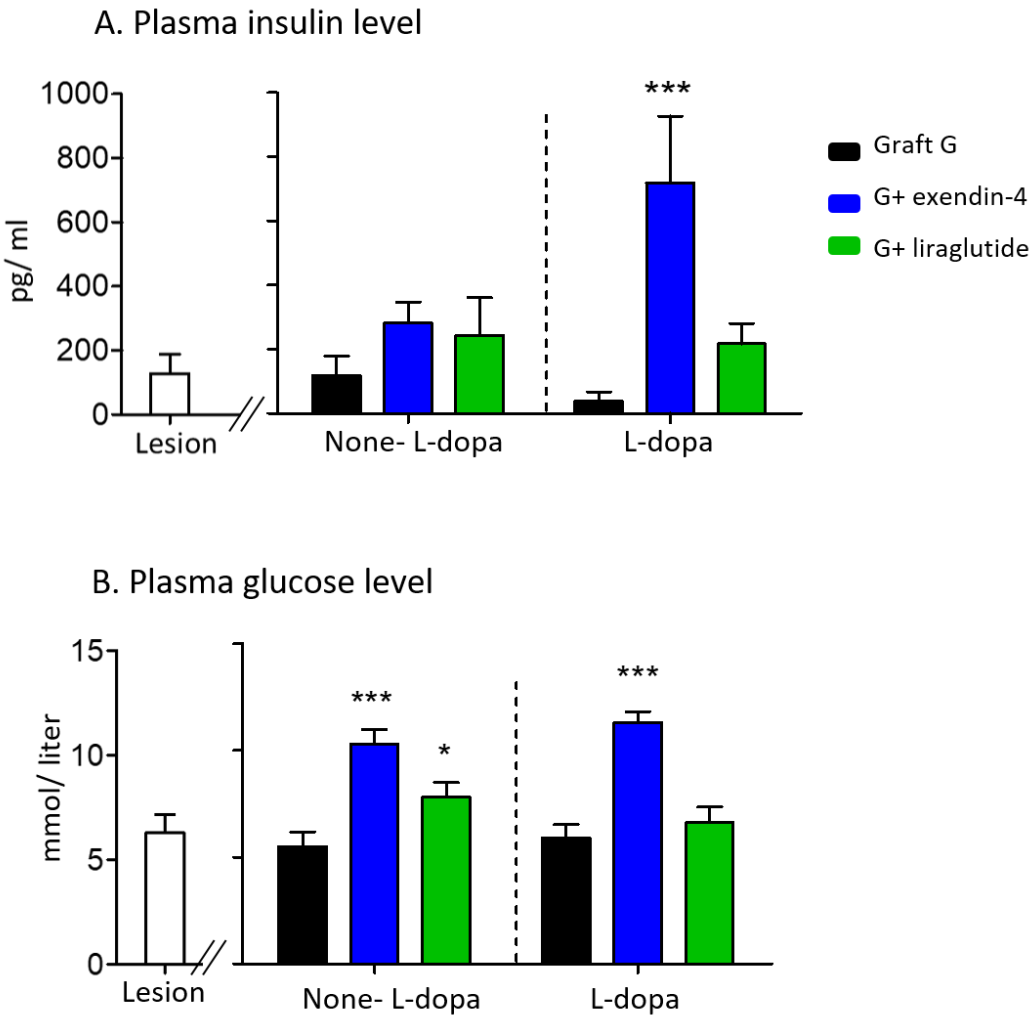


Figure 31 plasma level of insulin and glucose: exendin-4 increased the level plasma insulin level significantly in L-dopa treated group (two way ANOVA, ***p<0.001) and it has overall effect to increase the glucose level (two-way ANOVA, p<0.001); liraglutide has no effect on insulin level but it has overall effect to increase glucose level (two-way ANOVA, p<0.05).

4.3.12 Phosphorylated insulin receptors in the graft

The phosphorylation of insulin receptors investigated by IRS-1 pS^{1011} immunohistochemistry was used to identify the insulin receptor resistance on the grafted cells. One subject of each group was investigated in this test. A positive staining of IRS-1 pS^{1011} confined to the nuclei was detected in the graft of all groups. However only the group treated with in exendin-4 and L-dopa showed a high density of cytoplasmic IRS-1 pS^{1011} positive staining while it was not detected in the graft of any other group.

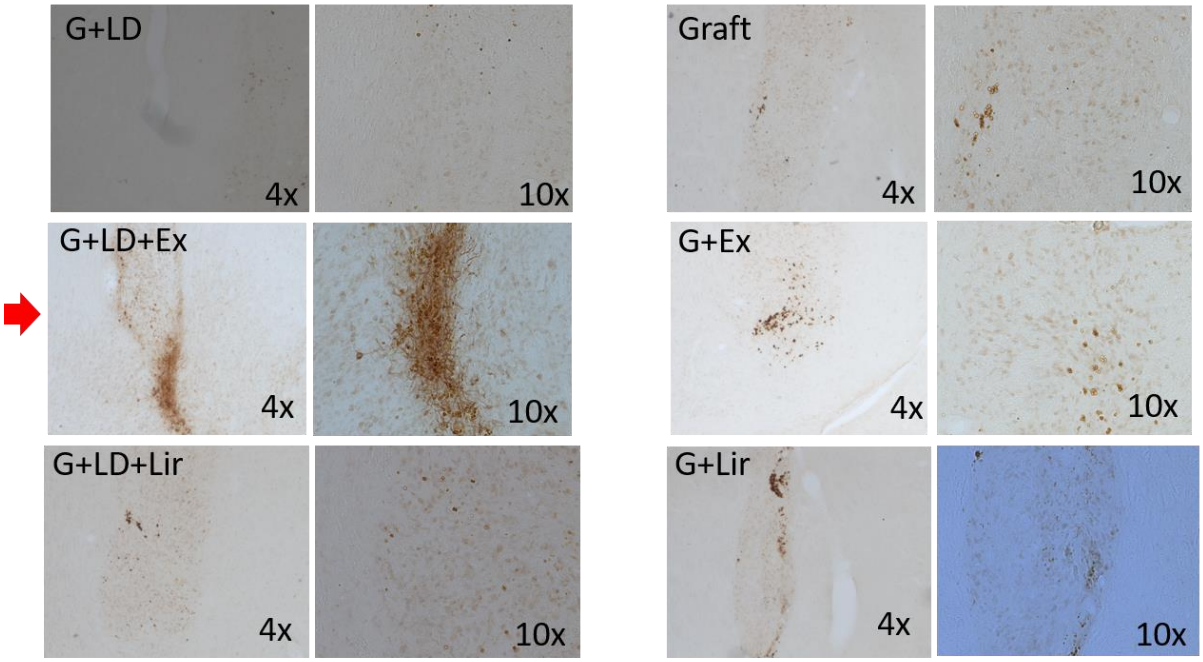


Figure 32 phosphorylated insulin receptors labelled with (IRS-1 pS1011) in the graft: IRS-1 pS1011 identified confined to the nuclei of grafted cells in one rat of all groups. However, the cytoplasmic detection was only in exendin-4 plus L-dopa treated group. The images were captured on 4X and 10X magnification. (G= graft, LD= L-dopa Ex = exendin-4, Lir= liraglutide).

4.3.13 Fat accumulation analysis in the liver

Fat accumulation in the liver was explored as a further investigation to explore the signs of abnormal insulin and glucose levels recorded in the group treated with exnendin-4 and L-dopa.

The Oli Red-O (ORO) staining for liver sections illustrated that the lesion only control group had a high percentage of fat accumulated in the liver representing 17.33 ± 6.5 % of liver surface area.

In the grafted groups: L-dopa, exendin-4 and liraglutide have no effect on the level of accumulated fat level in the liver (max: L-dopa: $F_{(1,45)} = 1.3$, n.s) (two-way ANOVA, between subject factor include: L-dopa, exendin-4 and liraglutide). A direct comparison between the lesion group and graft group which received exendin-4 showed that the later had a significantly lower percentage of accumulated fat in the liver compared to lesion control (t-test, two tailed, *$p<0.05$).

Haematoxylin and Eosin staining was used as another method to visualise fat accumulation in the liver. The test confirmed the ORO staining results as high accumulation of fat droplets was seen in the cytoplasm of cells that had a high percentage of ORO staining while few or no fat droplets were detected in the liver sections that had a low percentage of ORO (image B and D in Figure 33).

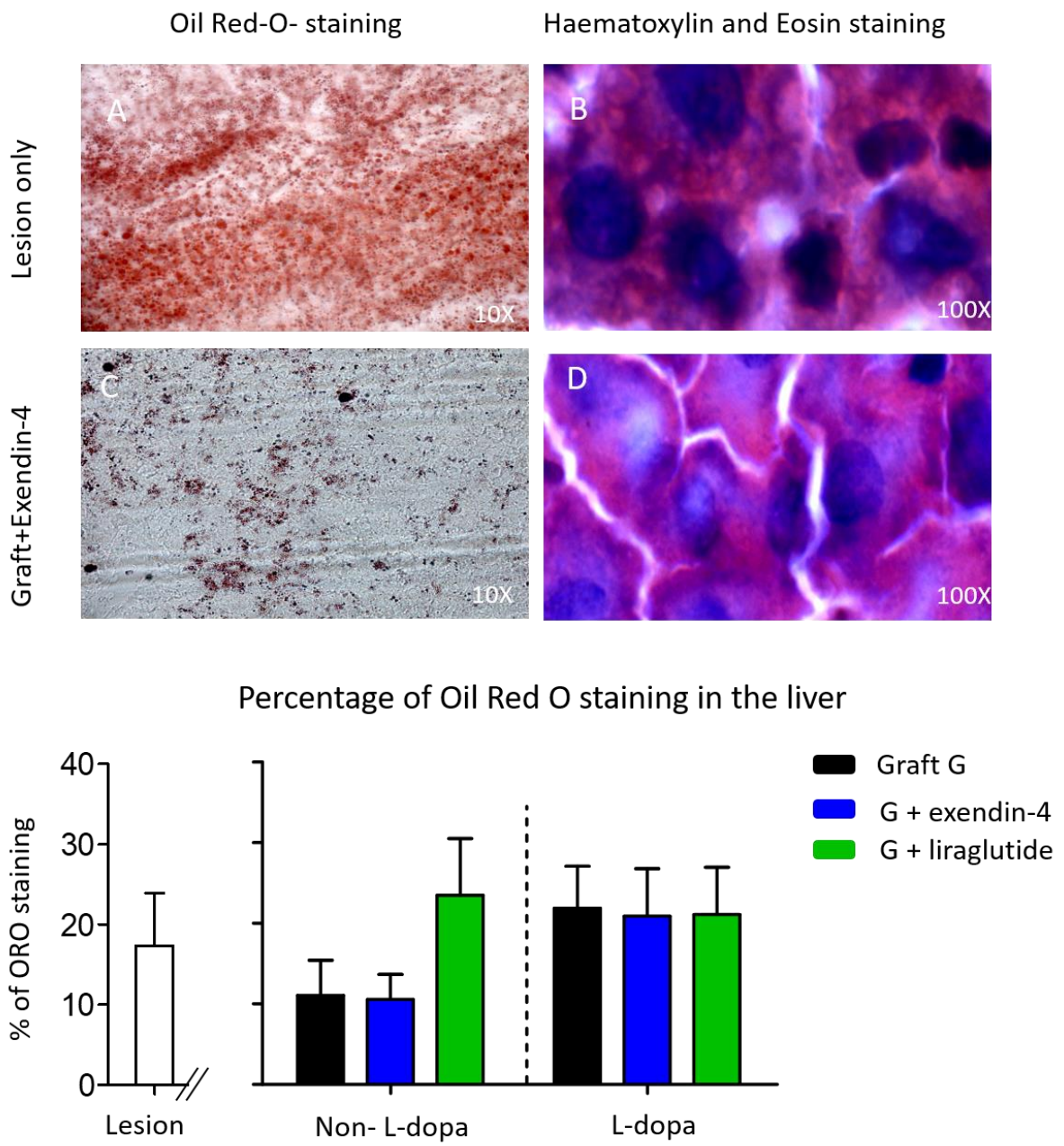


Figure 33 Percentage of fat accumulation in the liver: high percentage of fat accumulated in the liver of the lesion group representing 17.33% of liver surface area. There is overall effect of L-dopa, exendin-4 and liraglutide on the level of the accumulated fat in the grafted groups (two-way ANOVA). Image (A) shows a high level of Fat droplets in the liver of a rat of the lesion group stained by Oil Red-O and confirmed by H & E staining, image (B). Image (C) shows a low percentage of fat droplets in the liver of grafted rats treated with exendin-4 and confirmed by H & E staining, image (D). (ORO = Oil Red-O)

Table 6 summary for the results of exendin-4 and liraglutide compared to the graft control either in L-dopa or non-L-dopa treated groups.

test		Exendin-4		Liraglutide	
		saline	L-dopa	saline	L-dopa
Behavioural tasks	Amphetamine	Improved	No	No	Improved
	Cylinder	Improved	No	No effect	
	Stepping	No effect (tendency)		Improve	
	Vibrissae	No effect		Improve	
	L-dopa rotations	-	No effect	-	No
	L-dopa induced AIMs	-	No effect	-	No
Histological characteristics	TH count	Increase	No effect	No effect	
	TH volume	No effect		No effect (tendency)	
	TH fibres density	Increase	Reduce	Increase	
Plasma analysis	Insulin level	No effect	Increase	No effect	
	Glucose level	Increase		Increase	
Insulin resistance	IRS-1 pS^{1011}	Not detected	Detected	Not detected	
Inflammatory markers	Microglia CD11b	No effect	Increase	No effect	
	Leukocyte CD45	No effect		No effect	Increased
Blood vessels	Surface area	No effect		No effect	
	Diameter	No effect		Increase	
A9 / TH^+ ratio	-	No effect		No effect	
Serotonergic neurons count 5-HT	-	No effect		No effect	
percentage of fat accumulation in the liver	-	*Reduced compared to lesion group	No effect	No effect	

4.4 Discussion

The survival rate of the transplanted E14 VM cells in the current experiment was consistent with its survival in previous experiments in chapter 3. The data illustrated that only around 190 cells of the dopaminergic neurons (TH^+) had survived in the graft of control group (received saline only) after transplanting of two thirds of VM cells. By assuming that number of dopaminergic neuron precursors in each rat embryo's VM section is between 30,000 – 40,000 cells (Brundin et al. 2000), then the survival rate in this group was less than 1%. The graft was functional and present efficiency on reducing rats' asymmetric rotations after amphetamine administration. This indicates releasing the dopamine by the graft in the depleted side to counterbalance the dopamine level in the intact side, the data confirmed a complete degeneration for the endogenous nigral dopaminergic neurons to conclude this recovery is from the graft. The graft also ameliorated the motor deficit in the cylinder test but it was not effective in reversing deficits in stepping and vibrissae tests. This limitation was mainly attributed to the small size of the graft, as previous works clarified that wider innervation to the striatum and a certain threshold of graft size are required for better motor recovery (Hagell & Brundin 2001; Falkenstein et al. 2009).

The overall effect of L-dopa treatment on the grafted groups showed it has no influence on either graft function or survival unless it interacted with GLP-1R agonists. The only effect of L-dopa recorded in this experiment is increasing level of microglia and reducing rats' performance in cylinder test. The data showed that the effect on cylinder test was only in presence of exendin-4 treatment which it has been suggested that the latter in presence of L-dopa may deteriorate the effect of the graft (discussed below). L-dopa effect on microglia was also affected by exendin-4 treatment as higher level of microglia was recorded in presence of exendin-4 and no effect in absence of exendin-4. The effect of L-dopa on the graft in this study is consistent with previous studies which clarified that L-dopa has no effect on the survival or function of the VM cell transplantation but it has interference with the microglial inflammatory reaction toward the graft (xenograft) (Breger et al. 2016). in the current study, the data revealed that the presence of L-dopa treatment influenced GLP-1 agonist effects on graft survival and function (discussed below).

Exendin has neuroprotective effects in PD but the mechanism through which this may occur is unclear. Given that the same neurochemical processes are present in the striatum at the

time of transplantation, it was relevant to consider whether the same degree of protection might be afforded to the transplanted neurons. This study confirmed the presence of GLP1 receptors on VM dopaminergic neurons (E14) at the time of transplantation suggesting availability of the mechanical components for the GLP1 agonist to produce their effect to support graft survival. Post-mortem analysis 13 weeks after transplantation also confirmed the persistence of this receptor on the transplanted dopaminergic neuron which lead to conclude the receptor were present in the graft throughout the experiment. The GLP1 agonists exendin-4 and liraglutide showed they have the supporting effect on the survival and functionality of the transplanted VM dopaminergic neurons. However, the presence of L-dopa interfered with their effects. As exendin-4 was successful in supporting the graft only in the non-L-dopa treated group, while liraglutide was supportive to the graft only in rats who received L-dopa.

The current data illustrated nearly a complete lesion of the nigrostriatal dopaminergic neurons exceeding 97% in all groups including GLP-1 agonists treated rats with or without L-dopa. This indicated that GLP-1R agonists did not reverse the complete nigral dopaminergic neurons lesion induced by 6-OHDA, consequently it has no effect on the behavioural recovery results due to interference with the endogenous nigrostriatal dopaminergic neurons. In addition, in chapter 6, the data illustrated that exendin-4 has no effect on behavioural results (drug-induced rotation test, stepping test, vibrissae test and cylinder test) of a completed nigral dopaminergic lesion 6-OHDA rat model. Concluding that the behavioural tests in this experiment is evaluating the effect GLP-1R agonists on the graft rather than other targets.

Impact of exndin-4 on the graft survival and functional capacity:

Exendin-4 illustrated capability on supporting survival of transplanted cells but this effect was determined by presence of L-dopa treatment. It caused significantly increasing total number of dopaminergic neurons labelled TH+ in the graft in absence of L-dopa treatment while it has no effect in presence of L-dopa. In absence of L-dopa, exendin-4 caused an increase in the mean number of transplanted cells significantly by 6 times more compared to the graft control. This increment ratio is competitive to other trial attempt to improve the survival rate of grafted cells. For instance, intra-striatal injection of neurotrophic factors GDNF or FGF with VM tissue increased the number of TH cells that survived to 2.6 and 10 fold respectively in a rodent model (Rosenblad et al. 1996; Takayama et al. 1995). Moreover, treating the VM cell

suspension with caspase inhibitor or lazaroid prior to transplantation enhanced cell survival by almost two-fold (Schierle, Hansson, et al. 1999b; Karlsson et al. 1999). The consequence of using younger VM cells (E12 in rats) lead to a 6 fold improvement in TH cell survival (Torres et al. 2008). Besides increasing cell survival, the TH density in the core of the graft (TH fibres and cell body) was also significantly higher with exendin-4 treatment. This suggested that more fibres innervated by the dopaminergic neurons in the core of the graft which increase chance of fibres integration with the host cells. Importantly, this effect in absence of L-dopa was reflected in the better motor and behavioural recovery which included significant reduction in amphetamine-induced rotations and significant recovery in cylinder test. This is consistent with previous studies which showed recovery in the motor and behavioural tests is correlated with improvement in the graft dopaminergic neurons survival and fibre density (Hagell & Brundin 2001) . Taken together, the initial hypothesis that exendin-4 in the absence of L-dopa treatment influences the support of VM transplantation therapy is valid via increasing cell survival, fibre density, and improving motor performances in amphetamine rotation test and cylinder test.

The emphasis in this thesis is on evaluating the cell therapy in an animal model including L-dopa treatment to mimic the real clinical condition of patients who received L-dopa before and after cell transplantation. Exendin-4 effectiveness on graft survival and function was abolished in presence of L-dopa, even more it was responsible on reducing TH+ (TH fibres and cell body) density in the graft. Moreover, it participated on enhancing the effect of L-dopa on increase microglial level around the graft**.** Clinical trial had suggested a relationship between the deficiency of motor recovery and the accumulation of microglia around and inside the graft of patients who had short term immunosuppression (Olanow et al. 2003). Previous work on animal models has shown that induction of systemic inflammation leads to an inflammatory response against allogenic VM transplantation in a PD model which affects graft survival and function negatively (Shinoda et al. 1995; Hudson et al. 1994). Similarly xenograft transplantation caused a strong immunological reaction leading to graft rejection (Duan et al. 1995)**.** Surprisingly, administration of exendin-4 with L-dopa caused elevation in the plasma levels of the insulin and glucose suggesting the development of insulin resistance in this group of rats. Thus, the data illustrated that L-dopa may contradict the protective effect of exendin-4 (discussed below).

Impact of liraglutide on cell survival and functional capacity:

Liraglutide showed it had no effect on increasing the number of dopaminergic neurons. This may suggest that liraglutide was not able to protect the dopaminergic neurons. Recently, a published study revealed that liraglutide has a limitation on protecting the nigral dopaminergic neurons or recovering amphetamine rotation test in partial and complete 6-OHDA unilateral nigral lesions in the rat model (Hansen et al. 2016). However, liraglutide in the current study improved rats' performances in the vibrissae test, stepping test and amphetamine rotation test significantly which may suggest it has the therapeutic potential to improve the motor performance even with a smaller graft. Liraglutide illustrated a significant effect to increase the graft TH+ density (TH fibres and cell body). In addition, it had tendency to increase graft volume which may reach the significant difference if larger rat number was used to reduce variability. These could be the reason for supporting graft function by liraglutide. Previous study illustrated that the increment in fibres density and the graft volume has a positive influence to improve graft function (Hagell & Brundin 2001).

The effect of liraglutide on amphetamine rotation test interacted with L-dopa treatment, as better graft function was in presence of L-dopa and no effect in absence of L-dopa. This suggested that L-dopa may potentiate the effect of liraglutide on supporting the graft function without interference on the graft survival, volume or fibres density. The data revealed that liraglutide increase infiltration of leukocytes around the graft in presence of L-dopa. Although this infiltered leukocyte had no detrimental effect on graft survival but it may have a role on this improvement in graft function (discussed below).

Liraglutide showed it has an effect on increasing the diameter of the blood vessels in the grafted striatum. A study has shown that liraglutide has an effect on increasing blood flow and it has vasodilation effect on peripherally blood vessels in adult patients with T2DM (Nandy et al. 2014)**.** In cerebral blood vessels, recent study showed that liraglutide has the ability to reinstate the integrity of cerebral blood vessels and decrease the degree of cerebral microanuerysms and leakage in the APP/PS1 mice model which characterised by microangiopathies (Kelly et al. 2015). This dilation of the striatal blood vessels in the current study possibly participate in infiltration of Leukocyte in L-dopa treated rats. Especially, Previous work showed that L-dopa caused a significant increase in the length of the blood vessels of the dyskinetic rats in the substantia nigra with reporting of dysfunctional signs of

the blood brain barrier (Westin et al. 2006). Taken together, could L-dopa and lirglutide affecting permeability of blood vessels and infiltration of leukocytes.

The data clarified that co-administration of liraglutide with L-dopa did not induce abnormal changes in plasma insulin level suggesting these changes may be specific to exendin-4. Although liraglutide treatment causes increase level of plasma glucose significantly but the average glucose level in these rats (7.2 ± 0.6 mmol/liter) was within normal range of glucose level in Sprague Dawley rats (7 – 9 mmol/ liter) (Havel et al. 2000; Buchanan et al. 1991).

Did L-dopa treatment halt the protection effect of exendin-4?

The histological and behavioural data showed that L-dopa may influence the neuroprotective capability of exendin-4. Analysis of plasma samples illustrated that exendin-4 + L-dopa suffered from hyperinsulinemia and hyperglycaemia. This suggested a possibility of developing insulin resistance in these rats. The preliminary data from staining one rat's brain sections of each group with phosphorylated insulin receptor IRS-1 pS^{1011} showed a distinctive high level of the phosphorylated insulin receptor in the nuclei and cytoplasm of the cells in the graft of exendin-4 + L-dopa compared to others which only confined to the nuclei (Figure 32). Previous work studied insulin resistance in Alzheimer disease showing that detection of phosphorylated insulin receptors in the cytoplasm is expressed with insulin resistant cells in the hippocampus of AD while the receptors which are located at the nuclei are expressed in the normal cells (Talbot et al. 2012; Talbot & Wang 2014). A recent study showed that knocking out insulin receptors specifically in the brain using NIRKO mice caused mitochondrial dysfunction with increasing level of reactive oxygen species and increased lipid oxidation in the striatum. In addition, it caused a reduction in the level of dopamine in the striatum and nucleus accumbens by increasing activity of mono-amino-oxidase enzyme (MAO) which was reversed by MAO inhibitors (Kleinridders et al. 2015). Moreover, Wang and colleagues illustrated that the nigral dopaminergic neurons in a ob/ob and db/db mice model (a well-accepted T2DM model) are more vulnerable to degeneration by MPTP toxin compared to wild type mice. They also showed that insulin signalling is not only impaired in the pancreas and liver in this model but also in the midbrain (Wang et al. 2014). Furthermore, Saller et al. showed intravenous glucose dose (15 mg/kg) which elevated blood glucose levels by 30% in SD rats produced a reduction of dopaminergic neuron activity in SN and reduce dopamine release by 60-80% during the period of glucose elevation while administration of glucose at

250 mg/kg dose IV caused a complete cessation of dopamine release (Saller & Chiodo 1980). In clinical trials, several studies suggested that there is an association between Parkinson disease and T2DA (reviewed in (Santiago & Potashkin 2013)). Taken together, developing the insulin resistance in the host environment may potentiate several factors leading to the promotion of death of the transplanted cells or a reduction in their function. So, it has been hypothesised that hyperinsulinemia and hyperglycaemia may participate in suppression of the protective effect of exendin-4 on transplanted dopaminergic neurons.

