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