Another possible reason for the halt in the protective effect of exendin-4 in presence of L-dopa is the reversing of the suppressive effect on the microglia levels. High plasma levels of insulin and glucose may have contributed to the increase in the host immunological response. Previous work illustrated that high glucose levels can stimulate glial activation via increasing oxidative stress leading to increased secretion of TNFα and monocyte chemotactic protein-1 (MCP-1) in rat microglia culture and elevation of IL-6 and IL-8 expression and secretion by astrocyte (Quan et al. 2011; Bahniwal et al. 2017). In addition, peripheral insulin resistance has been linked with inflammation including release of pro-inflammatory cytokines like TNF-α, IL-6, IL-1β that can cross blood brain barrier (De Felice & Ferreira 2014). L-dopa may also have a direct effect on driving more activated microglia around the graft. Breger and colleagues showed that there was a significantly higher number of microglia and leukocytes around the xenograft (but not allograft) in a similar model exploring the role of L-dopa in cell transplantation (Breger et al. 2016). However, this higher inflammatory response by L-dopa did not affect graft survival or function, a closer analysis suggested that microglial or leukocyte phenotypes were affected by L-dopa.

The possible interpretation for this paradoxical effect of exendin-4 in the presence of L-dopa treatment is that there may be an interaction between these treatments which leads to accumulation of the exendin-4 metabolite that has mild to moderate GLP-1 receptor antagonism (exendin (15-39) and exendin (16-39)). These metabolites are normally cleared by the kidney via glomerular filtration (GFR) and tubules secretion (Baggio & Drucker 2007). A previous study showed that benserazide, administered in combination with L-dopa, interferes with GFR as it is used to reduce the increase in GFR in case of infusion of amino acid solution or in case of diabetic inducing GFR when infused at rate of 30µg/min/kg (Pfeil et al. 2006; Miihlbauer et al. 1994). In this experiment, benserazide was given subcutaneously

in a ratio of 1:1 with L-dopa at a dose of (12 mg/kg) once daily or each alternate day. This dose is higher than the total infused benserazide dose over 4 hrs to reduce the GFR (7.2 mg/kg), in addition it is given at the same time as exendin-4 which has a half-life of 60-90 min and biological action for 4 hrs. Moreover, the used L-dopa/ Benserazide dose in this experiment was also higher than patient's maintenance regular dose which is in the range of 400-800 mg daily of L-dopa and in a ratio of 4:1 with benserazide (i.e benserazide dose range 1.4 - 5.7 mg/kg/day, assuming adult weight is 70kg). So, it was assumed that the relatively high dose of benserazide may have reduced excretion of the exndin-4 metabolite leading to their accumulation in the blood. These metabolites may halt the protective effect of the exendin-4 on the graft either directly by blocking the GLP1 receptor or indirectly through inducing pathophysiological changes.

Did L-dopa support the liraglutide effect on graft function?

Previous work showed that L-dopa treatment does not affect graft survival or functioning. However, a recent study did reveal that L-dopa contributed to increased infiltration of leukocytes around the xenograft with proliferation of more CD4 subtype (T helper lymphocyte) than CD8 (cytotoxic lymphocyte) (Breger et al. 2016). Similarly, the group treated with liraglutide and L-dopa here had a high leukocyte infiltration around the graft. Other experiments suggested that L-dopa is responsible for promoting the differentiation of a more T helper subtype 2 (Th2) cells which cause release of the anti-inflammatory cytokines rather than T-helper subtype 1 (Th1) which cause release pro-inflammatory cytokines. Furthermore, L-dopa induce Th2 cells to release neurotrophic factors like BDNF and IGF-1 and polarise the microglia toward the M2 phenotype which has neuroprotective effects and releases anti-inflammatory cytokines (Carr et al. 2003; Mori et al. 2013; Nakano et al. 2009). CD4 lymphocyte and anti-inflammatory cytokines like IL-4 could cause conversion of microglia from M1 state to M2 phenotype (Ziv et al. 2006; Butovsky et al. 2006)**.** Interestingly, GLP1 agonists have been reported to have the ability to polarise macrophages from M1 state to M2 via stimulation of STAT-3 pathway (Shiraishi et al. 2012). A possible interpretation of the graft function support of liraglutide in the presence of L-dopa could be that a synergistic effect between the two therapies leads to a predominance of the anti-inflammatory markers. This may explain the beneficial effect of L-dopa plus liraglutide on improving graft function with effect on graft size. In previous study comparing motor functions of small dose graft and large

dose graft, the data suggested that less inflammatory reaction around the graft was one of the factors that may enable small graft to have the same large graft functional performance (Bartlett et al. 2004). However, future studies are needed to characterise the type of inflammatory cells and kind of predominant cytokines around the graft in the presence of L-dopa and liraglutide.

Effect of the graft and GLP-1 agonist on L-dopa induced dyskinesia and L-dopa induced rotations.

Development of dyskinesia following chronic L-dopa administration occurs through the lack of dopaminergic neuronal control of dopamine release and turn over in the synapse, leading to accumulation of dopamine post-synaptically and causing an excessive stimulation of dopamine receptors. The long-term consequence is the development of dyskinesia. The replaced dopaminergic neurons of all graft groups successfully reduced LID from week 4 and L-dopa-induced rotations on week 10 and 11 of transplantation compared to pre-transplantation level. The reduction in LID is depended on the graft size where a large graft (graft volume = 3.16 mm^3 ; TH count = 17,400) caused a higher reduction in LID compared to a smaller graft (graft volume = 0.11 mm^3 ; TH count = 280) (Lane et al. 2006). In the current study, exendin-4 showed a limitation to support survival of dopaminergic neurons in presence of L-dopa treatment while liraglutide even it showed ability to improve fibres density but it also didn't have significant effect to improve cell survive. This limitation in improving graft size may the reason for ineffectiveness of GLP-1 agonists to reduce L-dopa induced AIMs and rotations. However, exendin-4 and liraglutide illustrated a tendency on getting a better reduction on the AIMs slope at the first two weeks of transplantation compared to the graft control (AIMs reduction slope: graft (-0.4); exendi-4 (- 7.7) and liraglutide (- 9.1)). This may suggest that GLP-1 agonist may accelerate the maturation of the graft and its ability to handle exogenous L-dopa.

Effect of the GLP-1R agonists on DA subtype and serotonergic neurons survival in the graft

The other factors investigated in this experiment are the effect of GLP-1R agonists on the ratio of A9 subtype-dopaminergic neuron and survival of the serotonergic neurons in the graft because they are important in determining the graft functionality and side effects. Studies clarified that A9 subtype is the phenotype responsible for the motor recovery in the graft

(Grealish et al. 2010) and the presence of serotonergic cells in the graft are suggested to have an important role in developing GID (Politis et al. 2011). The results demonstrated that both exendin-4 and liraglutide had no effect either on A9 proportions or on serotonergic neuron survival in the graft, either in presence or absence of L-dopa. Thus, the effect of these parameters on the graft outcome were less likely to be changed by administration of GLP-1R agonists.

Dose the liver status has interference with Parkinson Disease and cell transplantation efficacy?

The fatty status of the liver was explored in this experiment because of the evidence of abnormal metabolic changes which were recorded with insulin and glucose. This experiment was not designed to evaluate the changes in the liver in PD model in presence the graft or the treatments. There is a lack for normal rats' liver control and normal liver exposed to all the used treatment in this experiment and lesion control group exposed to these treatments. However, Interestingly, the current liver data may give insight into the possible involvement of the liver in Parkinson Disease and/or cell transplantation. Surprisingly, in the 6-OHDA lesion control group a high accumulation of fat droplets in liver sections was recorded while the graft with exendin-4 reduced this condition. From previous work the percentage of fat in the liver of the normal rat was in the range between 0-2% (Kho et al. 2016; Bang et al. 2017; Gu et al. 2017) while the mean fat accumulation in the lesion control was higher than 17%, which could suggest the abnormal fat accumulation in the Parkinson's liver. Consistent with this is the finding that exendin-4 is effective at reducing fat accumulation and steatosis condition in the liver of Non-Alcoholic Fatty Liver Disease (NAFLD) (Eguchi et al. 2015; Lee & Jun 2016; Liu et al. 2014). So, this may give some support to the hypothesis that the liver is affected in this particular model of PD. However, this needs to be clarified in a study designed for this purpose in future and exploring the clinical liver data base in PD to determine whether there is a clinically relevant effect.

4.5 Conclusions

GLP-1R agonists were effective in supporting survival and function of the VM graft in 6-OHDA rat model. However, their supportive effect was determined by adding L-dopa treatment to the model. Exendin-4 increased graft size and enhanced graft function, however this effect was halted by L-dopa treatment. Plasma investigations showed that the interaction between

L-dopa and exendin-4 caused elevation in insulin and glucose levels. Further analysis identified signs of insulin resistance in the graft. It was hypothesised that insulin resistance which was developed from interaction between L-dopa and exendin-4 hindered the protective effect of the later. On the other hand, liraglutide illustrated no effect on the graft survival, instead it increased TH+ density of the graft (cell body and fibres). However, liraglutide illustrated ability to support graft function. Liraglutide effect on graft function is also interacted with L-dopa treatment, in contrast to exendin-4, it supports graft function in presence of L-dopa. Histological investigation showed that the interaction between L-dopa and liraglutide caused a significant increase in leukocyte infiltration around the graft. This data suggests the drug combination may induce inflammatory modulation leading to supporting graft function.

Thus, this chapter has helped to identify neuroprotective agents that can support survival and efficacy of primary VM cell transplantation. In addition, the impact of L-dopa on determining the supportive effect of the neuroprotective agents on the graft, highlighted the importance of considering L-dopa in cell therapy evaluation in animal models. The next chapters will address another issue of cell therapy in PD which is understanding the effect of L-dopa treatment on the development and function of human embryonic stem cell derived dopaminergic neuron hESC-DA transplantation in same model. In addition, it will explore the effect of exendin-4 as a neuroprotective agent to support these grafts.

5 Chapter 5: characterisation of (H9) human Embryonic Stem Cells (hESC)-derived dopaminergic neuron transplantation in a 6-OHDA rat model of PD in the presence of L-dopa treatment and neuroprotective agent

5.1 Introduction

Recently studies have successfully generated dopaminergic neurons that have the substantia nigra/A9 specific markers using stem cells derived through different lines and with varying protocols. In some cases, transplantation of these cells typically into the 6-OHDA lesioned rats produced successful cell survival and some evidence of graft function. hESC-DA derived from H9 cell line showed successful survival, functional and safe transplantation in animal models. H9 (WA09) cell line is a clinical grade line and banked under GMP conditions (Wi Cell® labs). Kirkeby and colleagues treated these cells with dual SMAD inhibitors and glycogen synthase kinase 3 (GSK3) inhibitors followed by adding SHH-C2411 to get region specific neurons (Kirkeby et al. 2012). They successfully yielded cells expressing the specific VM cells markers (FOXA2 and LMX1A) and generated a high number of TH expressing dopaminergic neurons *in vitro*. These cells were transplanted into the 6-OHDA rat and compared with human VM cells for 6 weeks. The TH count in the graft of hESC-DA was 15 times higher than the hVM graft; and 81% of the hESC-DA expressed LMX1A and FOXA2 compared to 21% of the hVM. The morphology of the neurons was bipolar and angular cell bodies with elongated axons and the pattern of innervation was identical to hVM. Then, they evaluated the graft for 18 weeks' post-transplantation, they found that 54% of the hESC cell in the graft was TH labelled (TH/HuNu) with a large proportion of Girk2 labelled neurons indicative of the A9 phenotype. In addition, they found that the graft can release dopamine, reverse amphetamine-induced rotations and improve performance in the cylinder test (Kirkeby et al. 2012). A follow up study illustrated the successful synaptic integration of these cells with the host striatal neurons and the making of rapid and extensive connections with afferent and efferent neurons (Grealish, Heuer, et al. 2014). So, these cells showed remarkable success in animal models which make it a potential candidate for clinical trials.

However, to transfer stem cell-based transplantation therapy into clinical studies, it should be validated in an animal model that mimics the real conditions as closely as possible. One of the clinical factors which has never been considered in hESC-DA experiments is: first, that the patients typically receive a variety of anti-Parkinsonian medications, commonly L-dopa; second, mostly those patients have developed LID, the most common side effect of these medications. In addition, patients continue to use these medications after transplantation for a while till get a mature and functional graft (the table below (Table 7) shows the range of

medications used by patients prior to and following human VM cell transplantation in the TransEuro clinical trial). It is plausible that the presence of L-dopa following transplantation may have a direct effect on the programming, differentiation and development of these cells. A recent paper published by Belinsky and colleagues showed that dopamine receptors (D1-D5) are expressed at different stages of differentiation of DA neurons from hESC; they found that treating these cell with L-dopa during the early and later stages of differentiation had effects including more neuroepithelial colonies, more neuronal clusters and more TH clusters (Belinsky et al. 2013). The interaction between these medications and stem cell- based transplantation needs to be tested to validate the safety, survival and functionality of these cells. Previous works in animal models of PD illustrates that L-dopa causes changes in blood brain permeability (Westin et al. 2006) and induces an enhanced inflammatory reaction around striatal xenografts (Breger et al. 2016) such things could change the host environment around the graft.

The other important point that needs to be highlighted in stem cell derived dopaminergic neuron transplantation is understanding the capability of these cells to ameliorate LID and the possibility of these cells to generate GID. To test the dyskinesia profile in an animal model of PD, animals should be exposed to a chronic L-dopa treatment prior to transplantation to establish LID. This because L-dopa makes conformational changes in the striatal cells leads to the induction of LID, then the efficiency of the graft to alleviate LID can be evaluated. Considering all of this together, it is important to characterise stem cell transplantation in animal models treated with anti- parkinsonian medication to be able to evaluate the safety, survival, functional and dyskinesia profile of stem cell grafts before moving to clinical trials.

This study aims to:

Firstly, understand the effect of L-dopa on hESC-DA graft using the cells developed by Kirkeby and colleagues and resolve key questions before clinical trials:

- Does L-dopa affect graft maturation and development?
- Does L-dopa affect the functional efficacy of the graft?
- Are hESC-DA able to reduce LID?

Secondly, based on the findings of the neuroprotection produced by exendin-4 on the VM cells in chapter 4, it also appears relevant to determine whether exendin-4 could support hESC-DA graft survival and function.

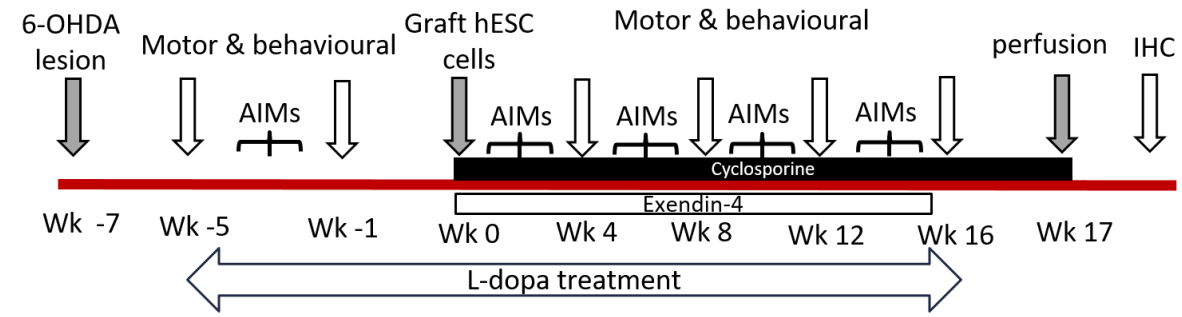
The objectives of this study therefore are:

1. Assessment the motor and behavioural recovery of the graft in the presence and absence of L-dopa.
2. Characterisation the histological features of the graft in the presence and absence of L-dopa.
3. Post-mortem detection of GLP-1 receptors expression in the graft of H9 hESC-derived dopaminergic neurons at the analysis.
4. Determination of the effect of exendin-4 on the behavioural and the histological measurments of the graft matured in the presence or absence of L-dopa.

Table 7 Parkinson Disease medications used by 12 patients at the time of VM cell transplantation (obtained from prof. Roger Barker, transEuro coordinator, personal communication) (abbreviations: AADC = aromatic amino-acid decarboxylase; COMT = catechol O methyl transferase).

Treatment class	number of patients out of 12	average daily dose
L-dopa + AADC Inhibitors (Stalevo, Madopar, Sinemet, Co-Beneldopa)	11	667 mg
D2 agonist (Ropinirole, Pramipexole)	9	ropinirole (15mg), Pramipexole (1.5 mg)
Monoaminoxidase inhibitors (Selegiline and Rasagiline)	10	Selegiline 10 mg; Rasagiline 1mg)
COMT inhibitor (Entacapone)	6	775 mg

5.2 experimental design and methods



45 rats divided into the following groups:

A. Lesion control
B. Graft
C. Graft+ exendin-4
D. Graft+ L-dopa
E. Graft+ L-dopa+ exendin-4

Figure 34 experimental time line and grouping

6-OHDA was infused unilaterally into the medial forebrain bundle in the right hemisphere (2.2.1) of 45 female Sprague Dawley rats. Two weeks later, amphetamine-induced rotations were assessed to determine the nigral dopaminergic lesion (rats who performed ipsilateral rotations ≥ 6 times/ min, were considered to have more than 90% lesioned of the right SN). Then the rats were divided into 5 groups (n=9): one group was lesion only control; four groups were transplanted with the ESC-DA into the depleted striatum (section 2.2.2.2.1). Groups were then allocated to a treatment group (two groups treated with saline ± exendin-4 and two groups with L-dopa ± exendin-4) (see Figure 34). The treatment with L-dopa started 5 weeks before cell transplantation surgery and continued for 15 weeks after transplantation. All the transplanted groups received immunosuppression using cyclosporine-A (10 mg/kg) 24 hrs before cell transplantation and continued daily i.p. until the end of the experiment (16 weeks). Exendin-4 treatment started 24 hrs before transplantation surgery and continued until week 15 post-surgery. The motor and behaviour tests including amphetamine-induced rotations, adjusting step test, vibrissae test, cylinder test and apomorphine-induced rotations were carried out throughout the experiment. These tests were applied on the following time points: prior to starting of L-dopa treatment; prior to transplantation surgery and every 4 weeks after graft surgery up to 16 weeks. The cylinder test was carried out only at the pre-L-dopa time point and at week 16, while the apomorphine test was carried out only at week 16.

Abnormal Involuntary Movements (AIMs) and L-dopa-induced rotation tests were recorded weekly before and after cell transplantation from the start of L-dopa treatment (section 2.4). L-dopa and exendin-4 treatments were stopped 2 days before each behavioural and motor test in order to wash out any functional effect. On week 17 of transplantation, all rats were perfused and the brains were collected for histological analysis. DAB-IHC was used to identify: TH to label dopaminergic neurons in the graft and the SN; HuNu to label human nuclei, Stem-121 to label human cytoplasm (ie incorporating projections); and CD11b to label the activated microglia around the graft (section 2.10.1). Double F-IHC was used to stain TH and GLP-1R (section 2.10.2).

5.2.1 Treatments

L-dopa in combination with benseraside was given to the rats 5 weeks before the transplantation surgery. It was started with a dose of 12 mg/ kg of each of L-dopa and benserazide and given as single s.c. injection every day for 3 week then reduced for 6 mg/kg of L-dopa + 12 mg/kg of benserazide for two weeks. The latter dose was continued in use after transplantation surgery every day for 16 weeks. L-dopa treatment was stopped 2 days before graft surgery and 2 days before each behavioural and motor tests.

Cyclosporine treament commenced 24 hrs before graft surgery and continued every 24±1 hrs until killing the rats. It was used at a dose of 10 mg/kg intraperotineally. Cyclosporine was obtained from Heath hospital pharmacy manufactured by Novartis in 50 mg/ 1ml ampoules. The ampoules were diluted with 0.9% normal saline and used immediately or kept refridgorated for no longer than 3 days.

Exendin-4 was given (i.p.) a dose of 0.5 µg/ kg twice daily for the first two weeks after cell transplantaton. Then because of poor rodent health (significant weight loss), it was stopped for 5 days and then commenced with single daily doses untill week 15. Exendin-4 was ordered from Tocris Bioscince as a 1mg reconstituted powder. It was dissolved in 1 ml of 0.9% normal saline and aliquoted (stored on -20 C^0 for a maximum 30 days).

5.2.2 Statistical analysis

The statistical analysis of the data was preformed using IBM SPSS. The behavioural data analysis was separated into two sets. First was between graft alone (in the absence of exendin-4 and L-dopa) and lesion groups to characterise the effect of the graft without treatments using repeated measure analysis (time as within subject factor; group (graft or

lesion) as between subject factor). The second set was between the grafted groups to evaluate the effect of exendin-4, L-dopa and their interaction on the graft throughout the experimental time using repeated measure analysis (time as within subject factor; exendin-4 treatment factor and L-dopa treatment factor as between subject factors). Repeated measure analysis was also performed on L-dopa rotation and AIMs score data (time as within subject factor, exendin-4 as between subject factor). Histological data was analysed using two-way ANOVA to find the effect of L-dopa, exendin-4 or their interaction on the graft (between subject factor included exendin-4 treatment factor; and L-dopa treatment factor). Fibre outgrowth medially or laterally was analysed using repeated measure analysis (distance from the graft as within subject analysis; exendin-4 factor and L-dopa treatment factor was between subject factor). Bonferroni was used for multiple pairwise comparison. Two tailed t-test was used to analysis the difference between the graft and the lesion at week 16 in apomorphine results and it is also used to analyse the difference between the medial and lateral fibre innervation in the graft alone (without treatment). The data was considered significant if P value < 0.05.

5.3 Results

5.3.1 Percentage of the nigro-striatal dopaminergic neurons lesion

TH cells counted in the SN showed that the percentage of dopaminergic neuronal loss on the lesioned side was more than 98% compared to the intact side in all experimental groups. This indicated an almost complete lesion of nigral dopaminergic neurons. It was therefore interpreted that any change in performance was due to the graft, not the treatment.

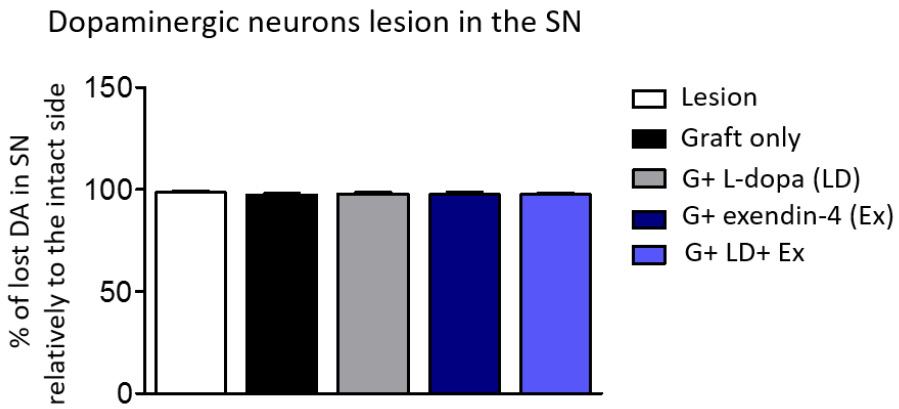


Figure 35 percentage of the lost dopaminergic neuron in the lesioned hemisphere compare to the intact side: more than 98% of the nigral dopaminergic neurons were lost in all groups.

5.3.2 Motor and behavioural results

5.3.2.1 The effect of the graft alone

The graft of H9 hESC-DA produced a significant overall recovery in amphetamine-induced ipsilateral rotation and this effect has significant interaction with the time (graft $F_{(1,15)} = 7.4$, **p<0.01; time*graft, $F_{(5,75)} = 16$, p<0.001); the significant difference was in week 12 and 16 ($F_{(1,15)\ min}$ week 12 = 7, p<0.01) (repeated measure ANOVA). it also has significant effect on apomorphine-induced contralateral rotations (week 16) (t-test, two tailed **p<0.01) compared to the lesion only control group. In the sensorimotor tests, the graft only group produced a high overall significant recovery in vibrissae tests and this effect has significant interaction with the time (graft, $F_{(1,15)} = 11$, p<0.01); graft*time $F_{(5,75)} = 5.4$, p< 0.001); it produced this significant difference only on week 16 ($F_{(1,15)} = 17.8$, p<0.001) (repeated measure ANOVA). Graft showed it has no significant effect in the stepping and cylinder test (max: cylinder test, $F_{(1,15)\ max} = 1.3$, n.s).

5.3.2.2 Effect of L-dopa and exendin-4 treatments on the graft

L-dopa treatment had no overall effect on amphetamine induced-rotations (repeated measure ANOVA, $F_{(1,30)} = 0.1$, n.s) and apomorphine-induced rotations, (two-way ANOVA, $F_{(1,30)} = 0.04$, n.s.). Exendin-4 also had no effect on graft function in either the amphetamine-induced rotations, (repeated measure ANOVA, $F_{(1,30)} = 0.58$, n.s;) or apomorphine-induced rotations (two-way ANOVA, $F_{(1,30)} = 1.6$, n.s.).

In the vibrissae test, the effect of both L-dopa treatment and exendin-4 had an overall significant effect to improve responses and there is a significant interaction between the treatments (repeated measure ANOVA: L-dopa, $F_{(1,30)} = 5.6$, $^{+}p< 0.05$; exendin-4, $F_{(1,30)} = 9.4$,

**p< 0.01; L-dopa x Exendin-4 $F_{(1,30)} = 5.6$, p< 0.05). This interaction showed that L-dopa effect was significant in absence of exendin-4 ($F_{(1,30)} = 9.8$, p<0.01) and had no effect in presence of exendin-4 ($F_{(1,30)} = 0$); similarly, exendin-4 was effective in absence of L-dopa ($F_{(1,30)} = 14$, p<0.001) and had no effect in presence of L-dopa treatment ($F_{(1,30)} = 03$, n.s). This interaction between L-dopa and exendin-4 was also interacted with the time (time x L-dopa x exendin-4: $F_{(5,150)} = 4.1$, p<0.001); on week 4, exendin-4 was effective in presence of L-dopa ($F_{(1,30)} = 5$,p<0.05) and not effective in saline group ($F_{(1,30)} = 1.7$, n.s), while on weeks 8, 12 and 16, exendin-4 was effective in saline groups (min: week 16: $F_{(1,30)} = 7.8$, p<0.01) and not effective in L-dopa group (max: week 16: $F_{(1,30)} = 2.4$, n.s).

In addition, in vibrissae test, there was a significant interaction between exendin-4 and the time (exendin-4 x time: $F_{(5,150)} = 3.8$, p<0.01); further analysis showed that exendin-4 was effective in weeks 4, 8 and 12 (min: week 4: $F_{(1,30)} = 6.5$, p<0.05) but it had no effect on week 16 ($F_{(1,30)} = 0.7$, n.s). There was a high tendency for interaction between L-dopa and the time but it was not significant (L-dopa x time: $F_{(5,150)} = 1.9$, p=0.09); the close analysis showed that L-dopa was effective on week 8, 12 (min: week 8: $F_{(1,30)} = 4.5$ p<0.05) but no effect on week 16 ($F_{(1,30)} = 0.7$, n.s)

In cylinder tests, L-dopa and exendin-4 had no overall effect on the test (max: exendin-4 $F_{(1,30)} = 2.5$, n.s). However, there is a significant interaction between the time, L-dopa and exendin-4 (time x L-dopa x exendin-4: $F_{(1,30)} = 7.8$, p<0.01), on week 16, L-dopa had significant improving effect in absence of exendin-4 ($F_{(1,30)} = 15.8$, p<0.001) and had no effect in presence of exendin-4 ($F_{(1,30)} = 0.01$, n.s); while exendin-4 had significant reducing effect in L-dopa group ($F_{(1,30)} =5.5$, p<0.05) and had no effect in absence of L-dopa ($F_{(1,30)} = 2.9$, n.s).

In stepping test, there was no significant effect for L-dopa, exendin-4 or their interaction (max: exendin-4: F (1,30) = 0.4, n.s).

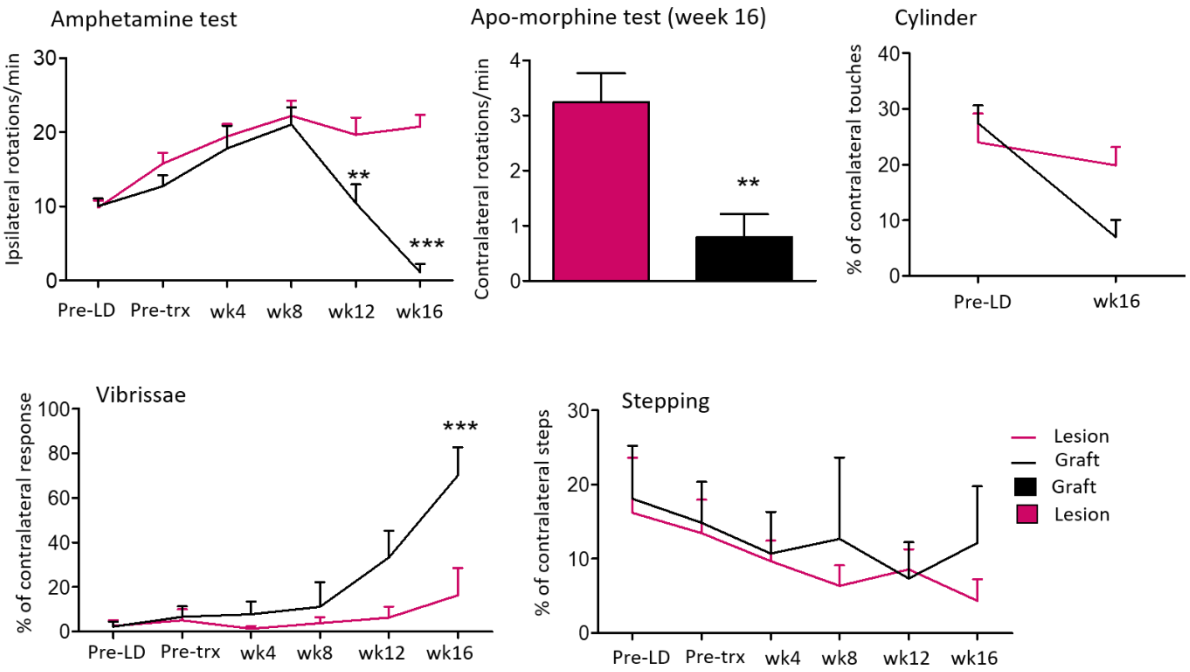


Figure 36 The effect of H9 hESC derived dopaminergic neurons graft on behavioural results: the graft effect was significant on reducing number of amphetamine rotations (repeated measure, ** p<0.01); on reducing number of apo-morphine rotations (univariant analysis, **p<0.01); on increasing rats' response in vibrissae test (repeated measure, **p<0.01). the graft had no effect on the stepping and cylinder tests.

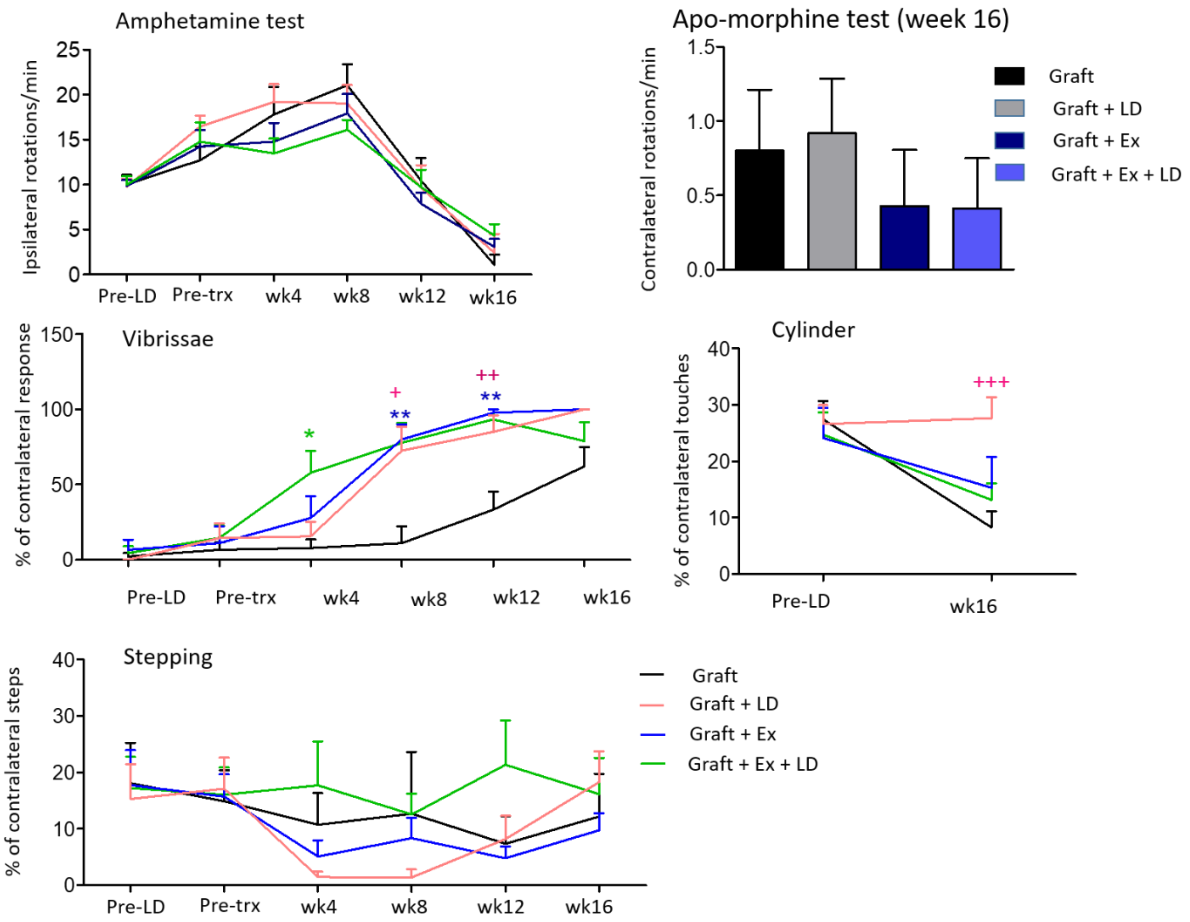


Figure 37 effect of L-dopa and exendin-4 on the motor and behavioural results: L-dopa and exendin-4 showed they had no effect on the amphetamine rotation tests, stepping test (repeated measure ANOVA) and apomorphine test (two-way ANOVA). In vibrissae test, both treatment had overall significant effect with significant interaction between them (L-dopa, $^{+}p< 0.05$, $^{++}p< 0.01$; exendin-4, *p< 0.05, **p< 0.01). In cylinder test, L-dopa has improving effect on week 16 only in non-exendin-4 group ($^{+++}p<0.001$).

5.3.3 L-dopa induced dyskinesia and rotations

Before transplantation, the dose of 12 mg/kg of L-dopa induced a fast upregulation in the level of contralateral rotations and total AIMs score through the 3 weeks of the treatment, as a result dosing was reduced and maintained for two weeks at a dose of 6 mg/kg.

After transplantation, total AIMs scores steadily reduced through the 15 weeks following transplantation and the pairwise comparison with time showed that the highly significant reductions started from week 4 compared to the base line time point one week before transplantation (repeated measure analysis, **p<0.01, ***p<0.001). Exendin-4 had no effect on the total AIMs score (repeated measure analysis, $F_{(1,12)} = 0.003$, n.s.). Similarly, L-dopa induced contralateral rotations reduced significantly and the pairwise comparison within the time showed that the significant difference started from week 5 compared to one week prior

to transplantation (repeated measure analysis, *p<0.05). Exendin-4 had no effect on L-dopa-induced rotations (repeated measure, $F_{(1,13)} = 1.1$, n.s).

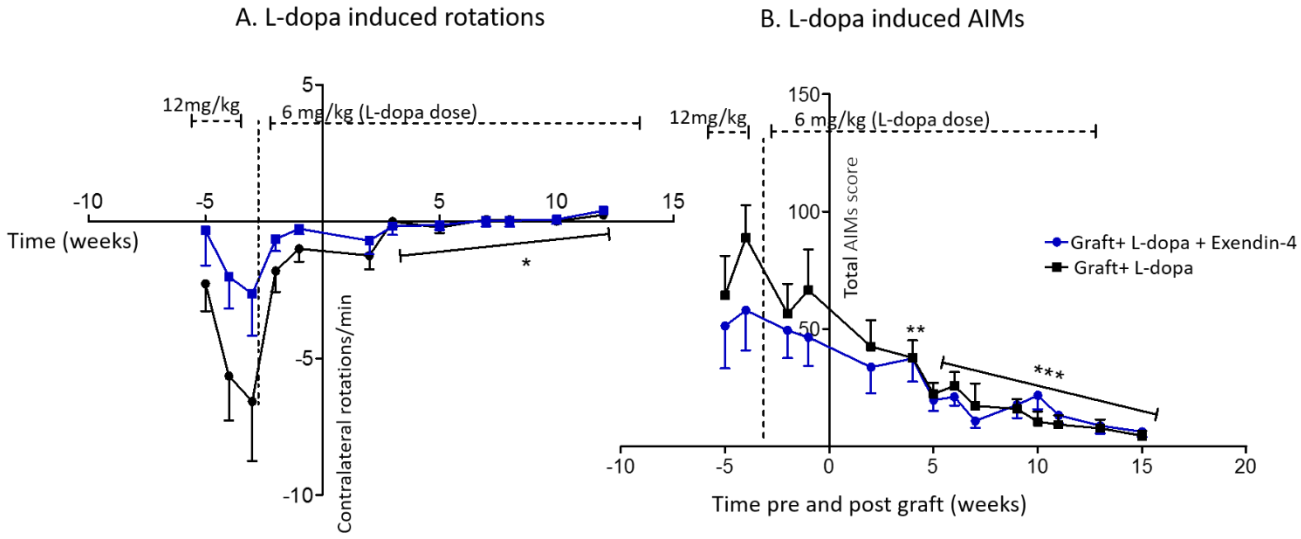


Figure 38 L-dopa induced rotation and Abnormal Involuntary Movements AIMs: (A) L-dopa induced contralateral rotations reduced significantly after transplantation since week 5 of the graft compared to baseline time point week -1(repeated measure analysis, *p<0.05); (B) total AIMs score reduced in high significance since week 4 of transplantation compared to week -1 (repeated measure analysis, **p<0.01, ***p<0.001). exendin-4 had no interference on number of L-dopa rotations or AIMs score.

5.3.4 Expression of GLP-1R on the dopaminergic neurons at the graft

Post-mortem analysis of the transplanted H9 hESC derived dopaminergic neurons in the lesioned striatum showed co-expression of the GLP-1 receptor with the dopaminergic neurons 17 weeks following transplantation.

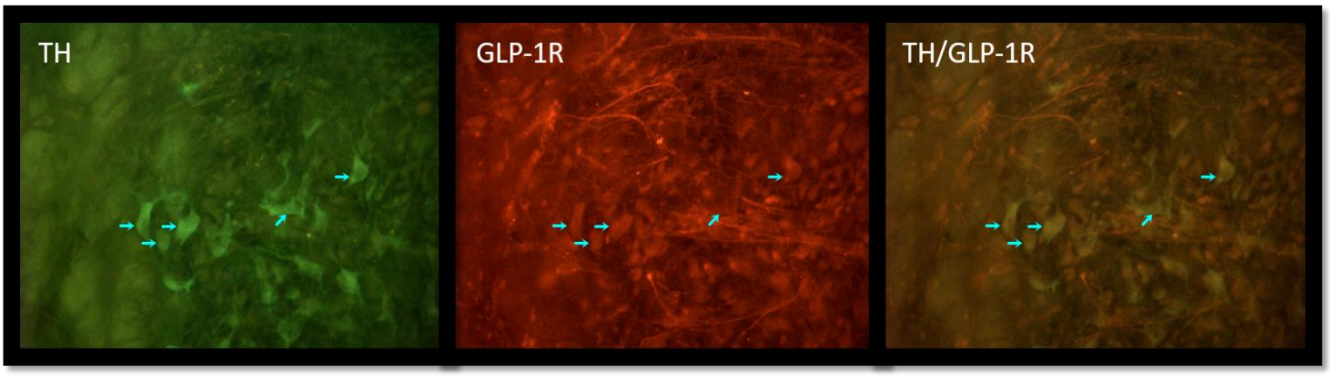


Figure 39 expression of GLP-1R in the graft: double fluorescent IHC illustrated co-expression of TH markers (green) with GLP-1R marker (red) in the 17 weeks graft of H9 hESC derived dopaminergic neurons graft.

5.3.5 Graft cells survival and volume

In the graft only group (absence of L-dopa and exendin-4), total graft cell survival identified by HuNu was 72,300 ± 5500 cells representing 24% of the transplanted cells (300,000) and the graft volume identified with stem-121 was 1.3± 0.18 mm^3. The dopaminergic neurons identified with TH immunohistochemistry showed a robust survival, the mean of the total dopaminergic neurons was (16118 ± 5370 cells), the volume of the dopaminergic neurons graft occupied an average of 1.27 ± 0.42 mm^3 of the striatum, and the dopaminergic neuron density was 13,461± 4,400 cells per mm^3. Compared to the total graft, the dopaminergic neurons represented around one fifth (22.2% ± 7 %) of total cell population and 86%± 28% of the total graft volume.

The adding of L-dopa and/or exendin-4 treatment to the graft had no effect on the grafts' characteristic features in terms of dopaminergic cell survival, dopaminergic graft (TH) volume, total cell population in the graft count, total cell graft volume, proportion of the dopaminergic neuron count to the total cells count and the dopaminergic graft volume to the total graft volume (two-way ANOVA analysis).

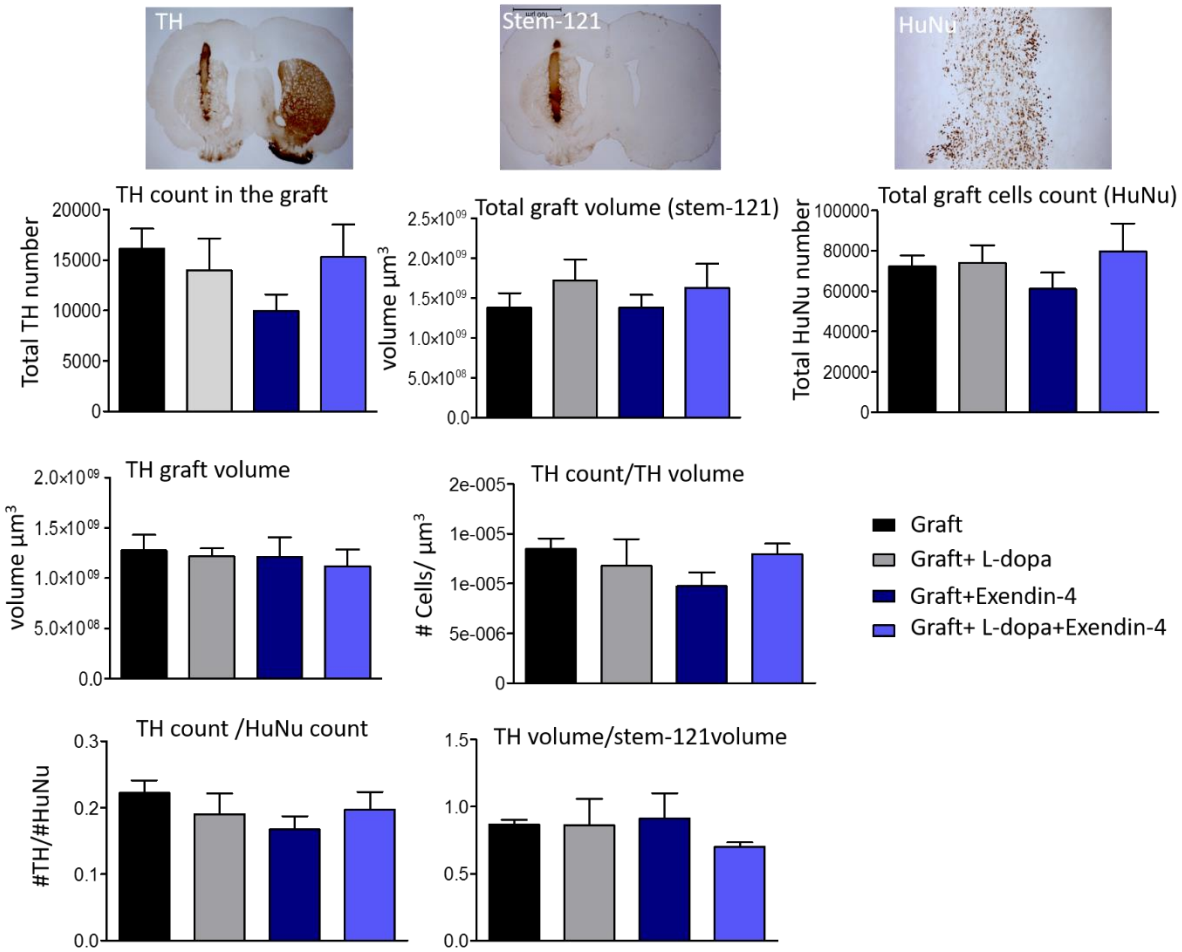


Figure 40 graft analysis: the graft in all groups labelled with TH to mark the dopaminergic neurons, HuNu to label the nuclei of all human cells in the graft and stem-121 to label all the cytoplasm of all human cells in the graft. There was significant effect for the exendin-4 and L-dopa on the graft and there was interaction between the groups on the following measurements (dopaminergic neurons count, dopaminergic neurons volume, total cells population count, total graft volume, the proportions of dopaminergic neurons count or volume to the total graft cells count or volume) (two-way ANOVA).

5.3.6 Pattern of fibres outgrowth

In the graft group (in the absence of L-dopa and exendin-4), within 600 µm distance of graft centre medially and laterally, the percentage of the striatum covered by the graft fibres was 49.6 % toward the medial side and 30.7 % at extending from the lateral side. This bias in sending fibres towards the medial side was significantly different from the lateral side (t- test, two tailed, * p< 0.05).

The effect of L-dopa and exendin-4 on graft innervation, A relative surface area of the fibres at distances of 200, 400, and 600 µm medially and laterally of the graft core showed a clear tendency of the graft to send more fibres toward the medial side compared to the lateral side.

The results showed that L-dopa and exendin-4 had no effect on fibre innervation on the medial and lateral side and there was no interaction between the treatments ($F_{(1,28)\ max}$ exendin-4, medially = 2.6, n.s) (repeated measure ANOVA, distance was the between subject factor). However, the data analysis at only 600 µm distance point showed that exendin-4 has a significant effect on increasing the percentage of striatal area covered by the graft fibres (two-way ANOVA: exendin-4, $F_{(1,30)} = 4.9$, $p<0.05$).

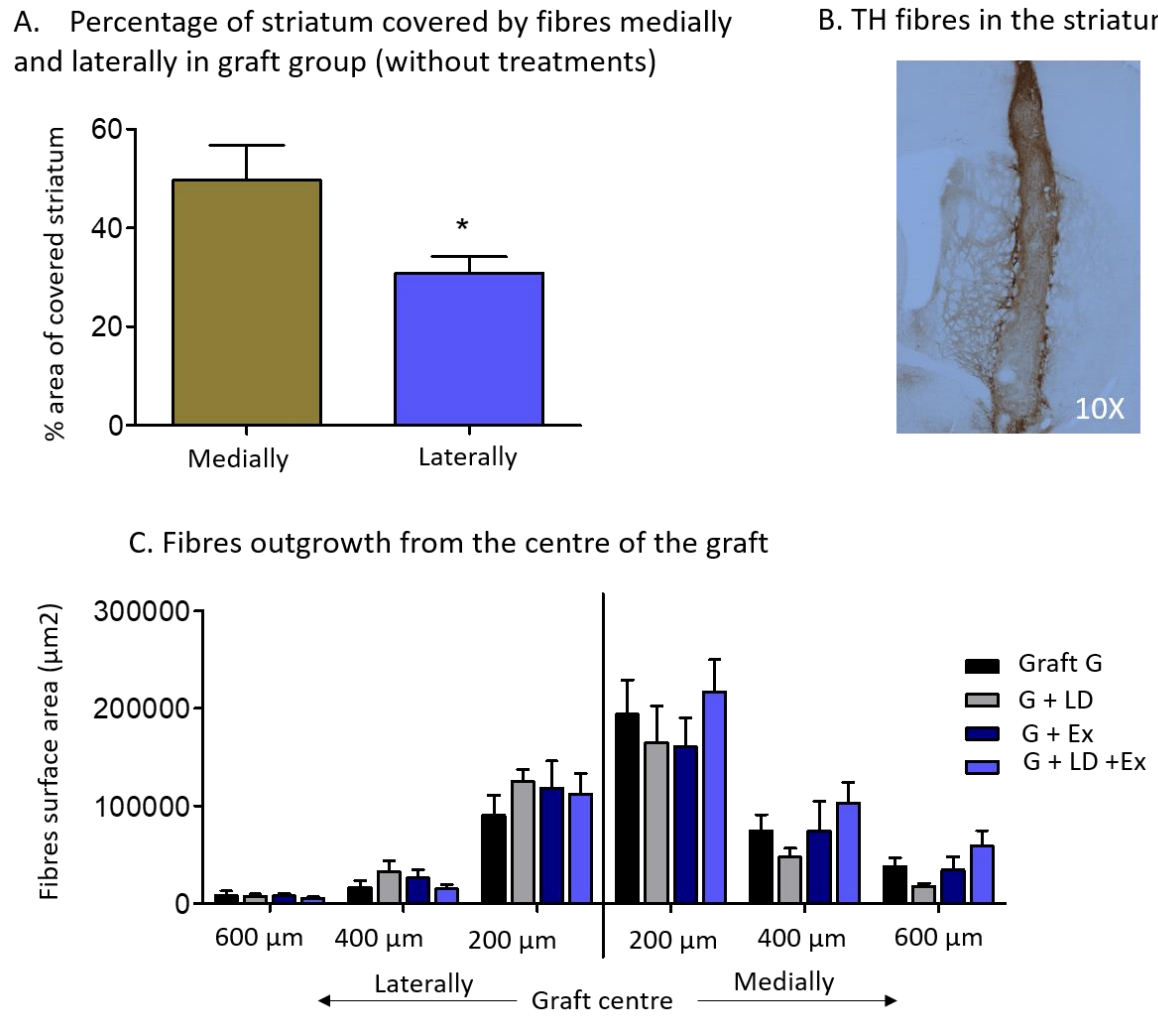


Figure 41 fibres outgrowth pattern: (A) in graft without treatments, the mean percentage of fibres covered the striatum throughout 600 µm of graft centre was 49.6 % medially and 30.7 % laterally with significant difference (t-test, $p<0.05$; (B) image for the graft showing pattern of TH innervation in the striatum; (C) effect of treatments on fibres surface area at distances of 200, 400, 600 µm of the graft centre medially and laterally showed there is no overall significant effect of the L-dopa and exendin-4 on fibres outgrowth and no interaction between the treatments (repeated measure analysis). However, at 600 µm distance point, exendin increase fibres surface area significantly (two-way ANOVA analysis, exendin-4, $p<0.05$, L-dopa, n.s.) G= graft, LD = L-dopa, Ex = exendin-4

5.3.7 Microglial density around the graft

The ratio of microglia stained with CD11b around the graft relative to the intact side was not significantly difference between the grafted groups, either in the presence or absence of L-dopa. Similarly, the addition of exendin-4 did not affect the level of activated microglia around the graft (two-way ANOVA, $F_{(1,27)\ max}$ L-dopa = 0.7, n.s).

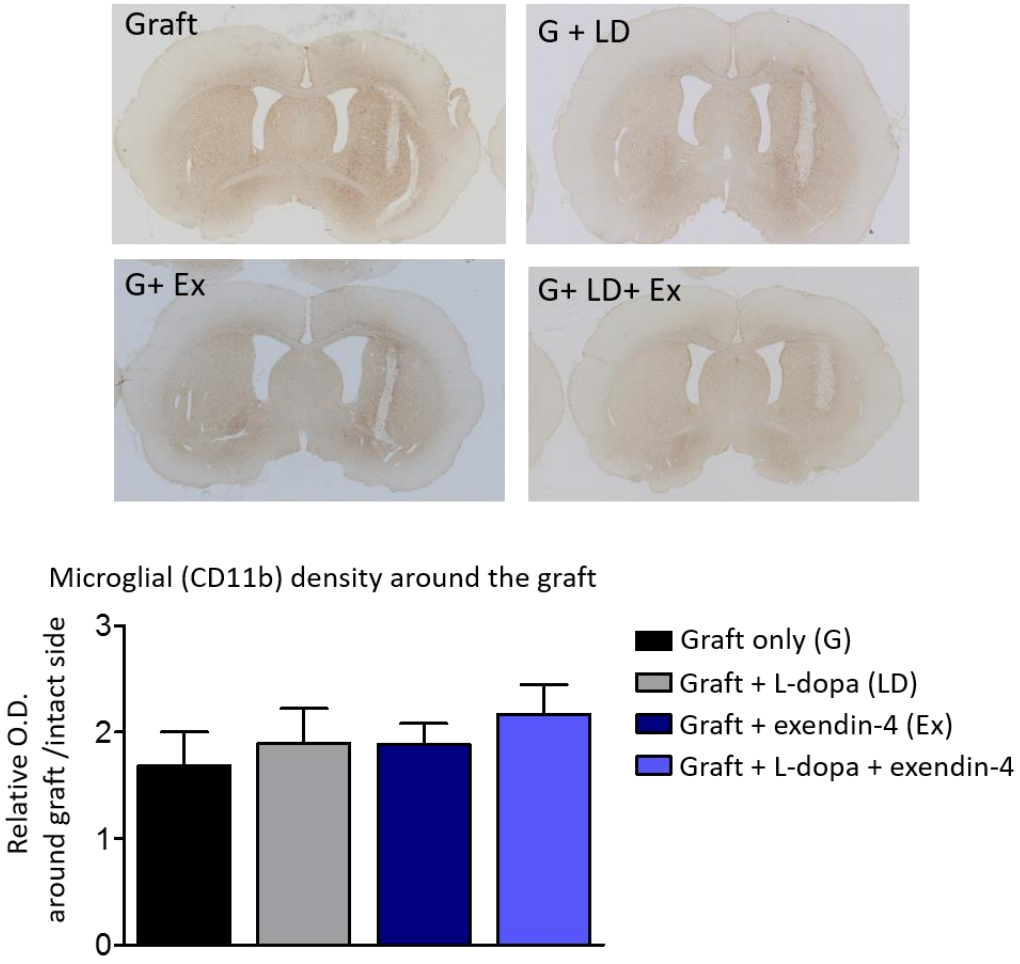


Figure 42 microglia labelled CD11b density around the graft: the measurement of the optical density of CD11b stain around the graft compared to the intact side of the striatum showed there was no significand difference in microglia density around the graft between all the transplanted groups (two-way ANOVA, n.s).

5.4 Discussion

H9 hESC derived dopaminergic neurons graft (without treatments) characterisation:

The histological features of the graft illustrate a robust survival of total cells in the graft (HuNu) at a rate exceeding 23% of the cells prepared for transplantation and the dopaminergic neurons (TH+) constituted about 22% of the cells in the graft (around 15,000 cells). These histological features were consistent with Kirkeby and colleagues, which used the same cell source and they even reported a greater yield of dopaminergic neurons from transplanted cells compare to the present study (around 18,000 per each 100,000 transplanted cells) (Kirkeby et al. 2012). The current histological findings were consistent with the functional efficacy of the graft which was evaluated using different behavioural and sensorimotor tests throughout this study. The confirmation that there was almost complete depletion of endogenous dopaminergic neurons demonstrated that the graft alone produced all motor function improvement. In the amphetamine-induced rotation test, which is used to assess the functional ability of the graft to release dopamine, a clear decline in the number of rotations since week 12 reflected the high dopaminergic population in the graft at post mortem. This is consistent with Kirkeby and colleagues who similarly reported the functionality of the same hESC-DA graft from week 12 of transplantation (Kirkeby et al. 2012) in the same assay. In a more detailed behavioural analysis the apomorphine test clarified whether the graft could normalise the sensitivity of the striatum created by the dopaminergic depleting lesion. Apomorphine is a non-selective dopamine receptor agonist which induces contralateral rotations in the unilaterally 6-OHDA rats due to super-sensitivity of the dopamine receptors on the lesioned side due to the lack of dopamine. So, the data confirmed that the pooled dopamine by the graft was efficient to partially reverse this sensitivity. So, both amphetamine rotation and apomorphine rotation data demonstrated the functionality of the H9 hESC graft.

Despite a robust survival of the dopaminergic cells in the graft and functionality of the graft to release dopamine, of all the sensorimotor tests, only the vibrissae test showed an improvement in motor function while deficits failed to recover in any of other tests (cylinder or adjusting step) recovered. This may suggest that 16 weeks was not long enough for the graft to have developed full functional therapeutic value. However, the Kirkeby study, which used the same cell source for transplantation, showed that the graft can alleviate number of

touches in the cylinder test significantly on week 14 of transplantation (Kirkeby et al. 2012). The limited functional recovery could be attributed to the low tendency of the graft to send fibres toward lateral side of the striatum while spreading the fibres favourably toward the medial side. Previous work clarified that better recovery for the sensorimotor tests is achieved from wider spread of the graft in the striatum (Falkenstein et al. 2009). In particular, the motor parts of the cortex send projections primarily to the dorsal and lateral portion of the striatum (Haber 2014), of which the latter had the lower innervation in the present study. Kriks et al transplanted H9 hESC-DA into the 6-OHDA-lesioned rat, which produced a graft that had histological features similar to the present study in terms of cell survival and volume. A published image (figure 4e in the paper) in this study shows an extensive graft innervation at the lateral side of the striatum suggested that this graft may have a preferable innervation toward lateral side. The grafts in this study reportedly alleviated stepping and cylinder tests significantly from week 12 following transplantation (Kriks et al. 2011) which is contrary to the present study. The developed dopaminergic neurons in both the Kriks et al study and the current study started from the same cell line (H9 hESC) and carry the essential protein expressions of midbrain dopaminergic neurons, but they were developed using different differentiation protocols (Kriks et al. 2011; Kirkeby et al. 2012). So, the difference in the motor recovery between current study and Kirks et al study could be related to the pattern of innervation in the lateral side of the striatum or possible a difference between the transplanted dopaminergic neurons due to the difference in the generating protocols.

Effect of graft on alleviation of L-dopa induced dyskinesia and rotations

The current study shows, for the first time, that hESC derived grafts can ameliorate L-dopa induced dyskinesia. The graft reduced AIMs scores at early post transplantation stages, even earlier than relieving motor and behavioural complications. The capability of the graft to alleviate LID in rodent model has previously been shown with foetal cells but has not been attempted with hESC-DA until now. Clinical cases report that VM cell transplantation can increase “on” time without dyskinesia (Kordower et al. 1998). In animal models, several studies from this lab have confirmed the capability of a VM graft to reduce AIMs scores (Breger et al. 2016; Lane et al. 2006). Different mechanisms have been suggested for the development of LID (reviewed in section 1.5.2), the most common theory is the dysregulation of synaptic dopamine levels after administration of exogenous L-dopa causing excessive and

pulsatile stimulation to the striatal dopamine receptors. Chronic L-dopa administration leads to long term plasticity in the dopamine receptors which increases sensitivity to dopaminergic stimulation. Uncontrolled synaptic dopamine levels are mostly due to the lack or reduction of the dopamine transporter DAT, a compensatory mechanism due to the degeneration of dopaminergic neurons. Recently the action of DAT on reducing LID was confirmed by a study showed that transplantation of non-dopaminergic cells (C17.2 cells) expressing DAT can reduce the dyskinesia score (Tomas et al. 2016). So, it is expected that stem cells controlled the LID dyskinesia via controlling the dopamine in the synapse via DAT or probably via reducing the striatum sensitivity by reversing the abnormal cell signalling and the genomic change that leads the dyskinesia, however the full mechanism remains to be elucidated.

Similarly, L-dopa-induced rotations were reduced by the stem cell grafts in time parallel to the reduction in AIMs score where the rats reached a static state of locomotion by week 4. The mechanism by which the graft reduced the L-dopa rotations is also dependent on termination of the dopamine action in the synapse by DAT. Without DAT, the stimulation of the dopamine at the DA receptors on the lesioned side is higher than the intact side causing the contralateral rotations. The ability of the hESC-DA to ameliorate L-dopa induce dyskinesia and rotations confirmed the functionality of these cells and relatively at early stages.

Effect of L-dopa treatment on the graft function, efficacy and histological characteristics:

Importantly, this data indicated for the first time that a graft transplanted into an environment receiving regular L-DOPA is not impaired in terms of survival or functionality. L-DOPA had no effect on the amphetamine-induced rotation test, the pattern of reduced rotations was identical in the presence or absence of L-dopa throughout the experimental time. This indicated that L-dopa did not affect the pattern of graft releasing dopamine throughout the graft maturation over 16 weeks. It also did not change the effectiveness of the graft on reversing dopamine receptor sensitivity as the apomorphine test showed no difference in the action of the graft in the presence or absence of L-dopa treatment.

The histological data clarified that L-dopa had no effect on the dopaminergic neurons survival, volume and fibres outgrowth. However, Interestingly, L-dopa had an overall supportive effect on improving the efficacy of the graft in the vibrissae test, which is the only parameter that recovered by H9 hESC-DA graft in this study. In addition it has improvement effect in cylinder

test in absence of exendin-4 treatment. A possible reason for this improvement could be related to improve graft maturation or accelerate graft maturation. The data showed there was a tendency for interaction of L-dopa effect with the time ($p = 0.09$). A close analysis illustrated that L-dopa effect reduced with the time, it has a significant effect on week 8 and 12 but no effect on week 16. This interaction tendency could be significant if longer experimental time was run, as further graft maturation may end with no more difference of L-dopa effect on the graft function.

The other interesting finding of L-dopa effectiveness on behavioural tests is it has interaction with exendin-4. The data showed that L-dopa was non-effective in vibrissae test in presence of exendin-4 at weeks 8, 12 and 16 and in cylinder test in week 16. This interaction is consistent with the interaction of exendin-4 and L-dopa in chapter 4, which involved developing insulin resistance leads to reduce exendin-4 effect of graft survival and function (discussed in chapter 4). Similar changes in the glucose and insulin homeostasis could be happened from this interaction in this chapter lead to reduce the effect of L-dopa. However, further histological and plasma analysis needs to support this hypothesis.

The effect of exendin-4 on the functionality and histological characteristics of the graft

Exendin-4 has neuroprotective effects in VM dopaminergic neurons transplanted in the depleted striatum of 6-OHDA rat model (chapter 5). Given that the H9 hESC-DA has the same midbrain dopaminergic neurons criteria, it was relevant to investigate whether the same degree of protection can be applied on H9 hESC-DA transplantation. The histological results indicate expression of the GLP-1 receptors on the dopaminergic neurons of the graft, thus the molecular machinery for exendin-4 to produce an effect was also available. However, exendin-4 did not have an effect on graft survival or volume neither in dopaminergic neurons nor on other cells in the graft indicating it has no protective effect on the transplanted cells. it also showed it has no effect on the graft fibres outgrowth. This was reflected in the behavioural data which showed no impact of exendin-4 on any of the drug-induced motor tests.

However, exendin-4 may have therapeutic value in the support of graft function as it did have a significant effect on recovering graft function in the vibrissae test. This improvement could be a result of improving or accelerating graft maturation. The data showed a significant

interaction between exendin-4 and the time and lost its effectiveness in the last time point (week 16).

Similar to L-dopa, the effectiveness of exendin-4 on behavioural tests was also affected by interaction with L-dopa. In vibrissae test, at early time point (week 4) the presence of L-dopa was supportive to the exendin-4 action as the latter produced better effect in presence of L-dopa. However, in the later time points (weeks 8, 12 and 16), L-dopa reduced the action of exendin-4. Furthermore, in week 16, exendin-4 had deteriorating effect in the cylinder test in presence of L-dopa. This may suggest that the L-dopa has potentiate the effectiveness of exendin-4 on graft at early time point but then possible physiological changes in insulin haemostasis resulted from both drugs interaction may lead reduce the effect of exendin-4. This adds further weight to evidence that exendin-4 may have a beneficial effect on graft function but that this is negated by the presence of L-DOPA.

The possible reasons why exendin-4 did not illustrate those same robust effects on graft function and survival as shown on the primary cell transplantation (chapter 4) include: 1) the lower dose used in this experiment, as exendin-4 used here was lower in dose frequency (0.5 µg/kg) once daily instead of twice daily due to the poor health of the animals (rapid weight loss was observed in treated animals requiring us to alter the protocol for animal health reasons); 2) A possible interaction between cyclosporine and exendin-4 could lead to the former counteracting the action of the later. It has been explained in chapter 4 that exendin-4 metabolism in the kidney produces metabolites that have mild to moderate antagonistic effects on the GLP1 receptors and both exendin-4 and its metabolites are excreted via the kidney. Studies showed that cyclosporine at a dose of 5mg/kg for a long period (9 weeks) caused nephrotoxicity characterised by tubulointerstitial lesion and reduction in the glomerular filtration rate GFR (Sereno et al. 2015; Sereno et al. 2014).

The present study reports a severe reduction in the weight of the rats who were treated with exendin-4 after 2 weeks of starting the exndin-4 treatment, especially in group with L-dopa. This reduction in weight is believed to be related to the exendin-4 treatment rather than the graft as it was only present in groups treated with exendin-4 and all rats improved after reducing the dose of exendin-4. In particular, it is well recognised from pre-clinical and clinical studies that GLP-1 and GLP-1 receptor agonists promote satiety and weight loss (Baggio & Drucker 2007). A possible interaction between exendin-4 and cyclosporine-A could also be

the cause of the rats' weight reduction by exendin-4. In chapter 4 cyclosporine treatment was not necessary and none of the grafted rats treated with exendin-4 had weight loss. In chapter 6 with the same protocol, a severe weight loss was similarly observed in the exendin plus cyclosporine treated animals. This consolidates a possible interaction between exendin-4 and cyclosporine-A. This is important for clinical translation because patients will be receiving both L-dopa and cyclosporine for the first few months' post-transplantation at a minimum.

Impact of exendin-4 and L-dopa on the microglia reaction around the graft

Exendin-4 and L-dopa had no effect on the levels of microglia around the graft. In contrast, L-dopa had a notable effect on increasing the level of microglia around allogenic VM grafts and this effect was exaggerated in the presence of exendin-4 chapter 4 (section 4.3.8). It has been suggested that developed insulin resistance in rats treated with exendin-4 and L-dopa leads to induce level of microglia around the graft (discussed in chapter 4). Possible reasons for the absence of this effect of L-dopa could be related to the presence of the immunosuppressant agent cyclosporine or to the reduced dose of exendin-4.

Safety, stability and graft side effect

The current study showed that the transplantation of dopaminergic neurons derived from H9 hESC utilising the Kirkeby protocol produced no evidence of any tumour over 17 weeks of transplantation neither in the presence nor absence of L-dopa. Importantly, this is the first study to have attempted to determine whether GID could be induced by hESC derived grafts. No evidence of GID was observed either in the presence of L-DOPA, amphetamine or in their spontaneous behaviours. Amphetamine stimulation is needed to evaluate GID risk in animal models (Lane et al. 2006). At week 16 none of the rats showed any signs of GID when tested under the influence of amphetamine. So, the present data suggests that the grafts either with or without L-dopa are safe, stable and without GID throughout the 16 weeks of the experiment.

5.5 Conclusion

This chapter demonstrates that the H9 hESC- derived dopaminergic neurons can survive and function in an animal model exposed to the chronic administration of L-dopa treatment in the same manner as a non-L-dopa exposed model. Furthermore, L-dopa has supportive effects on some of the motor tests performed and this effect could be a result from either supporting graft function at early stages post-transplantation or due to acceleration of graft maturation. H9 hESC-DA also ameliorated LID and L-dopa-induced rotations at early stages post-transplantation, earlier than relieving other motor deficits. This chapter also demonstrated that exendin-4, the neuroprotective agent, has no effect on protecting or improving survival of the H9 hESC-DA cells. However, it demonstrated the ability to improve some motor tasks, this effect may be due to improve or accelerate graft maturation. The current chapter answered important questions about the effect of L-dopa and exendin-4 on hESC-DA. However, other questions are raised about if this result is unique to this cell line or could be applied to another hESC line; given that some of this data is consistent with foetal cell studies in chapter 4 but some elements are different. So, the next chapter will explore the effect of L-dopa and exendin-4 in grafts derived from an alternate hESC line.

6 Chapter 6: the impact of L-dopa and exendin-4 on transplanted RC17 human embryonic stem cell (hESC)- derived dopaminergic neurons

6.1 Introduction

The translation of stem cell therapy from animal models through to clinical trials requires the use of dopaminergic neurons generated from clinical grade and genetically stable stem cell lines. These directed lines then require differentiation with clinical grade materials to generate dopaminergic neurons prior to transplantation into people with Parkinson's disease. The genetic stability of the cell line is an essential factor to be consider in stem cell therapy to avoid the possibility of these cells developing a carcinogenic phenotype – continuous, unchecked proliferation. Some pluripotent stem cell lines have multipotency and self-renewal characteristics similar to cancer cells and some others gain chromosomes when cultured for a long time (Stephenson et al. 2010; Baker et al. 2007; Draper et al. 2004). Recently, Canham and colleagues screened the genetic stability of 25 hESC lines which they are established under clinical grade Good Manufacturing Practice (GMP) conditions in the UK (the genetic stability of the H9 cell line used in chapter 5 has not been explored yet). They used a whole-genome single nucleotide polymorphism (SNP) array analysis to determine copy number variation (CNV) and copy-neutral loss of heterozygosity (CN-LOH) in isolated genome of the cell lines. They found 9 of these cell lines have structural genomic variance identical to the health cells while 15 cell lines have CNV greater than 100 kb, however most of these variations were found to be commonly occurring in health individual when checked in the genomic variant database (Canham et al. 2015). RC17 cell line was one of the hESC lines which was screened by Canham and colleagues and they reported that RC17 cell line have CNV equal to 144 kb which is commonly occurring in healthy cells in a ratio of 1:25.

Tilo Kunath at Edinburg University has generated dopaminergic neurons carrying midbrain markers from RC17 hESCs. They adapted a protocol modified from Kirkeby and colleagues which depends on dual SMAD inhibition to direct cells towards a neural fate, followed by adding SHH and activation of WNT signal to yield the midbrain criteria dopaminergic neurons. Clinical grade materials are used to direct the RC17 stem cells to create the dopaminergic neurons which made these cells clinically applicable (Tilo Kunath, Personal Communication, NECTAR 2014). These cells were recently transplanted into the 6-OHDA lesioned rat by the Brain Repair Group at Cardiff University. These cells were capable of surviving and functioning in the dopamine depleted striatum. Interestingly, the size of this graft was relatively small and characterised by long fibre projections within the striatum which may make them efficient to

cover the striatum with less dopaminergic neurons (Mariah Lelos, Personal communication, NECTAR 2016).

In chapter 5, I clarified the importance of testing cell transplantation in an animal model exposed to anti-parkinsonian medication and for that purpose H9 hESC derived dopaminergic neurons were evaluated in the 6-OHDA lesioned rat model exposed to regular L-dopa treatment. In addition, in chapter 4, I also demonstrated that exendin-4 behaves as a neuroprotective agent that supported the survival and efficacy of primary cells transplantation in the 6-OHDA lesioned rat. It is important to determine whether these findings translate to other dopaminergic neurons derived from other hES cell lines and therefore can be more generalizable. Given that RC17 grafts appear to have a different histological phenotype after transplantation and are generated via different a protocol. I decided to use this line, in collaboration with Kunath and colleagues, which also has the added advantage of matching the clinical grade criteria. So, I hypothesised that L-dopa and exendin-4 can change the efficacy and histological features of the RC17 hESC-derived dopaminergic neurons graft in unilateral 6-OHDA lesioned rat model of Parkinson's disease.

Aims:

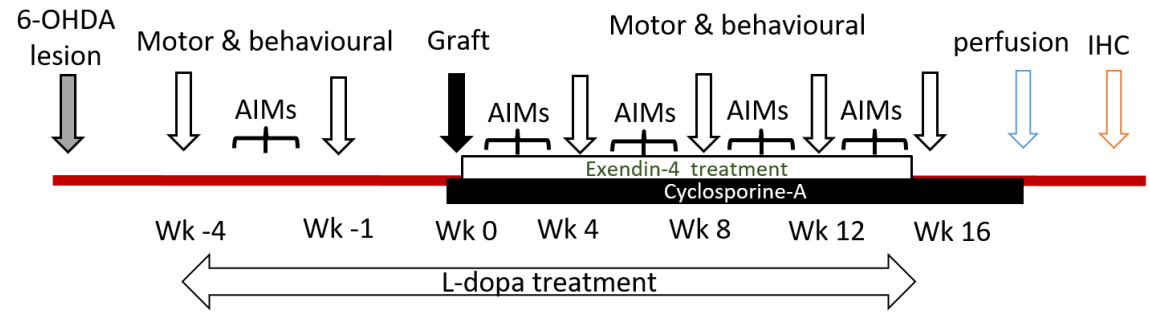
- Determine the efficacy and survival of RC17 hESC-derived dopaminergic neurons graft in 6-OHDA rat model.
- Understand the potential of exendin-4 to protect late stage degeneration and/or transplanted dopaminergic neurons to increase survival.
- Understand the extent and pattern of L-dopa effect on the efficacy and survival of the graft.

The objectives of the study are to use the unilateral 6-OHDA lesioned rat model to determine:

1. whether exendin-4 has neuroprotective effects at prolonged post lesion intervals.
2. the ability of transplanted RC17 hESC- derived dopaminergic neurons to ameliorate motor and behavioural deficits followed by characterisation of the histological features of the graft.
3. the effect of L-dopa on the survival and function of the hESC derived graft.
4. the ability of the graft to ameliorate L-dopa induced dyskinesia.

5. the presence of GLP-1 receptors on the RC17 hESCs- derived dopaminergic neuronal graft.
6. the therapeutic value of adding exendin-4 to the grafted groups on supporting the behavioural results and its effect on the histological features of the graft.

6.2 Experimental design and methodology



57 rats divided into the following groups:

A. Lesion only (n= 9)
B. Lesion + exendin-4 (n= 9)
C. Graft (n= 9)
D. Graft + exendin-4 (n= 10)
E. Graft+ L-dopa (n= 10)
F. Graft+ L-dopa+ exendin-4 (n= 10)

Figure 43 experimental time line and the grouping

57 female Sprague Dawley rats underwent 6-OHDA unilateral lesion surgery to the right medial forebrain bundle (section 2.2.1). Three weeks later, the extent of the nigral dopaminergic lesion was evaluated by amphetamine rotation, the successfully lesioned rats were selected based on more than 6 ipsilateral rotations per min. The lesioned rats were then divided into 6 groups: lesion only control group; lesion plus exendin-4; and four groups were transplanted with the RC17 hESC derived dopaminergic neurons into the depleted striatum (section 2.2.2.2.2) and divided into the following: graft only; graft plus exendin-4; graft plus L-dopa; graft plus L-dopa and exendin-4 (see grouping in Figure 43). A range of motor and behaviour tests, including amphetamine rotations, adjusting step and vibrissae tests were conducted one week before transplantation and at 4 time points (every 4 weeks) after transplantation surgery, the cylinder test was performed prior to transplantation and at week 16 while apomorphine rotations were carried out only at week 16. Abnormal Involuntary Movements (AIMs) and L-dopa-induced rotations were tested once a week before and after cell transplantation from the initiation of L-dopa treatment (section 2.4). Both L-dopa and exendin-4 treatments were stopped 2 days before each behavioural and motor test to wash out their pharmacological effects. On week 17 of transplantation, all rats were perfused and the brains were collected for histological analysis. DAB-IHC was used to determine: the number of dopaminergic neurons in the graft and confirm the degree of loss in the SN (TH); the number of human cells surviving (HuNu staining human nuclei) and Stem-121 to label

human cytoplasm; and density of microglia around the graft (CD11b) (section 2.10.1)**.** Double F-IHC was used to co-localise TH and GLP-1R (section 2.10.2).

6.2.1 Treatments

L-dopa was started 4 weeks before cell transplantation surgery and continued for 15 weeks after transplantation. Rats were immunosuppressed with Cyclosporine-A (10 mg/kg), and was started one day before cell transplantation and continued every 24 hrs until the end of the experiment. Exendin-4 treatment was started immediately after transplantation surgery and continued until week 15 post-transplantation.

L-dopa was used at a dose of 6 mg/kg/ day in combination with a double proportion of benserazide and administered daily, s.c.. Exendin-4 was given at dose of 0.5 µg/kg (i.p.) twice daily for the first two weeks after cell transplantaton. Severe weight loss in some animals led to a reduction in frequency to once daily administration. L-dopa and exendin-4 treatment were stopped 2 days before each behavioural and motor test.

6.2.2 TH fibre outgrowth measurement

Fibre outgrowth was analysed in this chapter by two different methods. The first method aimed to compare the fibre measurement between the groups. The images of the grafts were captured at 10X magnification using an Olympus B 50 microscope, the images were then inverted to the dark field using ImageJ to allow easy recognition of the stained TH fibres from the background. A grid was applied across the image on dimensions of 100*100 µm. Then the number of fibres intersecting with the vertical line of the grid at distances of 200, 400 and 600 µm were counted manually across the medial and lateral side of graft borders (Figure 44). The second method aimed to measure the percentage of the striatum that was covered by the graft fibres medially and laterally within 600 µm distance of the graft centre in the graft only group. ImageJ software was used to adjust the image threshold selectively to the fibres area then the proportion of area that was covered in the striatum expressed as a percentage of the striatal area was calculated. The process included reducing the image background and enhance the threshold selectivity to the fibres by using the following steps (Second method, Figure 44): inverting the image to dark field then enhancing the image contrast (image B); re-inverting the image to the brightfield (image C); using IHC tool to select DAB colour and remove other colours (image D); inverting image to dark field again (image E) and applying the image filter process using Gaussian blur option (image F); re-inverting the image to white

field and converting the image to the grey scale (8 bit) then the image threshold selection was applied (image G) followed by calculating the percentage of the threshold surface area in the selected image.

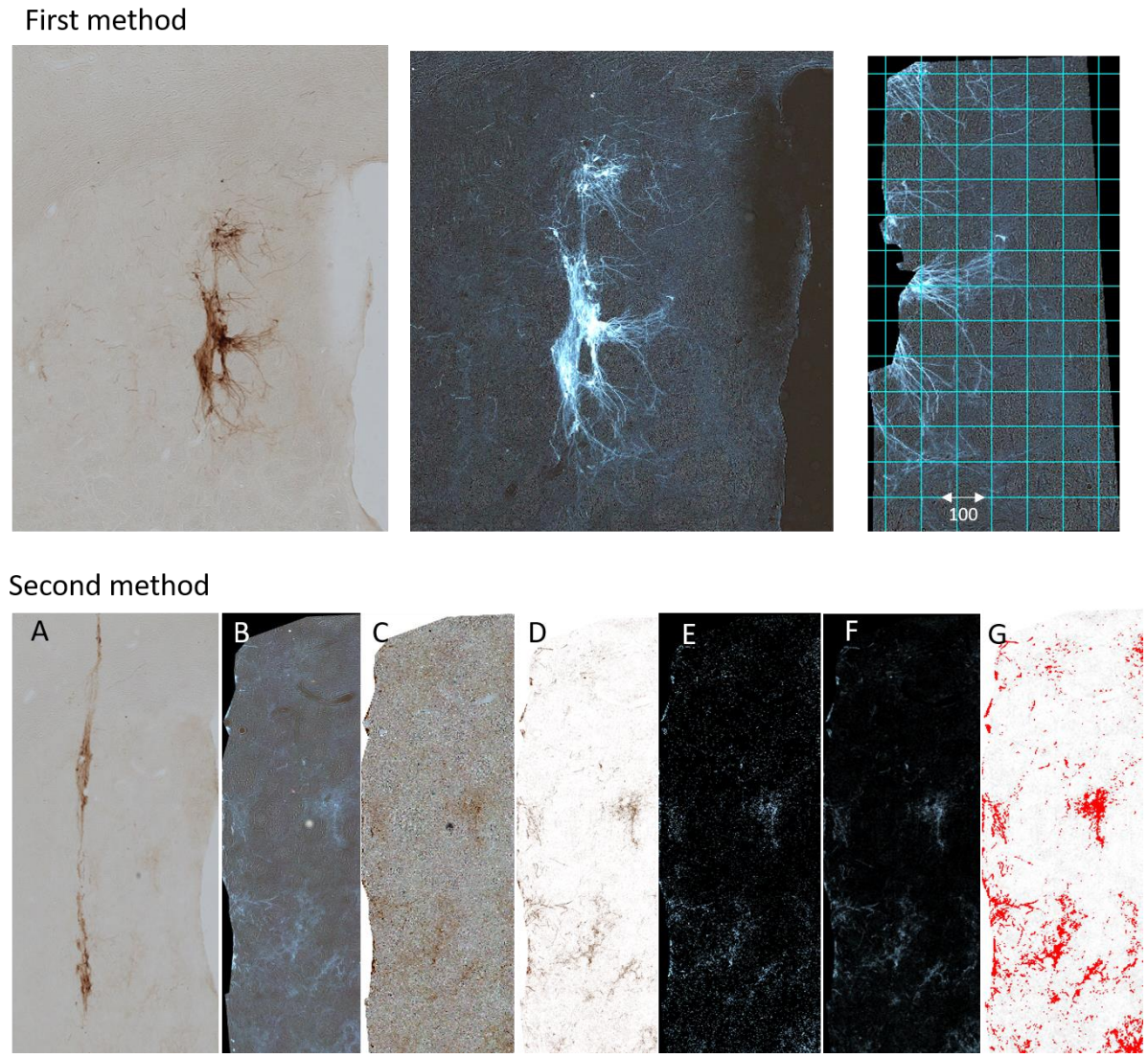


Figure 44 fibre outgrowth analysis methods using ImageJ software: first method included inverting the image to dark field followed applying 100*100 grid then the fibres intersected with vertical line of grid counted at distances 200, 400, 600 µm; second method included a process of reducing image background (A to G) then the percentage of threshold surface area in the selected image was counted.

6.2.3 Statistical analysis

The data was analysed using SPSS. Repeated measure analysis was performed in the behavioural results to determine the effect of time, graft, and drug treatments (L-dopa, exendin-4 and their interaction). Two-way ANOVA was used to analyse the effect of L-dopa, exendin-4 and their interaction on the graft in the apomorphine-induced rotation results and the histological analysis of the graft. AIMs score and L-dopa rotation were analysed using repeated measure analysis. Bonferroni post hoc was selected for pairwise comparison. Selected T-test analysis was used to analyse the apomorphine results comparing graft and lesion or comparing lesion to lesion plus exendin-4.

6.3 Results

6.3.1 Nigrostriatal dopaminergic lesion

The initial amphetamine-induced rotation test showed that all lesioned rats rotated ipsilateral more than 6 rotations per minute indicating that more than 90% of the nigral dopaminergic neurons were degenerated. The histological data confirmed this and showed that more than 97% of the nigral dopaminergic neuron were depleted in the right hemisphere (Figure 45). This confirms that the behavioural tests were evaluating the graft effect on the behavioural recovery outcomes and nigrostriatal dopaminergic neurons were not affected by the use of the different agents.

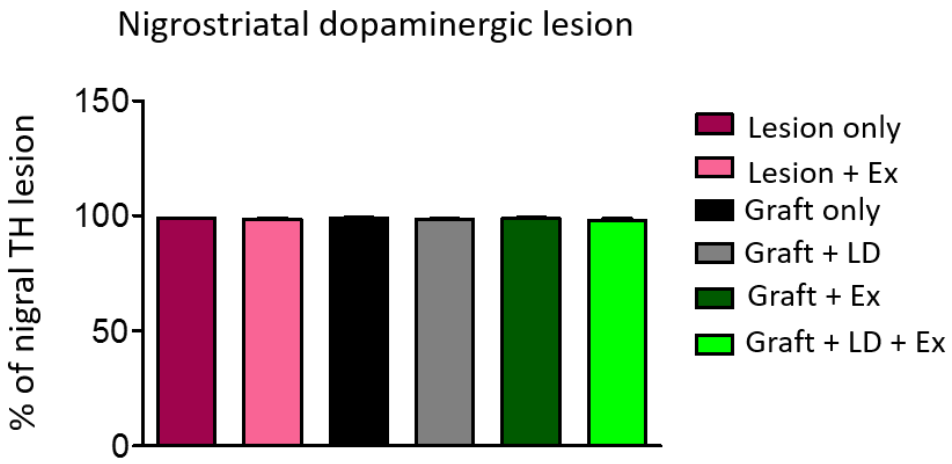


Figure 45 nigrostriatal dopaminergic lesion: percentage of the lost nigrostriatal dopaminergic neurons in the right hemisphere compared to the intact side was more than 97% in all groups

6.3.2 Behavioural results

6.3.2.1 Effect of exendin-4 on the lesion group

The purpose of testing effect of exendin-4 to lesion control group is to confirm that exendin-4 alone has no effect on this late post lesion interval. The behavioural tests (amphetamine-induced rotation test, apomorphine-induced rotation test, stepping, vibrissae, and cylinder tests) confirmed that exendin-4 had no effect on the behavioural outcomes of the lesion group throughout the 16 weeks of treatments (repeated measure ANOVA).

6.3.2.2 The effect of graft alone (absence of L-dopa and exendin-4)

The presence of the RC17 hESC derived graft had no significant effect on any of the motor and behavioural results (amphetamine rotation, apomorphine rotation, stepping, vibrissae and cylinder tests) over the 16 weeks following transplantation; However, there was a trend for a reduction in the amphetamine-induced rotations but without statistical difference (repeated measure ANOVA, max: amphetamine test, $F_{(1,16)} = 2.98$, $p = 0.1$, n.s; apomorphine: t-test, 2-tailed, n.s). A more detailed look at the pattern of amphetamine-induced rotations on pre-transplantation and week 16 showed that the grafted rats started rotation at a lower level than the lesion group in week 16. Analysis of the rotation through-out first 40 min of amphetamine injection at both time points showed that the overall effect of the graft was not significant ($F_{(1,16)} = 1.9$, n.s) but it has interaction with time (time*graft, $F_{(1,16)} = 8.2$, $p<0.01$); the graft has significant reducing effect on week 16 ($F_{(1,16)} = 5.14$), $p<0.05$) (repeated measure ANOVA, group was between subject factor; time was within subject factor).

6.3.2.3 Effect of L-dopa and exendin-4 treatments on the graft:

There was no significant effect of L-dopa, exendin-4 or their combination on amphetamine rotations throughout the 16 weeks on the graft, However, there was a tendency for amelioration in the number of rotations in response to L-DOPA (repeated measure ANOVA: L-dopa effect, $F_{(1,33)} = 3.1$, $p = 0.08$; exendin-4 effect, $F_{(1,33)} = 0.06$, n.s; exendin-4 and L-dopa interaction, $F_{(1,33)} = 0.152$, n.s). In the apomorphine rotation test, there was no significant effect of treatment, however there was a tendency for exendin-4 to reduce the number of rotations (two-way ANOVA: exendin-4 effect, $F_{(1,33)} = 4$, $p = 0.052$; L-dopa effect, $F_{(1,33)} = 1$, $p = 0.3$; interaction, $F_{(1,33)} = 0.02$, n.s). Similarly, treatments had no significant effect on stepping, vibrissae and cylinder tests (repeated measure analysis).

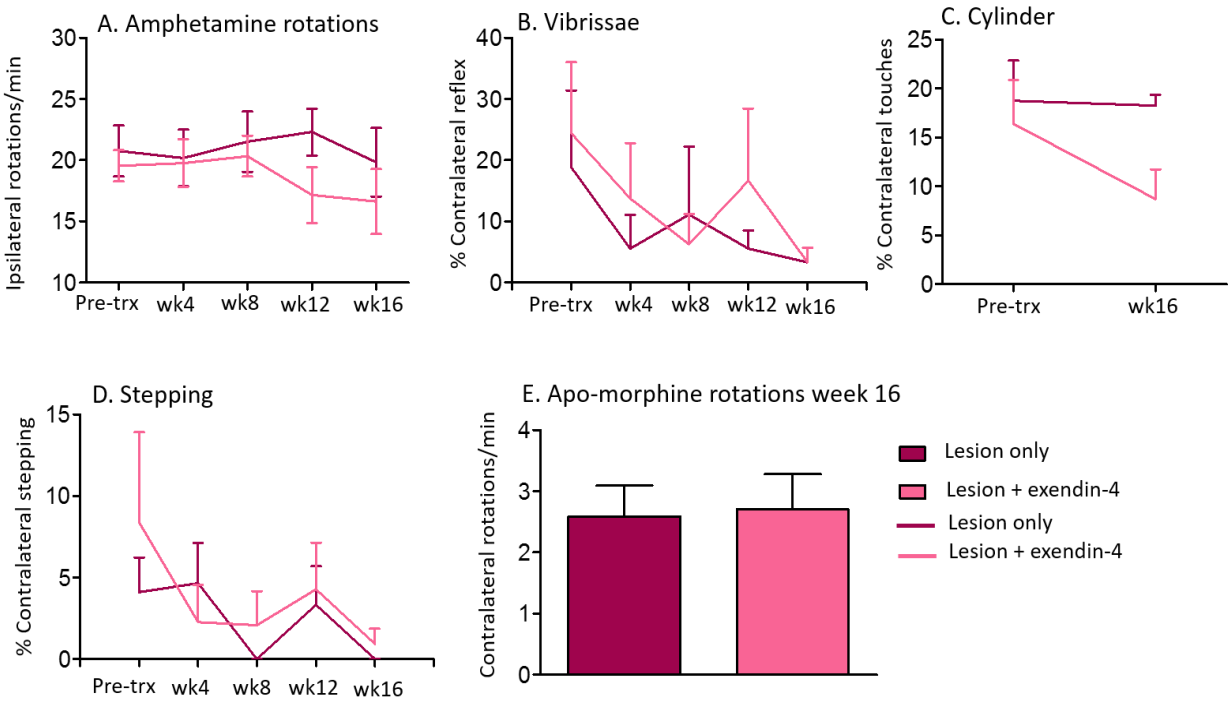


Figure 46 effect of exendin-4 on behavioural outcomes of 6-OHDA lesion rat model: exendin-4 had no significant effect on the lesion control group in any of behavioural results amphetamine-induced rotation test (A), vibrissae test (B), (cylinder test), stepping test (D), and apomorphine-induced rotation test (E) (repeated measure analysis).

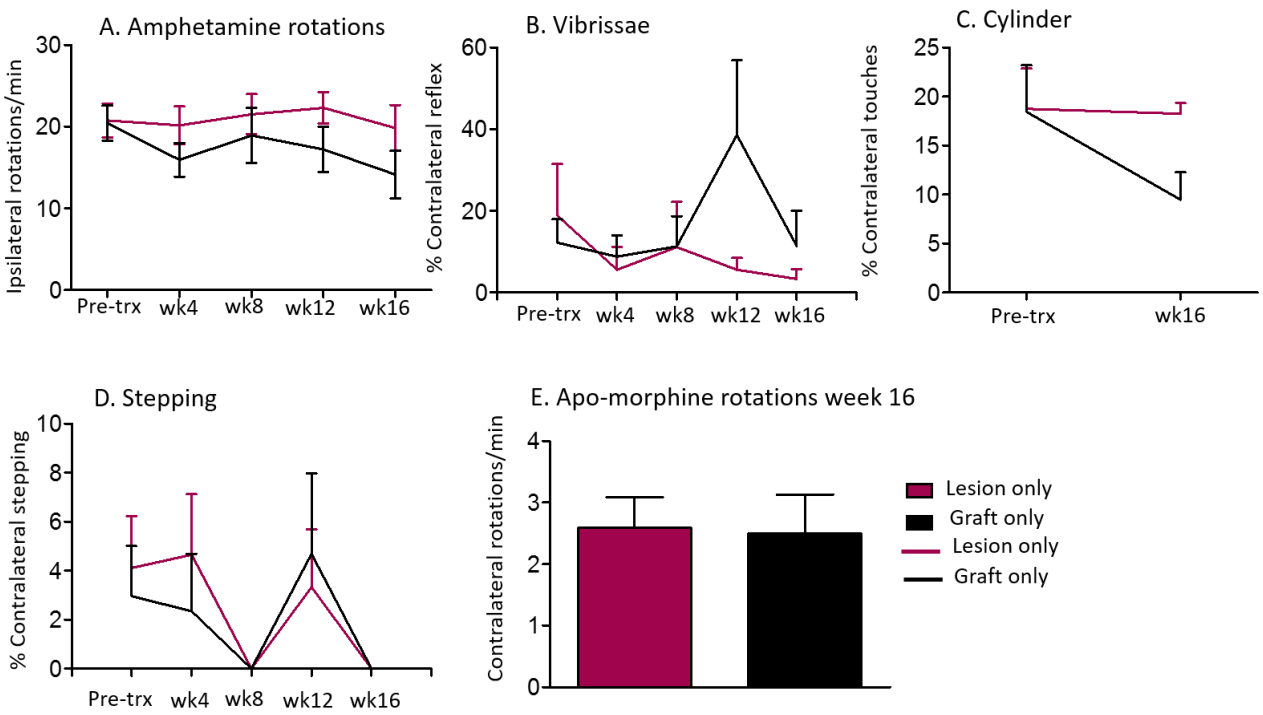


Figure 47 effect of RC17 hESC-derived dopaminergic neurons graft (without treatments) on behavioural and motor results: RC17 hESC-DA graft had no significant effect on the lesion control group in any of behavioural results amphetamine-induced rotation test (A), vibrissae test (B), (cylinder test), stepping test (D), and apomorphine-induced rotation test (E) (repeated measure analysis).

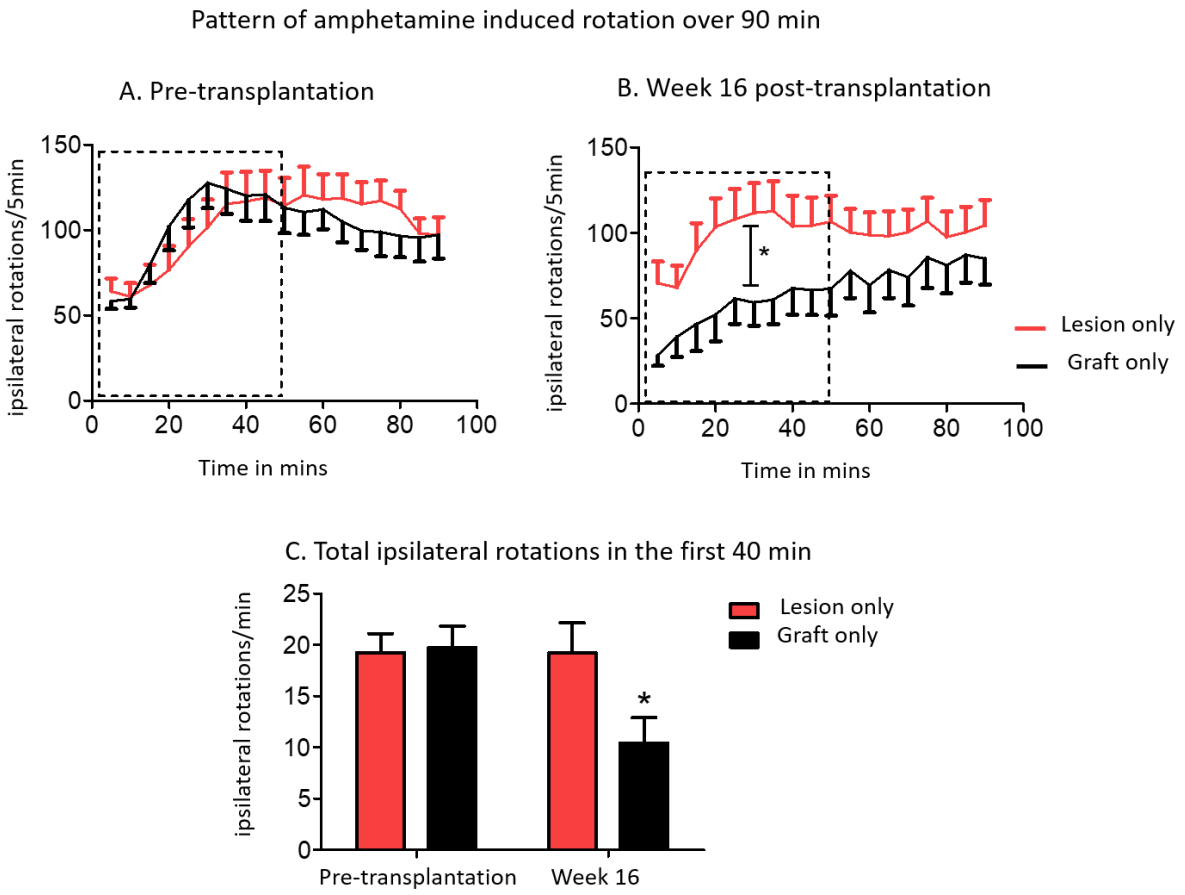


Figure 48 pattern of amphetamine-induced rotations of the graft (without treatment) throughout 90 min pre-transplantation (A) and on week 16 post- transplantation (B). figure (C) showed significant reduction in the ipsilateral rotation through-out first 40 of amphetamine injection (repeated measure analysis, *p<0.05).

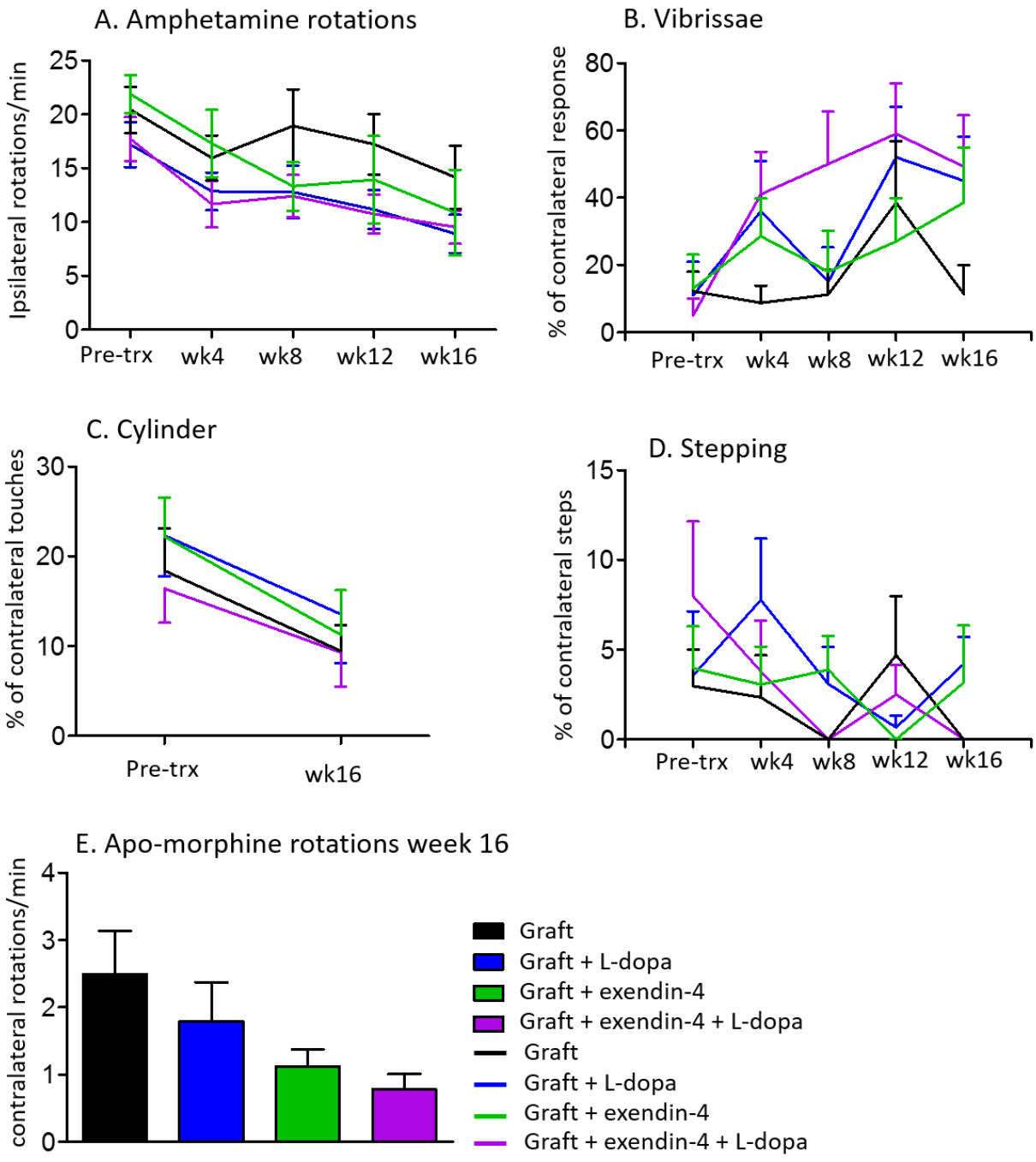


Figure 49 effect of L-dopa and exendin-4 of the behavioural results of the graft: L-dopa showed a tendency to ameliorate amphetamine induced rotations without significant difference (figure A) (repeated measure analysis); neither L-dopa nor exendin-4 had effect on the vibrissae (B), cylinder (C), stepping (D) (repeated measure analysis) and apomorphine rotation tests (E) (two-way ANOVA).

6.3.3 L-dopa induced Abnormal Involuntary Movements (AIMs) and rotations

L-dopa-induced dyskinesia, reflected in the total AIMs score gradually reduced throughout the 16 weeks following transplantation. The overall effect of the graft to reduce the AIMs score was highly significant and pairwise comparison to the base line time point (week -1) showed a significant reduction from week 3 post transplantation (repeated measure ANOVA, ***p< 0.001). Exendin-4 has no effect on AIMs score post transplantation (repeated measure ANOVA, $F_{(1,18)} = 0.185$, $P = 0.696$).

The number of L-dopa-induced contralateral rotations was significantly reduced in both graft and graft plus exendin-4 groups. The overall effect of the graft, throughout the experimental time, on reducing the number of contralateral rotations was significant; the pairwise comparison with base line time point before transplantation (week -1) showed a significant difference from week 7 following transplantation (repeated measure ANOVA, *P< 0.05). There was no additional effect of exendin (repeated measure ANOVA, $F_{(1,18)} = 1.55$, $P = 0.229$).

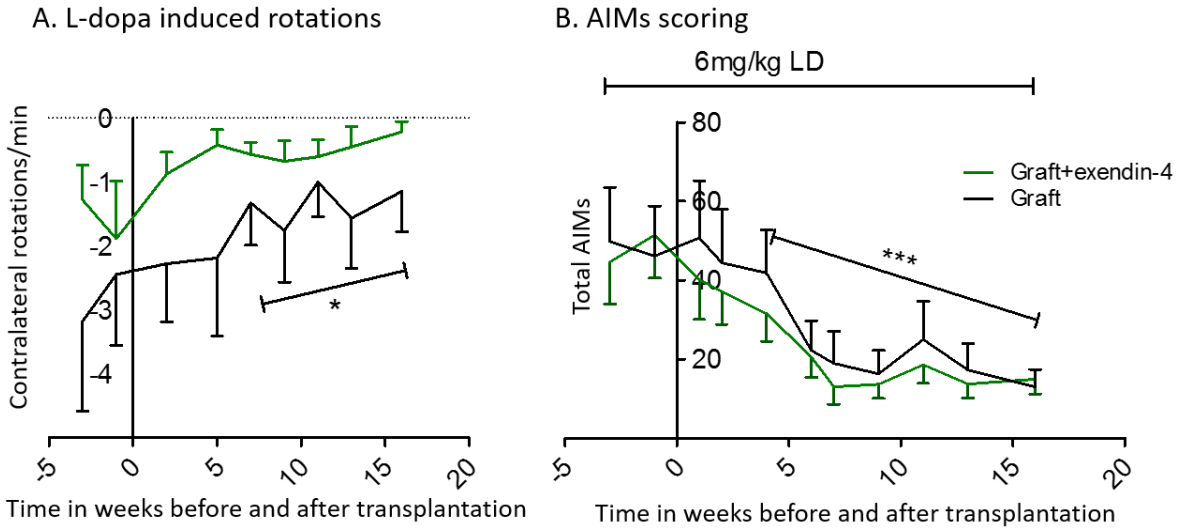


Figure 50 L-dopa- induced rotation and AIMs: figure (A) the overall effect of graft on reducing number of contralateral rotations was significant throughout 16 weeks of transplantation and without statistical difference between the graft and graft plus exendin-4 groups (repeated measure analysis, *p<0.05). Figure (B) the overall effect of graft on reducing AIMs score was highly significant throughout 16 weeks of transplantation without statistical effect for the exendin-4 treatment (repeated measure analysis, ***p<0.001).

6.3.4 Expression of GLP-1 receptor on the graft

GLP-1 receptors were colocalised with on TH labelled cells in the graft of RC17 hESC derived dopaminergic neurons after 17 weeks of transplantation.

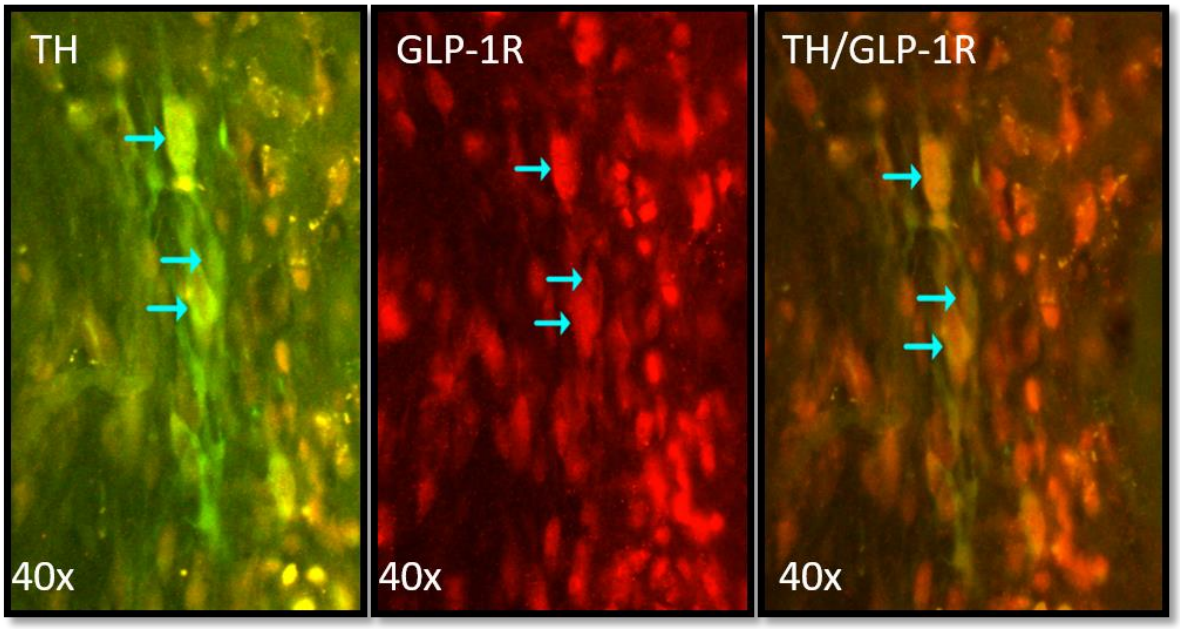


Figure 51 expression of GLP-1R in RC17 hESC graft: double fluorescent IHC showed co-localisation of TH markers (green) with GLP-1R marker (red) in 17 weeks' graft.

6.3.5 Histological analysis of the graft

The histological characteristics of the graft alone (absence of L-dopa and exendin-4): The average number of dopaminergic neurons in the graft, identified by TH immunohistochemistry was 532 ± 88 cells; the average volume of the TH positive graft was 0.066 mm^3; with a density of 8000 cells per mm^3. To examine the total grafted cell population, we labelled all the grafted human cells with HuNu. The average graft size with human cells was 2004± 88 cells; the average of the total graft volume labelled with stem121 was 0.08 ± 0.016 mm^3. This gave an average number of dopaminergic neurons as a proportion of the total cell population in the graft as 46% with an average proportion of TH volume to the total graft volume of about 88%. The percentage of the yielded dopaminergic neurons in the graft was about 0.1% of the number of the cells transplanted in this experiment (500,000 cells).

L-dopa, exendin-4 and their combination had no effect on the number and the volume of the dopaminergic neurons TH^+ in the graft (two-way ANOVA). They also had no effect on the total grafted cells population number labelled HuNu or volume labelled stem121 (two-way ANOVA, L-dopa, $F_{(1,29)} = 0.2$; exendin-4, $F_{(1,29)} = 0.12$, n.s.). The proportions of dopaminergic neurons count TH^+ to the total number of cell populations in the graft HuNu (TH count/ HuNu count) was not changed significantly by either L-dopa or exendin-4 and without significant interaction between both treatments; however, exendin-4 illustrated a trend to reduce TH/HuNu ratio (two-way ANOVA, exendin-4, $F_{(1,29)} = 1.8$, n.s.). While, the proportions of dopaminergic neurons volume TH^+ to the total cell populations graft volume stem121 (TH volume/ stem121 volume) was reduced significantly by exendin-4 (two-way ANOVA, $F_{(1,29)} = 4.212$, $p < 0.05$).

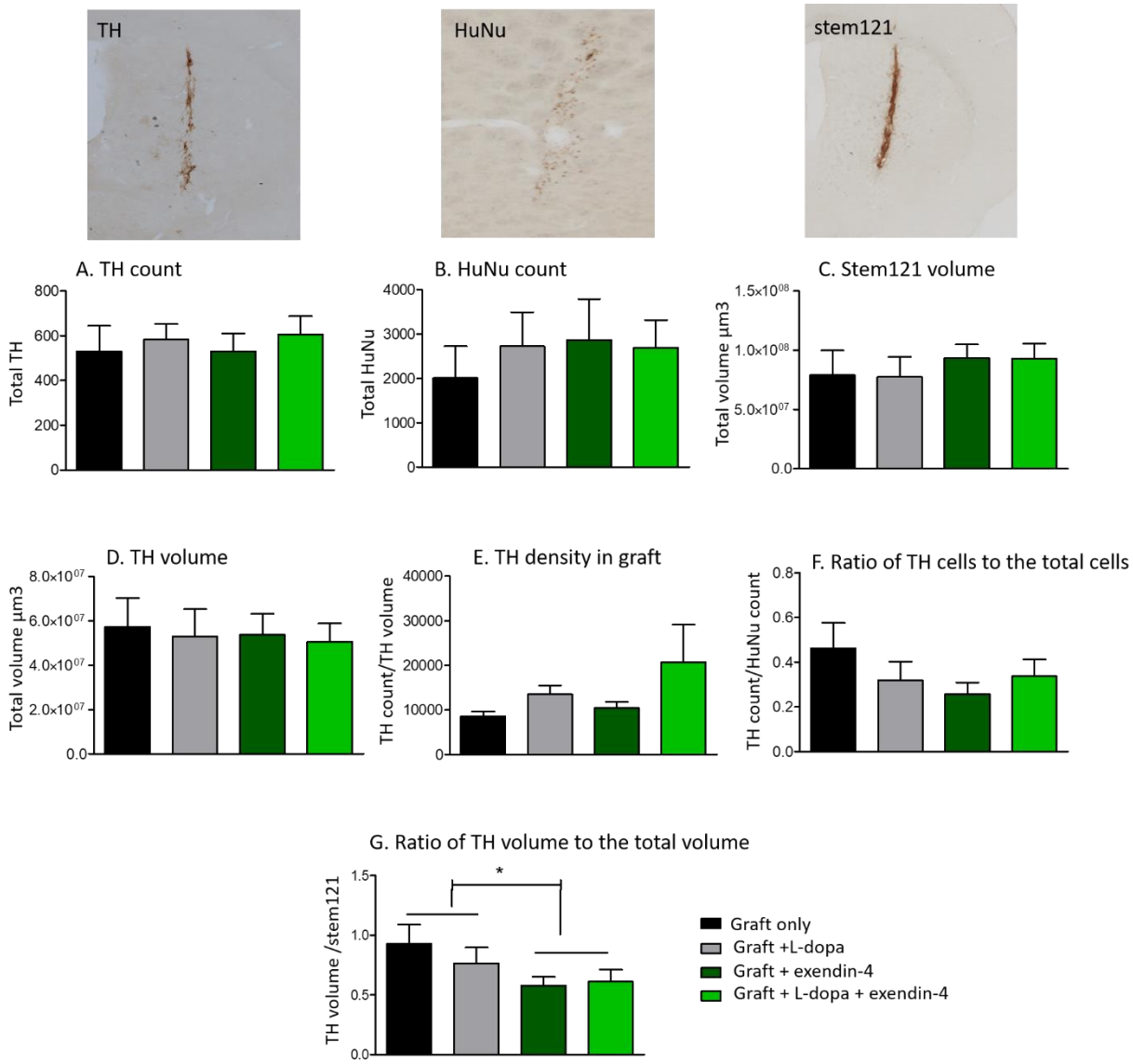


Figure 52 histological analysis of the graft: two-way ANOVA analysis used to determine the effect of L-dopa, exendin-4 and their interaction on the: both treatments and their interaction had no effect on (A) TH count; (B) HuNu count; (D) TH volume; (C) stem121 volume; (E) TH count/TH volume; (F) TH count/HuNu count; while exendin-4 showed a significant overall effect on reducing the Proportion of TH volume to the total graft volume stem121 (TH volume/ stem121 volume) (G) (two-way ANOVA analysis, *p<0.05).

6.3.6 Fibres outgrowth pattern of the graft

In the grafted group (absence of L-dopa and exendin-4), the percentage of fibres covering the striatum at a distance 600 µm distance from the graft centre was about 5 % towards the medial side and 1.84% toward the lateral side of the striatum. The tendency of sending fibres medially was significantly different from the lateral side (t- test, two tailed, * p< 0.05).

We evaluated the *effect of the treatments on the graft innervation*, counting the number of fibres at the distances of 200, 400, and 600 µm from the graft centre medially and laterally. This showed that the bias for medial project was unchanged by the addition of L-dopa and / or exendin-4. The statistical analysis showed that both treatments and their interactions has no significant effect on fibre projections at either of the 3 distance points from the graft centre medially and laterally (Repeated Measures ANOVA).

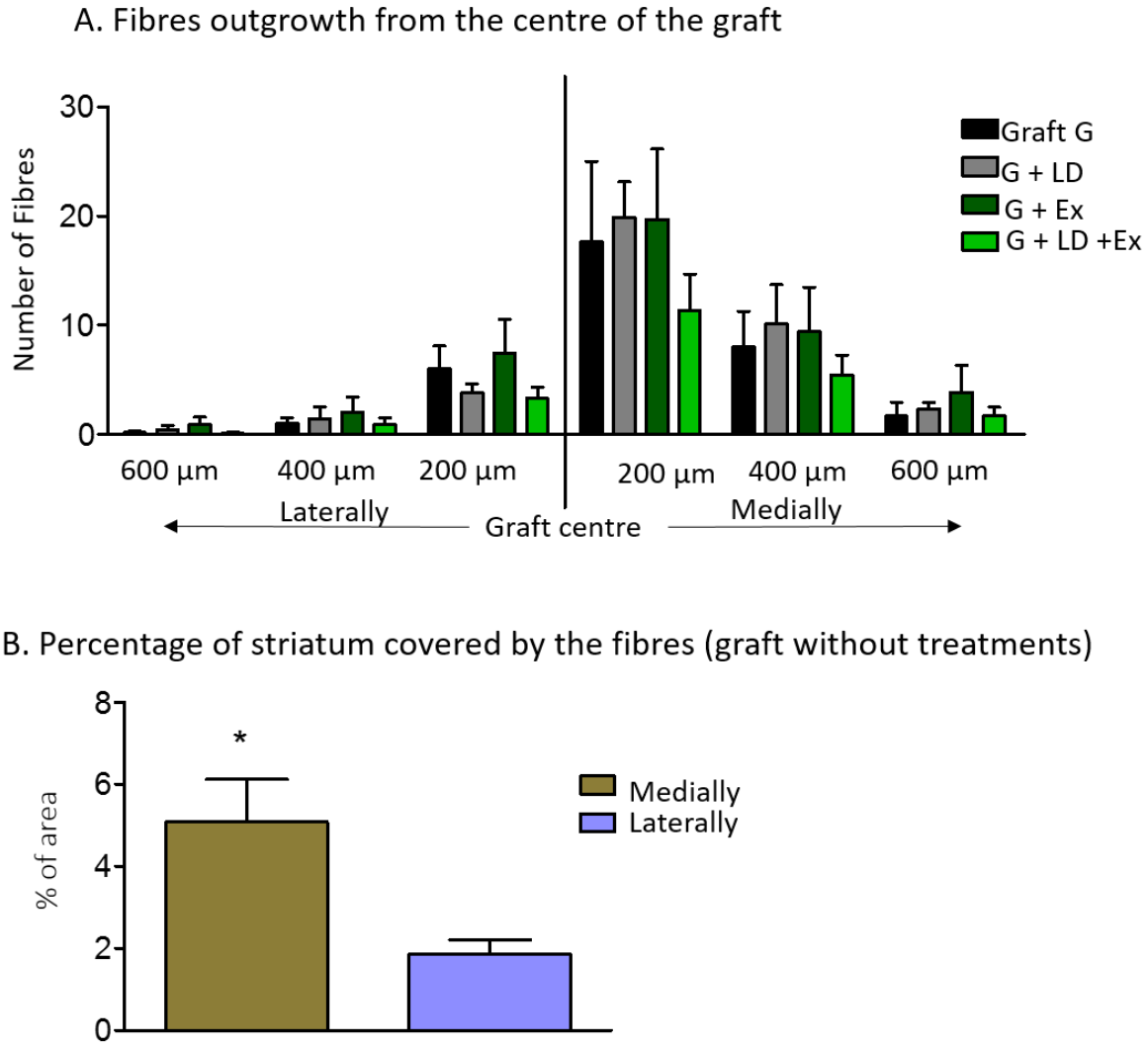


Figure 53 fibres outgrowth measurement: (A) number of counted fibres at distances of 200, 400, 600 µm of the graft centre medially and laterally showed there is no significant effect of the L-dopa and exendin-4 on fibres outgrowth (repeated measure analysis). (B) in graft only group, the mean percentage of fibres covering the striatum was 5% medially and 1.8 % laterally within 600 µm distance from the graft core; there is significant bias toward medial projections (t-test, $p < 0.05$). (G= graft, LD = L-dopa, Ex = exendin-4)

6.3.7 Microglial level around the graft

Measuring the optical density of microglia stained CD11b around the graft relative to the intact side illustrated that the graft and the lesion control group have similar levels of inflammation. There was no effect of L-dopa, exendin-4 and their combination on the level of microglia around the graft. However, L-dopa induced a trend to increase the level of microglia around the graft without statistical difference (two-way ANOVA: L-dopa, $F_{(1,31)} = 4.09$, $P = 0.052$; exendin-4, n.s.; interaction, n.s.).

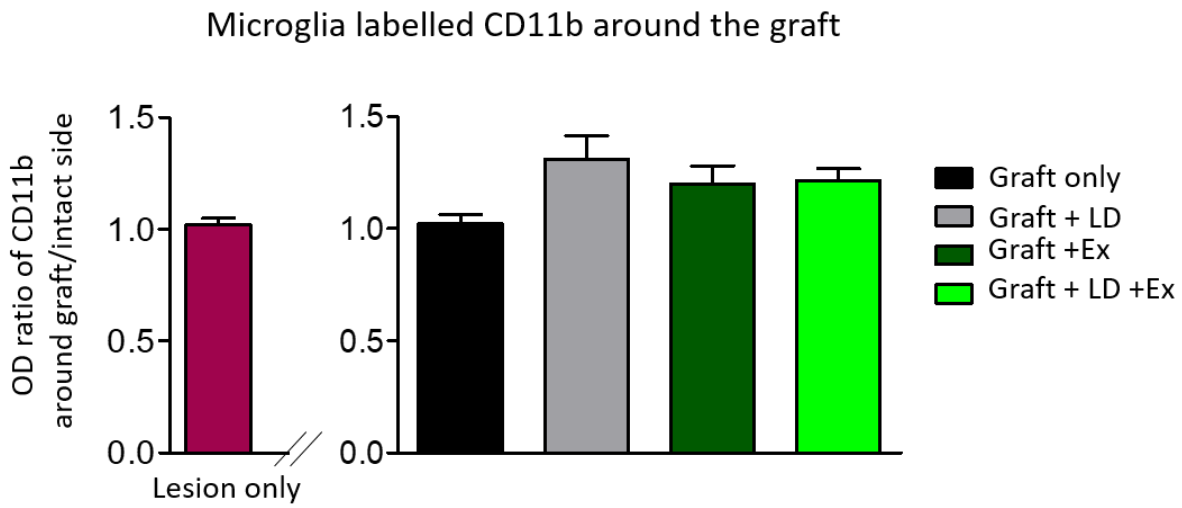


Figure 54 relative optical density of activated microglia labelled CD11b around the graft compared to the intact side of the striatum: L-dopa and exendin-4 or their interactions had no effect on the level activated microglia around the graft.

6.4 Discussion

Therapeutic value and histological characterisation of RC17 hESC – derived dopaminergic neurons graft (in absence of L-dopa and exendin-4):

The behavioural data illustrated that the graft has only a trend to recover amphetamine-induced rotations without an effect on other behavioural tests up to 16 weeks of transplantation. However, the data from analysing the pattern of amphetamine rotations on week 16 illustrated that the graft was functional at the early phase of the amphetamine response and the graft reduced the number of rotations significantly over the first 50 min of the assessment. This early phase rotation reduction is likely to be the most relevant to the initial burst of dopamine release (Lane et al. 2006). This may suggest that the graft is functional but not mature enough at this stage to release sufficient dopamine in response to amphetamine effect. The pattern of rotations showed that the difference in number of rotations between the groups was narrowed at the second hour of the test and consequently the difference in the net rotation between the groups was insignificant. The response to apomorphine confirmed that dopamine receptor super-sensitivity was not affected by the graft, also indicative that this was too immature to release sufficient dopamine to normalise striatal receptor dysregulation. Previous experiments conducted by the Brain Repair Group at Cardiff University (unpublished work) using the same cell source (RC17 – derived dopaminergic neurons) transplanted into the 6-OHDA rat model showed that the graft was functional and able to ameliorate deficits in motor tests at week 20 following transplantation. This would appear to confirm that 16 weeks post transplantation was not a sufficient duration for the graft to be significantly functional. Due to the combined administration of immunosuppression and pharmacological agents, animal welfare was adversely affected and whilst normally studies can continue to 20-24 weeks, this study had to be terminated at week 16. Future studies, using the neonatal desensitisation model (Heuer et al. 2016; Kelly et al. 2009) would allow longer experiments to be conducted without the need for immunosuppression.

The histological data confirmed the behavioural data that a low number of dopaminergic neurons survived and their fibres covered only a small area of the striatum which may represent the main reason for the limitation in the graft functionality at 16 weeks post transplantation. In comparison to the H9 hESC- DA grafts in chapter 5 (see

Table 8) which were also terminated at week 16 post transplantation, H9 hESC- DA graft produced effectiveness in recovery of motor function from week 12 following transplantation. However, histological comparisons showed that the RC17 graft had 30 times fewer dopaminergic neurons, 19 times smaller volume and its fibre coverage of the striatum was less medially by 10 times and laterally by 15 times than H9 hESC- DA graft. On the other hand, to compare the efficiency of the graft on covering the striatum regardless its graft size, the ratio between the percentage of the striatum covered by the fibres and graft volume was calculated. The comparison showed that RC17 projected fibres had the relative ability to cover striatal area two times more than H9. This infers that RC17 hESC derived neurons are more powerful in term of fibres outgrowth than H9 hESC graft. The current histological data is consistent with the data presented by the Brain Repair Group who showed a small graft and relatively long fibres with a functional graft at week 20 (unpublished work).

The histological analysis of the graft demonstrated that the dopaminergic neurons constituted about 46 % of the total number of cell populations in the graft labelled with HuNu. This is considered relatively large proportion of the total surviving cells compared to the same ratio in H9 hESC graft which represented about 22% of the total cells population. Although the graft was smaller, this suggests that neurons derived from the RC17 cell line are more likely to retain their dopaminergic phenotype compared to the H9 cell line under the used protocols. This would be considered an advantage for RC17 graft as one of the aims in stem cells transplantation is getting more purified dopaminergic neurons graft with less cells heterogeneity. The identity of other cells in the graft of these stem cells is unknown and their presence could have an impact on graft functionality, safety or existence of graft side effects. Previous experiments in which human-induced pluripotent stem (hiPS) cell derived dopaminergic neurons were transplanted into a rat model demonstrated the presence of undifferentiated cells (positive for the marker Nestin) within the grafted cell population which may carry the possibility of continuous cell division and the possibility of tumour formation (Cai et al. 2010). On the other hand, from primary cell transplantation, it has been clarified that the heterogeneity of transplanted cells in clinical trial, like presence of serotonergic neurons, is one of the factors that could drive the emergence of GID (Politis 2010). So, it is important for future studies to identify the other type of cells in stem cells graft and to

understand what possible effect they can have in term of graft development, function and safety.

The current data also showed that the graft had no effect on inducing microglial reaction and the level of microglia around the graft was identical to its level in the lesion control. In the large graft of the H9 hESC-DA (Chapter 5), the graft was surrounded by a higher level of the microglia compared to the RC17 hESC-DA graft in the current experiment. This difference in microglial reaction between the two grafts is possibly to be related to the difference in the graft size as the smaller size of RC17 hESC-DA graft has a low tendency to induce microglial reaction. This could be another advantage of getting a small and effective (well innervating) graft as inflammation may interfere with graft survival, function and the development of graft induced dyskinesia.

The comparison between the small and large graft of hESC-DA in this thesis is comparable to previous comparisons between the small graft and large graft of VM cells transplantation. Bartlett and colleagues demonstrated that smaller graft (TH^+ count around 600 cells) induces less inflammatory reaction and has longer fibre outgrowth compared to a graft the 4 times larger. They also showed that the small graft can produce the same function as larger graft but it is time dependent, as longer periods of time are required by small grafts to reach the same effect of larger graft (Bartlett et al. 2004).

Effect of the graft on L-dopa-induced dyskinesia and rotations

Importantly, even with a small graft with limited functional efficiency there was a clear efficiency in amelioration of LID. The graft reduced both the number of contralateral rotations induced by L-dopa and the expression of AIMS. This suggests that the presence of the graft establishing dopamine responsive negative feedback system that control the L-dopa-derived dopamine levels and therefore reducing AIMs and rotations because the level of dopamine in the striatum can be reduced. This data not only confirmed that the stem cells transplantation can reduce L-dopa induced dyskinesia but also this amelioration can be achieved by small grafts and at early stages of transplantation. Taken together with amphetamine-induced rotation results (some recovery) and apomorphine-induced rotations (no effect), the graft was able to release the dopamine and establish a dopamine responsive negative feedback system without normalising the super-sensitivity of the dopamine receptors.

The effect of L-dopa on graft survival and function:

As with the previous chapter, this work for the first time examines the effect of L-dopa administration on the development of the graft. Determining the effect of L-dopa on the functionality of the graft was not fully tested in this experiment because of the limited size of the graft. However, L-dopa tends to ameliorate the amphetamine-induced rotation response at a faster rate than in the absence of L-dopa (without reaching statistical difference, probability for the null hypothesis $P = 0.08$). This effect is likely to be from the effect of L-dopa on the graft rather than the host brain as previous studies confirmed that L-dopa has no effect on the amphetamine-induced rotation test of 6-OHDA lesioned rat models (Steece-Collier et al. 2009; Breger 2013). So, this data could suggest that L-dopa is unlikely to be toxic to the grafts and in fact may support graft functionality or accelerate graft maturation. Rats treated with L-dopa alongside the graft did also appear to begin to show a reduction in rotations in response to apomorphine which may also indicate that the L-dopa increases the ability of the graft to normalise striatal sensitivity. However, no data is available to confirm that this change in apomorphine-induced rotations does not result from the impact of L-dopa on the 6-OHDA lesion rather than the graft. This make the interpretation of the apomorphine response of the effect of L-dopa in the graft uncertain without having lesion plus L-dopa treatment control group.

Critically L-dopa administered once daily has no effect on the survival of the dopaminergic neurons derived from hESC even in a small graft. This is an important development in our understanding of the possible effects of L-dopa. Previous experiments in cell culture illustrated that L-dopa has neurotoxic effect on neural stem cells via increasing the oxidative stress and inducing the apoptotic pathways when used at high doses (Park et al. 2011; Jang et al. 2015; Liu et al. 2004; Maharaj et al. 2005). The evidence suggested that L-dopa possibly causes further degeneration of the nigral dopaminergic neurones in PD. However, studies on transplanted allogenic VM cell transplantation showed no effect of L-dopa on cell survival (Breger et al. 2016). The current data extends our understanding show that the hESC-DA grafts can survive in the host brain primed with L-dopa regardless of graft size as the results of Chapter 5 clarified that L-dopa also had no effect on the survival of the massive graft of H9 hESC-derived dopaminergic neurons. However, the impact of L-dopa on graft survival also need to be explored in multiple dose administration of L-dopa. Because of logistical

complexities, the dose frequency of L-dopa used in this thesis was once daily or on alternate days which is less than what the patients usually received (3-4 times a day)**.** L-dopa also had no interference on the survival of other cells in the graft allowing a constant ratio between the number or the volume of the dopaminergic neurons to the total cells population in the graft which is consistent with the results of the effect of L-dopa on H9 hESC (chapter 5).

The effect of exendin-4 on the 6-OHDA lesion model:

Exendin-4 is able to reverse motor deficits in a partially lesioned MPTP mice and 6-OHDA lesioned rats resulting from the protection of the nigrostriatal dopaminergic neurons (Li et al. 2009; Harkavyi et al. 2008). In the present study exendin-4 had no effect on the motor and behavioural outcomes of the 6-OHDA lesioned rats who had lost more than 97% of their nigrostriatal dopaminergic neurons unilaterally. This supports that the effect of exendin-4 on the behavioural recovery tests in grafted rats' results from its effects on the graft and not from affecting other brain regions. However, exendin-4 illustrated trend to reduce amphetamine rotations but this was marginal and noticed only after prolonged drug administration and not observed in other tests (apomorphine-induced rotation, stepping, vibrissae and cylinder). Because the confirmation of almost a complete loss of the TH^+ cells in SN of the right side, this slight reduction in rotations could have result from the long-term effect of exendin-4 on dopamine release in the intact striatal side. Studies in mice have shown that exendin-4 attenuates dopamine release in nucleus accumbens after nicotine stimulation (Egecioglu et al. 2013). Even though this was in a different model it points to the possibility that exendin-4 has similar long term effects on reducing dopamine release from the dopaminergic neurons in the intact striatum.

Effect of exendin-4 on RC17 hESC-derived dopaminergic neurons graft

As in the previous chapters, GLP-1 receptors were localised on the dopaminergic neurons of the graft. Nevertheless, it was found that exendin-4 has no effect on TH survival or fibre projections. Given that the grafts in this experiment were small, any effect on supporting/enhancing cell survival would be more noticeable than in a large graft. this is consistent with the effect of on the survival and fibres outgrowth of the H9 hESC derived graft when used at similar dose**.** However, with H9 hESC-DA, exendin-4 did improve graft function in vibrissae test (discussed in chapter 5) which it was not the case with the RC17 graft. This

difference is possibly attributed to the difference between the cells, even though both are dopaminergic in phenotype and expressed the GLP-1 receptor.

The other noticeable finding on the effect of exendin-4 on RC17 hESC- derived dopaminergic neurons graft is that it produced a significant reduction in the ratio of dopaminergic neuron graft volume to the total grafted cells volume (TH volume/ stem121 volume) and had a tendency to reduce the ratio of dopaminergic neurons counts to the total cell population count in the graft (TH/ HuNu). These changes in the ratio resulted from an improvement in volume and count of other cells in the graft by exendin-4 (without significant difference) with no or slight effect on the dopaminergic neurons leading to cause the reduction in the proportions of TH^+ compared to the total cells population. This suggest that exendin-4 may have effect on other cells in the graft, like increasing their fibres density consequently diffuse the graft volume or it may improve the survival or increase proliferation of other non-dopaminergic cells in the graft. Such events could reduce the purity of the graft which may have implications on the graft. For instance, the ratio of serotonergic neurons to dopaminergic neurons in the graft is thought to be an important factor in triggering the development of GID (Politis et al. 2011). A further study may be needed to sort out the kind of cells in the graft, especially serotonergic neurons, to determine whether exendin-4 had effect on them.

The effect of L-dopa and exendin-4 on microglial reaction around the graft

The present data also showed that L-dopa tended to increase the level of activated microglia around the graft (the p value of null hypothesis was 0.052). In Chapter 5, the large graft of H9 hESC-DA has higher level of microglia around the graft compared to the intact side and L-dopa has no further effect on the level of microglia. However, in this chapter, the small RC17 hESC-DA graft has no effect on microglial level around the graft compared to the intact side but L-dopa has a clear trend to increase the level of microglia around the graft. This may suggest that either the graft size and/ or the type of stem cell source have influence on the impact of L-dopa to inflammatory responses. In chapter 4 results of this thesis, the data showed that L-dopa increase level of microglia around the allogenic VM graft. Previously, Breger and colleagues (2016) used the same model and demonstrated that L-dopa increased the levels of microglia around the xenografts but not allografts following VM cells transplantation which pointed to the importance of the histological compatibility on determining the L-dopa effect

on microglial reaction. They hypothesised different mechanisms by which L-dopa induced the inflammatory reaction around the graft including that: L-dopa may stimulate proliferation or activation of the inflammatory cells; L-dopa may interact with cyclosporine-A lead to reduce the later action in the brain; L-dopa may alter blood brain barrier function leading to influx of the immune cells (Breger et al. 2016). They also showed that this increment induced by L-dopa had no deteriorating effect on graft survival or function of the graft which is consistent with the current results that illustrated that L-dopa has not reduced graft survival or function but rather it has tendency to improve graft function in the amphetamine-induced rotation test (discussed above).

Like its effect on H9 hESC-DA graft (chapter 5), the current data showed that exendin-4 has no effect on microglial level around the RC17 hESC-DA graft and the interaction between L-dopa and exendin-4 does not change the effect of L-dopa on microglial level. This in contrast with the results of chapter 4 which showed that exendin-4 enhance the effect of L-dopa on increasing the level of microglia around the allogenic VM graft significantly (discussed in chapter 4)**.** The same reasons suggested in chapter 5 could be also responsible for the non-further elevation of microglial level around the RC17 hESC-DA grafts in presence of exendin-4 including: the presence of cyclosporine-A in the model may interact with exendin-4 lead to halt the action of the latter on the microglia; the reduced dose of exendin-4 may reduce its effectiveness on the microglial level**.**

6.5 Conclusion

The current data demonstrated that RC17 hESC-derived dopaminergic neurons can survive in the striatum of 6-OHDA rat model throughout 16 weeks of experimental time, but it has low survival rate and produce small graft. The data also showed that the duration of the experiment was not long enough for the graft to reach maximal efficacy in reversing the deficits in most of the motor tasks. Insufficient graft maturation was suggested to be the reason for the incomplete graft function. Importantly the current data, consistent with chapter 5 results, confirmed the ability of hESC-derived dopaminergic neurons to reduce L-dopa induced abnormal involuntary movements despite the small graft size. It also confirmed, taken together with results of chapter 5, that the transplantation of dopaminergic neurons derived from different hESC cell lines can survive and function in in the 6-OHDA lesioned rat model exposed to chronic L-dopa treatment, regardless of graft size. The present chapter also demonstrated that administration of exendin-4 has no effect on graft survival and function. Although, it supported H9 hESC-DA graft function and fibre outgrowth to a degree suggesting that the supportive action of exendin-4 depends on the source of transplanted cells.

Table 8 summary of the behavioural and histological findings from H9 and RC17 hESC transplantation in 6-OHDA rat model. In addition to the summarise the effect of L-dopa and exendin-4 treatments on these findings.

Tested Factor		RC17 hESC graft	H9 hESC graft	L-dopa effect on		Exendin-4 effect on	
				Rc17	H9	RC17	H9
Behavioural recovery tests	Amphetamine rotation	Trend to recover (week 16)	Recovered (week 12)	Trend to reduce	No	No	No
	Apomorphine rotation	No	Recovered	No	No	Trend to reduce	No
	Stepping, vibrissae, cylinder	No	Improve vibrissae		Increase in vibrissae and cylinder	No	Increase in vibrissae
AIMs reduction		Reduced	Reduced			No	No
L-dopa rotation		reduced	reduced			No	No
TH count		532	16,118	No	No	No	No
HuNu count		2004	72,000	No	No	No	No
TH volume		0.066mm^3	1.27mm^3	No	No	No	No
% of survival		0.1%	5%				
% of striatum covered by Fibres	Medially M	5%	50%	No	No	No	Trend to increase
	Laterally L	2%	29%	No	No	No	No
% of Fibres M / graft volume		75	39				
TH/HuNu		0.46	0.22	No	No	Trend to reduce	No
TH/stem121		0.88	0.8	No	No	Reduced	No
Microglial level (around graft/ intact side)		1	1.7	Trend to increase	NO	No	No

7 Chapter 7: General discussion

Cell transplantation is one of the promising therapies in the treatment of Parkinson disease but it is still facing significant logistical challenges in terms of cell survival and efficacy. This thesis aimed to identify neuroprotective agents that would support transplanted cells and importantly understand the effects of the most commonly used drug in PD, L-dopa, on determining neuroprotective agents' efficacy on the graft. Two families of agents were investigated in this thesis, ghrelin receptor agonists and glucagon-like peptide 1 receptor agonists. Both had previously showed evidence of neuroprotective capability to protect dopaminergic neurons in cell culture and animal models. Generally, both may have neuroprotective effects on transplanted dopaminergic neurons through peripheral administration that would improve cells survival and support graft function. The data in chapter 3 clarifies that peripheral administration of ghrelin receptor agonists have limited efficacy to support transplanted allogenic VM cell (discussed in chapter 3). In contrast, results in chapter 4 illustrated that peripheral administration of GLP-1R agonists could support graft survival and function, however their efficacy was determined by the presence of L-dopa treatment (discussed in chapter 4).

In the second part of the study I wanted to identify the consequence of the co-administration of L-dopa and the neuroprotective agents on the hESC-derived dopaminergic neuronal grafts. These are considered as one of the alternative cell sources for transplantation in PD that is expected to supersede primary foetal VM cells transplantation as a more reproducible source of cells. So, it was important to characterise survival, function and safety of these cells in an animal model that more closely mimicked the reality of patient, by exposing them to anti-parkinsonian medications. In addition, it was important to consider the ability of the neuroprotective agents identified in first part of study to support survival and efficacy in this new model design. The results from chapter 5 and 6 demonstrate that hESC-DA neurons can survive and function in the presence of L-dopa and the addition of exendin-4 provided some support to these cells.

This thesis identified that GLP-1 agonists (exendin-4 and liraglutide) have supporting effects on both primary and hES cell transplantation, and it is also recognised that L-dopa had a considerable impact on determining the protection status of these agents. In addition, the addition of L-dopa to the animal model answered important questions allowing more

clinically relevant evaluation of the functional capability and survival of hES-derived cell transplantation. This thesis made steps forward in the use of GLP-1R agonists, exendin-4 and/ or liraglutide, for clinical trials to support cell therapy in PD and raises issues of the importance around using L-dopa in the modelling of PD in animal models to evaluate cell therapy and both of these key issues are discussed in detail below.

7.1 Are GLP-1 agonists ready to support cell therapy in clinical trials of PD?

As described in the introduction of chapter 4, exendin-4 and liraglutide are already used clinically to treat type 2 diabetes mellitus T2DM and their safety issue was approved for clinical use for T2DM treatment. However, the GLP-1 analogues were put under tier 2 classification in the American Diabetes Association and European Association. This category is for treatments that are less well validated, in this case because the GLP-1R signalling pathways of these analogues are not fully understood and have different clinical sequelae in the severity of the side effects that are produced and the therapeutic value of each (Nathan et al. 2009; Weston et al. 2014). Currently, exendin-4 is also in clinical trials to treat PD patients and it has a beneficial effect on patients with Parkinson's disease determined as an improvement in the Movement Disorders Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) score compared to the placebo control group. Additionally, the safety assessments of exendin-4 in PD patients showed that it was generally well tolerated, the main notable side effects were reduction in body weight and gastric disturbances. The mean body weight reduction was 3.2 kg over 12 months compared to 0.8 kg loss in the placebo group. This reduction affected 19 patients out of 20 (the dose of exendin-4 was reduced in two patients to reverse this reduction in body weight after 10 month and one of them withdrew because of this issue) (I Aviles-Olmos et al. 2013).

The effectiveness of GLP-1 agonist to support cell therapy in PD was explored in animal models throughout this PhD. Indeed, GLP-1 agonist was effective in supporting transplanted cells survival, improving fibre outgrowth and enhancing graft efficacy in behavioural tasks. This suggests that there may be the potential to use exendin-4 or liraglutide to support cell therapy in the clinic specifically exendin-4 which was safe and well tolerated by Parkinsonian

patients. However, this thesis raised some issues needed to be clarified before considering GLP-1 agonists (exendin-4 or liraglutide) for clinical trials as a support for cell therapy:

1. The effectiveness was influenced by the presence of L-dopa treatment (chapter 4).
2. Development of an insulin resistance due to interaction between exendin-4 and L-dopa (chapter 4).
3. A considerable body weight loss was recorded when co-administered with cyclosporine immunosuppression (chapter 5&6).
4. The pattern of graft support was dependent upon the cell source for transplantation (chapters 4, 5, 6).

The PD patients who undergo cell transplantation will be highly likely to already be on L-dopa treatment and will be administering immunosuppressants (mainly cyclosporine-A), to prevent graft rejection in the immediate post-graft period. Both these medications appeared to interfere with either efficacy or safety of exendin-4. The co-administration of L-dopa and exendin-4 illustrated that the latter has no effect on support survival or function of embryonic VM cell transplantation while it supported the functional and survival of the graft in the absence of L-dopa. Again, in H9 hESC-DA transplantation, the efficacy of exendin-4 at ameliorating deficits in behaviour in the vibrissae test was reduced in the presence of L-dopa. The reduction of exendin-4 efficacy in the presence of L-dopa was accompanied by an increase in glucose and insulin plasma levels, in addition a sign of insulin resistance was also detected on the grafted cells. Based on these finding it has been suggested that L-dopa may interact with exendin-4 causing insulin resistance on the transplanted cells leading to cessation of the protection effect of exendin-4. This raises concern about the safety and ability of exendin-4 to support cell therapy in the clinic. However, in the clinical trial conducted by Aviles-Olmos and colleague, insulin resistance was investigated in PD patients receiving L-dopa and exendin-4 for 12 months using a glucose tolerance test. Only one participant reached the upper threshold of glucose levels out of 20 patients undergoing testing, all of whom were categorised as a moderately to severely parkinsonian. This suggests the possibility that the interaction between exendin-4 and L-dopa is less likely to appear in humans (Iciar Aviles-Olmos et al. 2013). However, the insulin resistance determined in this clinical study depended only on results from the glucose tolerance test, and no data available about insulin plasma levels or insulin resistance at the molecular level. In addition, the

interaction between L-dopa and exendin-4 could be different between PD patients receiving cell therapy or in the absence of cell therapy. An alternative hypothesis suggested in chapter 4 is that the insulin resistance developed could be related to the interaction between exendin-4 and benserazide (aminoacidic decarboxylase AADC inhibitor used in combination with L-dopa to avoid peripheral L-dopa conversion to dopamine) rather than L-dopa itself. In clinic, Parkinson disease patients use L-dopa in different combination with AADC inhibitors like benserazide and carbidopa. Thus, more studies maybe needed to understand the mechanisms that lead to develop this insulin resistance in animal model and understand its possible incidence in parkinsonian patients.

When co administered with cyclosporine, used to prevent rejection of transplanted cells, exendin-4 had a dramatic impact on rats' body weight in two separate experiments (chapter 5 & 6). In chapter 5, after 10 days of starting exendin-4 and cyclosporine treatment 16 out of 18 rats had severe weight loss in a short time, reaching more than 10% loss of body weight and in some cases reached to 20%, and similarly happened in chapter 6 experiment. However, this was remedied by using single daily dose of 0.5 µg/kg of exendin-4 rather than twice daily dose in both experiments. This is a concern for clinical translation, especially given that baseline weight loss was observed in the clinical trial in most of the PD participants and one of them withdrew from the trial after 10 months because of the severity of weight reduction. These patients were not on the additional cyclosporine required post-transplantation raising concerns that this could be problematic (I Aviles-Olmos et al. 2013). While this weight loss is considered beneficial in T2DM, it is much less acceptable as a side effect in PD. Clinically, this weight reduction recovered by reducing the dose or cessation of the exendin-4 administration (I Aviles-Olmos et al. 2013) and similarly by reducing the dose frequency in the 6-OHDA-lesioned rats in this thesis (chapter 5 and 6). The reduced dose of exendin-4 was still effective in supporting graft efficacy of H9 hESC-DA in the vibrissae test and improved fibre innervation of the striatum however this effectiveness was not reflected in improved cell survival rats or other behavioural tests. This suggested that body weight can be monitored by reducing the exeindin-4 dose with the possibility of maintaining its therapeutic efficacy.

Despite the fact that all cell types evaluated in this thesis (E14 VM cells, H9 hESC-DA graft, RC17 hESC-DA graft) expressed the GLP-1 receptor following transplantation, there was a clear difference in the impact of exendin-4 on graft survival and function. As an illustration, in

rat embryonic VM grafts (chapter 4), it improved dopaminergic neuronal survival and graft function; in H9 hESC-DA graft (chapter 5), it supported graft function in vibrissae test possible due to improve or accelerate graft maturation but without affecting cell survival or fibre outgrowth; in RC17 hESC-DA graft (chapter 6), it had no effect on graft survival, fibres outgrowth or graft function but rather it may have interfered with other cells in the graft rather than dopaminergic neurons (see Table 9). This difference in part may relate to the change in protocol required in the presence of cyclosporine in chapter 5 and 6 in which the dose frequency had to be reduced for welfare reasons; alternatively, a difference in the intracellular signalling pathway of the GLP-1 receptors may occur because of the different originating species of the cells (human and rat), development stage (primary VM and ESC-DA), or source of cell line (H9 and RC17).

Table 9 main impacts of exendin-4 on graft outcomes of different cell sources for transplantation

Main effects on the graft	Type of transplanted cells		
	E14 VM cells	H9 hESC-DA	RC17 hESC-DA
Graft function	Improved	Some improvement	No effect
Dopaminergic neurons survival	Improved	No effect	No effect

The other GLP-1 agonist, liraglutide, was also supportive to the function of VM graft. However, it had no effect on graft survival which raises the question about what is the mechanism involved to support graft function. The data showed that it may support graft function through improving TH+ fibres density in the graft with a tendency to improve graft volume. Interestingly, liraglutide effectiveness on graft function (amphetamine rotation test) also interacted with L-dopa, as liraglutide produced best function in presence of L-dopa which may provide an advantage to supportive the graft function in “real world model”. In addition, there was no evidence of insulin resistance from this combination with L-dopa. One of the suggestions that liraglutide produced better effectiveness in presence of L-dopa was through modulation of the inflammatory reaction around the graft as there was a significant increase in infiltered leukocytes in the striatum resulting from interaction between L-dopa and liraglutide (discussed in chapter 4). This mechanism could be affected by the presence of

cyclosporine which acts through inhibition of T cell activation and blocking transcription of cytokine genes like IL-2 and IL-4 (Matsuda & Koyasu 2000). Since patients received cyclosporine after cell transplantation, liraglutide needed to be tested in animal models exposed to cyclosporine-A. In addition, issues raised here from using exendin-4, such as weight reduction and variety in the pattern of effect by the difference in cell source also need to be considered before considering the use of liraglutide in clinical trial.

It was originally intended to use liraglutide alongside exendin-4 to continue to evaluate its effect on hESC-DA transplantation in chapters 5 & 6 but because of practical and financial constraints only exendin-4 was selected to be tested on hESC-DA therapy in this thesis. The reasons for selection of exendin-4 over liraglutide were: first, exendin-4 is in clinical trials to treat PD which will help to give clearer evaluation to its value for support cell therapy in clinical trials; second, liraglutide showed no effectiveness on protecting and improving cell survival; third, the intention was to replicate the exendin-4 effect to get a greater understanding of the mechanisms underlying the intriguing results of development of insulin resistance from interaction between exendin-4 and L-dopa (note: liver and blood samples were collected from chapter 5 and 6 experiments and further investigations on involvement of insulin resistance and liver abnormalities on cell therapy will be conducted as soon as financial support is obtained).

Taken together further studies need to be conducted to answer the validity of GLP-1R agonists on supporting cell therapy in clinical trials and it should consider the use the following:

1. 6-OHDA animal model received L-dopa and cyclosporine because both treatments are used by patients in clinic after transplantation and both interacted with the effectiveness of exendin-4 and possibly liraglutide.
2. normal (0.5 µg/kg twice daily) and reduced dose frequency (once daily) of exendin-4 to compare its effectiveness with minimum dose that may be needed to avoid weight reduction.
3. Carbidopa or other AADC inhibitors instead of benserazide in combination with L-dopa to avoid a hypothesised interaction between exendin-4 and benserazide. The later may be responsible for accumulation of exendin-4 metabolites that have an antagonistic effect on the GLP-1 receptors (discussed in chapter 4).

7.2 New insights of using L-dopa in animal models of cell therapy in PD

L-dopa treatments were used throughout this thesis to mimic the real clinical fact that administration of anti-Parkinsonian medications will continue for patients who receive cell therapy. It has been clarified previously that L-dopa is responsible for inducing changes at the host brain can affect evaluation of graft outcomes (Steece-Collier et al. 2009)(García et al. 2011a). For instance, priming the rats with chronic L-dopa treatment before, and continuing treatment after VM cells transplantation led to a significant reduction in the ability of the graft to alleviate LID compared to non-primed rats(Steece-Collier et al. 2009) This indicated importance of using chronic L-dopa to estimate the real ability of the graft on reversing LID. In addition, the same study showed that rats primed with L-dopa have significantly less fibre outgrowth compared to non-primed rats. Such effects would reduce the grafts ability to cover an appropriate area of the striatum, and consequently might affect graft function. Other experiments showed that the severity of GID depends on the severity of pre-existing LID as more severe pre-transplantation LID would develop severe GID later (which need L-dopa treatment to develop) (García et al. 2011a). Thus, adding L-dopa treatment to develop pre-existing LID would enable a real evaluation of the induction of the GID by the graft. Moreover, some studies conducted in cell culture and animal models suggested that L-dopa increases oxidative stress and has neurotoxic effects which would increase degeneration of the dopaminergic neurons (Maharaj et al. 2005; Jang et al. 2015; Liu et al. 2004; Park et al. 2011). This effect of L-dopa may change the host brain environment, a factor which would add complexity to determine the status of survival or function of transplanted cells dopaminergic neurons. Westin and colleague demonstrated that chronic L-dopa treatment in the 6-OHDA lesioned rat caused changes in blood brain barrier permeability and induced angiogenesis (Westin et al. 2006). This effect can also cause changes in the host brain environment as the changes in blood brain permeability may increase inflammatory cells infiltration that would affect graft function or survival. Breger and colleagues demonstrated that L-dopa treatment causes increases in the microglial level around the VM xenograft and increases in leukocyte infiltration (Breger et al. 2016). Similarly, in this thesis, a clear trend to increase the level of microglia by administration of L-dopa around RC17 hESC-DA graft was noticed (chapter 6).

However, although L-dopa is capable of inducing these wide ranging and significant alterations to the host brain, the studies presented here show that once daily L-dopa has no

direct effect on behavioural recovery or survival of embryonic VM transplantation ((Chapter 4) & (Breger et al. 2016; Steece-Collier et al. 2009)). Before this thesis work was conducted, there was no understanding of the effect of L-dopa on the hESC-DA grafts. Indeed, this PhD addresses an important question about the validity of hESC-DA grafts in the presence of L-dopa treatment. Furthermore, it has highlighted the importance of using L-dopa in animal models to evaluate cell therapy which include the hypothesised mechanisms of findings that:

1. L-dopa is being able to directly support to hESC-DA graft function or maturation.
2. L-dopa induced dyskinesia recovery using hESC-DA graft helps in early prediction of the graft function.
3. L-dopa induces or participates in inducing peripheral physiological changes that could impact on graft outcomes.

This PhD has not only revealed that hESC-DA grafts can survive and function in the presence of L-dopa regardless of graft size, but it also showed that L-dopa can even support graft efficacy. In H9 hESC-DA graft (large graft), L-dopa had a positive effect on the vibrissae test and cylinder test; in RC17 hESC-DA (small graft) L-dopa showed a high tendency to ameliorate amphetamine rotation tests throughout the experimental time. The mechanism of this effect is not clear as there is no change in cell survival or fibre outgrowth by the effect of L-dopa. This suggested that this improvement is not related to the graft size but other factors may be involved like dopaminergic neurons subtype or synaptic integration which is needed for further investigation. In chapter 5, the supportive effect of L-dopa has high tendency (but not significant) to interact with the time as its effect diminished with extended post graft times, which suggested that the L-dopa may be accelerating graft maturation. In a slightly different profile, the supportive tendency of L-dopa on RC17 hESC-DA graft continued to the same extent throughout the experimental time. Neither cell line is believed to be fully mature at 16 weeks, but the RC17 appeared to take longer to develop to their full potential. The L-dopa effect could result from an interaction with the host environment or from a direct pharmacological action on the dopamine receptors on the grafted cells. Dopamine receptors subtypes (1 to 5) were identified on H9 hESC in vitro at all the stages of differentiation toward a mature dopaminergic phenotype. Importantly, dopamine receptor agonists affected the neuronal differentiation process and the subtype of the dopaminergic neurons as they

increased the number of TH neurons produced but reduced GIRK2 expression, a marker of the desirable A9 subtype (Belinsky et al. 2013).

An unanticipated benefit to the use of L-dopa in the *in vivo* model is that it appeared to provide a very early indicator of hESC-DA graft function. LID and L-dopa-induced rotations were reduced weeks before a reversal of the motor and behavioural deficits was identified. This suggests that the cells are capable early on of re-uptaking extracellular dopamine, re-store and releasing it again under an autoregulatory mechanism to decrease the pulsatile excitation of dopamine receptors after L-dopa administration (Björklund 1992). This improved management of L-DOPA would reduce the behavioural response. Moreover, the stem cell graft reversed LID regardless of the graft size or the maturation time required to reduce behavioural deficits. Thus, it will increase the potential of identifying the functionality of small grafts or slowly maturing grafts whose function may be underestimated throughout the experimental time (see chapter 6). This is in contrast with primary cell transplantation which required a large graft size and extensive innervation to reduce the LID score (Lane et al. 2006). This may suggest that the hESC-DA graft is more efficient than primary cells grafts in controlling postsynaptic dopamine levels elevated by L-dopa administration.

The other notable effect of L-dopa was the development of the systemic changes of insulin resistance and increased peripheral inflammatory infiltration, only observed on co-administration with GLP-1 agonists (exendin-4 and liraglutide). This was accompanied by a modulation of the neuroprotective agents' effect on the graft. The presence of L-dopa converted liraglutide from inactive neuroprotective agent to an active agent while vice versa happened with exendin-4. Although the exact mechanism is unknown, we can speculate that these systemic changes have a role in determining graft outcome (discussed in chapter 4). This highlighted the importance of considering L-dopa in evaluating graft outcomes in the presence of other factors. The candidate patients for cell therapy transplantation receive a range of medications including anti-parkinsonian medications (see table 1 in chapter 5) as well as other neuro-active and non-neuroactive medications (see below, Table 10). This complex of medications increase the likelihood of a drug interaction with L-dopa and possibility of inducing systemic changes comparable to those observed with the interaction of L-dopa and GLP-1 agonists. Since the mechanism by which the L-dopa interact with exendin-4, the anti-diabetic drug, to induce insulin resistance is unclear, any medication may

interfere with glucose-insulin homeostasis could possibly have the potential to interfere with L-dopa. For instance, some clinical cases have reported that MAO- inhibitors, selegiline and rasagiline, caused increased insulin release, insulin sensitivity and hypoglycaemia in non-diabetic parkinsonian patients (Ibrahim et al. 2017; Rowland et al. 1994). In addition, developing insulin resistance is one of the side effects of the immunosuppressant regime used after transplantation like azathioprine and prednisolone (Prokai et al. 2012).

Throughout this thesis, the L-dopa treatments were used in a once daily dose regimen while in clinic the reality is that patients receive more frequent doses, up to 4-5 times a day. This in turn could make the effect of L-dopa on the graft outcomes more robust and obvious. However, replicating this frequency of drug administration is not feasible in a long-term study for animal welfare reasons but looking to replicate this with more constant dopamine receptor stimulation would be desirable in the future. Moreover, the possible sensitivity of the graft efficacy by the systemic changes underline that not only L-dopa treatment should be considered in evaluation of graft function but all possible medications used by the patient that have a tendency to induce insulin resistance or inflammatory modulation.

Table 10 neuronal and non-neural medications used by 12 parkinsonian patients at the time of VM cells transplantation in TransEuro clinical trial (obtained from prof. Roger Barker, transEuro coordinator)

Neuro medications (number of patients)	Non-neuro medications (number of patients
zopiclone 7.5 mg nocte (1)	Prednisolone (6)
citalopram 30mg OD (1)	Omeprazole (6)
flunitrazepam 1mg OD (1)	Trimoxazole (5)
Zolpidem 5mg OD (1)	Cyclosporine (6)
	Azathioprine (6)
	Alendronate (2)
	Anti-asthmatic medications (1) including: symbicort; terbutaline, fluticasone; cromoglicate

7.3 Experimental limitations

There are some limitations underlined different experiments in the thesis mainly due to financial or practical reasons. The balanced design of the experimental chapters was lacking to 6-OHDA unilateral lesion control group treated with an appropriate drug used within the experiments. For instance, in chapter 3, there was no lesion control group treated with either ghrelin or JMV-2894 (long acting ghrelin); in chapter 4, there was a lack for lesion group treated with exendin-4, liraglutide, L-dopa, exendin-4 plus L-dopa and liraglutide plus L-dopa; in chapter 5 and 6 there was absence lesion group treated with exend-4, L-dopa exendin-4 plus L-dopa, except in chapter 6 there was lesion plus exendin-4 group. The value of adding these treatments to lesion control group will help to confirm evaluation the effect of these treatment on the graft function and excluding any effect on the behavioural tests due to interaction with other targets. The behavioural data analysis was only depended on that there was a complete lesion for endogenous dopaminergic neurons (the main contributor that may change the behavioural outcomes in presence of these drugs) and assumed that these treatments had no interference with the behavioural tests through interaction with other systems rather than the graft. The effect some of these treatments was evaluated on the

behavioural tests of complete unilateral 6-OHDA lesioned rats in previous published papers, like L-dopa was tested in the stepping, cylinder, vibrissae and amphetamine rotation test (Breger et al. 2016); liraglutide was tested in amphetamine and apomorphine induced rotation tests (Hansen et al. 2016)), In addition exendin-4 which used in chapter 4, 5 and 6 was tested in chapter 6. All these treatments showed they had no effect on the behavioural outcomes of the long lasting complete lesion 6-OHDA rat model. However, the effect of these treatments is still need to be tested in the same experiment at the same condition for better evaluation. So, it is recommended for future study to include control lesion group treated with these agents.

The other limitation in this thesis was lacking adding cyclosporine-A treatment to the lesion control group in chapter 5 and 6. Cyclosporine-A was used in the grafted groups to avoid graft rejection and there was no need to added in the lesion control group where there is no graft. However, some papers showed that cyclosporine-A increase the rats' hyperactivity (Borlongan et al. 1999)**,** in addition it reduces the animal welfare with time. These may affect the rats' response in the motor and behavioural tests. The data analysis of the graft function compared to the lesion control in chapter 5 and 6 assumed there was no effect of cyclosporine-A on rats' response on the motor tests. However, for better evaluation, it is recommended to include cyclosporine treatment in the lesion control group in future study.

7.4 Thesis conclusion

This PhD work identified the GLP-1R agonists as neuroprotective agents capable of supporting optimisation of cell therapy in PD. It also clarified that hESC-DA can survive and function in the presence of L-dopa treatment which was added to the 6-OHDA rat model to mimic the reality of patients' clinical situation. It also verified that hESC-DA can ameliorate the L-dopa induced dyskinesia. This thesis confirmed the importance of adding L-dopa to the animal models to evaluate cell therapy in PD. It is recognised that L-dopa participated in inducing peripheral changes that hypothesised to have determine effect on graft function and survival, in addition L-dopa may have supportive effect to the function of the hESC-DA graft. This work also opened new questions need to address. For instance, GLP-1R agonists illustrated differences in their action and safety when co administrated with L-dopa and cyclosporine-A and when different cell sources were used for transplantation. These suggested further

studies need to validate the best GLP-1R agent's effectiveness and safety to support transplanted cells. This thesis also raised other issues may require further investigations including whether GLP-1R agonists have interaction with other medications used by parkinsonian patients that might compromise its effectiveness or safety; whether L-dopa has interactions with other medications used by the parkinsonian patients which might induce peripheral changes that would affect graft survival and effectiveness.

8 Appendixes

8.1 Appendix A: antibodies optimisation information and method of detection for the histological and cytological targets:

Target	Serum	Primary antibody	Secondary antibody	Method	Protocol optimisation notes
Dopaminergic neurons DA graft in striatum	Goat	Rabbit TH (1:1000)	Biotinylated anti- rabbit (1:200)	DAB-IHC	Standard*
Co-localisation of ghrelin receptor GHSR1a and DA in E14 VM cells	Horse, Goat	Mouse TH (1:400), rabbit GHSR1a (1:50)	Anti-mouse Alex flour 488 (1:500), biotinylated anti-rabbit (1:200), Texas red Avidin	Double F-ICC	GHSR signal maximised by using biotinylated secondary antibody
Co-localisation of ghrelin receptor GHSR1a and DA in the striatal graft	Goat	Mouse TH (1:1000), rabbit GHSR1a	Anti-mouse Alex flour 488 (1:500), biotinylated anti-rabbit (1:200), streptavidin Cy3	Double F-IHC	GHSR incubated for 72hr at RT with using biotinylated secondary.
Co-localisation of ghrelin receptor GHSR1a and stem cells in E14 VM	Donkey, Goat	Goat SOX2 (1:50), rabbit GHSR1a	Anti-goat Alex flour 488 (1:500), biotinylated anti- rabbit (1:200), Texas Red Avidin (1:200)	Double F-ICC	GHSR signal maximised by using biotinylated secondary antibody
Co-localisation of ghrelin receptor GHSR1a and neurons in E14 VM	Goat	Mouse BIII (1:50), rabbit GHSR1a	Anti-mouse Alex flour 488 (1:500), biotinylated anti-rabbit (1:200), Texas Red Avidin	Double F-ICC	GHSR signal maximised by using biotinylated secondary antibody
Co-localisation of Glp-1 receptor and DA in E14 VM cells	Horse, Goat	Mouse TH (1:400), rabbit GLP1R (1:200)	Anti-mouse Alex flour 488 (1:500), biotinylated anti-rabbit (1:200), Texas Red Avidin	Double F-ICC	GLP1R signal improved with biotinylated secondary antibody

Target	Serum	Primary antibody	Secondary antibody	Method	Protocol optimisation notes
Co-localisation of GLP1R and DA neurons on striatal graft	Goat	Mouse TH (1:1000), rabbit GLP1R (1:100)	Anti-mouse Alex flour 488 (1:500), biotinylated anti-rabbit (1:200), streptavidin Cy3 (1:200)	Double F-IHC	GLP1R incubated for 72hr at RT with using biotinylated secondary.
Co-localisation of GLP1R and stem cells on E14 VM cells	Donkey, Goat	Goat SOX2 (1:50), rabbit GLP1R (1:200)	Anti-goat Alex flour 488 (1:500), biotinylated anti- rabbit (1:200), Texas Red Avidin (1:200)	Double F-ICC	GLP1R signal improved with biotinylated secondary antibody
Co-localisation of GLP1R and neurons on E14 VM cells	Goat	Mouse BIII (1:50), GLP1R (1:1:200).	Anti-mouse Alex flour 488 (1:500), biotinylated anti-rabbit (1:200), Texas Red Avidin (1:200)	Double F-ICC	GLP1R signal improved with biotinylated secondary antibody
Ghrelin O Acyl Transferase GOAT and DA on E14 VM cells	Goat	Rabbit GOAT (1:200), mouse TH (1:1000)	Anti- rabbit Alex flour 594 (1:500), anti- mouse Alex Flour 488 (1:500).	Double F-ICC	Primary antibody optimisation
Ghrelin O Acyl Transferase GOAT and stem cells on E14 VM	Goat, donkey	Rabbit GOAT (1:200), goat SOX2 (1:50)	Anti- rabbit Alex flour 594 (1:500), anti- mouse Alex Flour 488 (1:500).	Double F-ICC	Primary antibody optimisation
Ghrelin O Acyl Transferase GOAT and neuros on E14 VM		Rabbit GOAT (1:200), mouse TH (1: 400)	Anti- rabbit Alex flour 594 (1:500), anti- mouse Alex Flour 488 (1:500).	Double F-ICC	Primary antibody optimisation

Target	Serum	Primary antibody	Secondary antibody	Method	Protocol optimisation notes
Activated microglia in the grafted striatum	Horse	Mouse CD11b (1:2000)	Biotinylated anti- mouse 1:200	DAB-IHC	Standard
Leucocyte in the grafted striatum	Horse	Mouse CD45 (1:500)	Biotinylated anti- mouse 1:200	DAB-IHC	Standard
Serotonergic neurons in the grafted striatum	Goat	Rabbit 5-HT (1:30,000)	Biotinylated anti- rabbit 1:200	DAB-IHC	Standard
A9 dopaminergic neurons with TH labelled DA in graft	Goat, horse	Rabbit GIRK2 (1:400), mouse TH (1:1000)	Anti- rabbit Alex flour 594 (1:500), anti- mouse Alex Flour 488 (1:500).	Double F-IHC	Primary antibody optimisation
A9 dopaminergic neurons in grafted striatum				DAB-IHC	Standard
Human nuclei in the grafted striatum	Horse	Mouse Hunu (1:2000)	Biotinylated anti- mouse 1:200	DAB-IHC	Standard

Target	Serum	Primary antibody	Secondary antibody	Method	Protocol optimisation notes
Human cytoplasm in the grafted striatum	Horse	Mouse stem121 (1:3000)	Biotinylated anti- mouse 1(:200)	DAB-IHC	Standard
Neuronal progenitor in hippocampus	Goat	Rabbit DCX (1:1000)	Biotinylated anti- rabbit (1:200)	DAB-IHC	Primary antibody optimisation
Striatal blood vessels	Horse	Biotinylated *Lycopersicon esculentum* (1:4000)	Streptavidin Cy3 (1:200)	Single F-IHC	Primary incubated for 72 hr at 4 ^{0}C, secondary incubated for 3 hrs at RT.
Ghrelin receptors in an extracted protein of VM, SN, Str, Hipp, Ctx*	5% NFM	Goat GHSR1a (1:200)	HRP anti- goat (1:5000)	WB	Optimisation of Primary antibody and quantity of the loaded protein
GLP-1 receptors in an extracted protein of VM, SN, Str, Hipp, Ctx	5% NFM	Rabbit GLP1R (1:500)	HRP anti- rabbit (1:5000)	WB	Optimisation of Primary antibody and quantity of the loaded protein
Phosphorylated insulin receptor	10 % horse	IRS-1 pS ^{1011} (1:100)	Biotinylated anti-mouse (1:200)	DAB-IHC	Increase serum concentration up to 10%; incubate the primary at 4 ^{0}C overnight

8.2 Appendix B: products information of antibodies, chemical and materials:

Chemicals, anti-bodies and instruments	Company	Product code
6-OHDA	Sigma	H116-5mg
Anti- goat Alex flour 488	Life Technologies	A-11055
Anti- mouse Alex flour 488	Thermofisher Scientific	A-11029
Anti-rabbit Alex flour 594	Thermofisher Scientific	A-11037
BCA protein assay kit	Thermoscientific Lab	23227
Benserazide	Sigma Aldarich	B7283-5GM
Biotinylated anti mouse	Vector	BA-2001
Biotinylated anti rabbit	Vector	BA-1000
Biotinylated Tomato lectin	Vector labs	B-1175
DAB	Sigma	D5637-16
DMEM-F12	Gibico	21331-020
Dornase alpha (Accutase)	Roche	Batch: N0202
DPX	Fisher	12658646
Exendin-4	Tocris	1933
Fine tip Forceps	F.S.T	11295.51
Fine tip Scissors	FST	15000-08
Goat anti-SOX2	Santacruz	Sc-17320
Hamilton syringe (removable needles)	Sigma	20697
Hamilton needles	Sigma	19132-u
HRP anti- rabbit	Vector	pI-1000
HRP anti-goat	Vector	PI-9500
Isopropanol	Fisher	P1274
Laemmli lysis buffer	Sigma	28733-5x 2 ml
L-dopa	Sigma Aldarich	D1507-5GM
Methylated spirit	Fisher	11482874
Mouse anti- BIII tubulin	Abcam	Ab18207
Mouse anti CD11b	Ab Serotec	MCA 275GA
Mouse anti HuNu	Millipore	MAB 1281
Mouse anti-CD45	Ab Serotec	MCA 43R
Mouse anti-stem121	Takara	Y40410
Mouse anti-TH	Millipore	MAB318
Nitrocellulose bloting membrane	GE- Healthcare	10600011
PLL	Sigma	P1274
Rabbit anti 5-HT	Immnuostar	20080
Rabbit anti DCX (doublecortin)	Abcam	AB18723
Rabbit anti GIRK2	Alomone Labs	APC-006
Rabbit anti- GOAT	Phoenix Pharmaceuticals	H-032-12
Rabbit anti- IRS1-pS^{1011}	Cell signalling Technology	2385S
Rabbit anti-GHSR1a	Alomone Labs	AGR-031
Rabbit anti-GLP-1R	Abcam	Ab188605

Chemicals, anti-bodies and instruments	Company	Product code
Rabbit anti-TH	Millipore	AB152
Streptavidin Cy3	Stratech	016-160-084-JIR
SuperSingle west Dura kit	Thermoscientific Lab	34075
Texas Red Avidin	Vector	A-2006
Trypan blue 0.4%	sigma	T8154-20ml
tryplE	Gibico	12604-013
Xylene	Fisher	10784001

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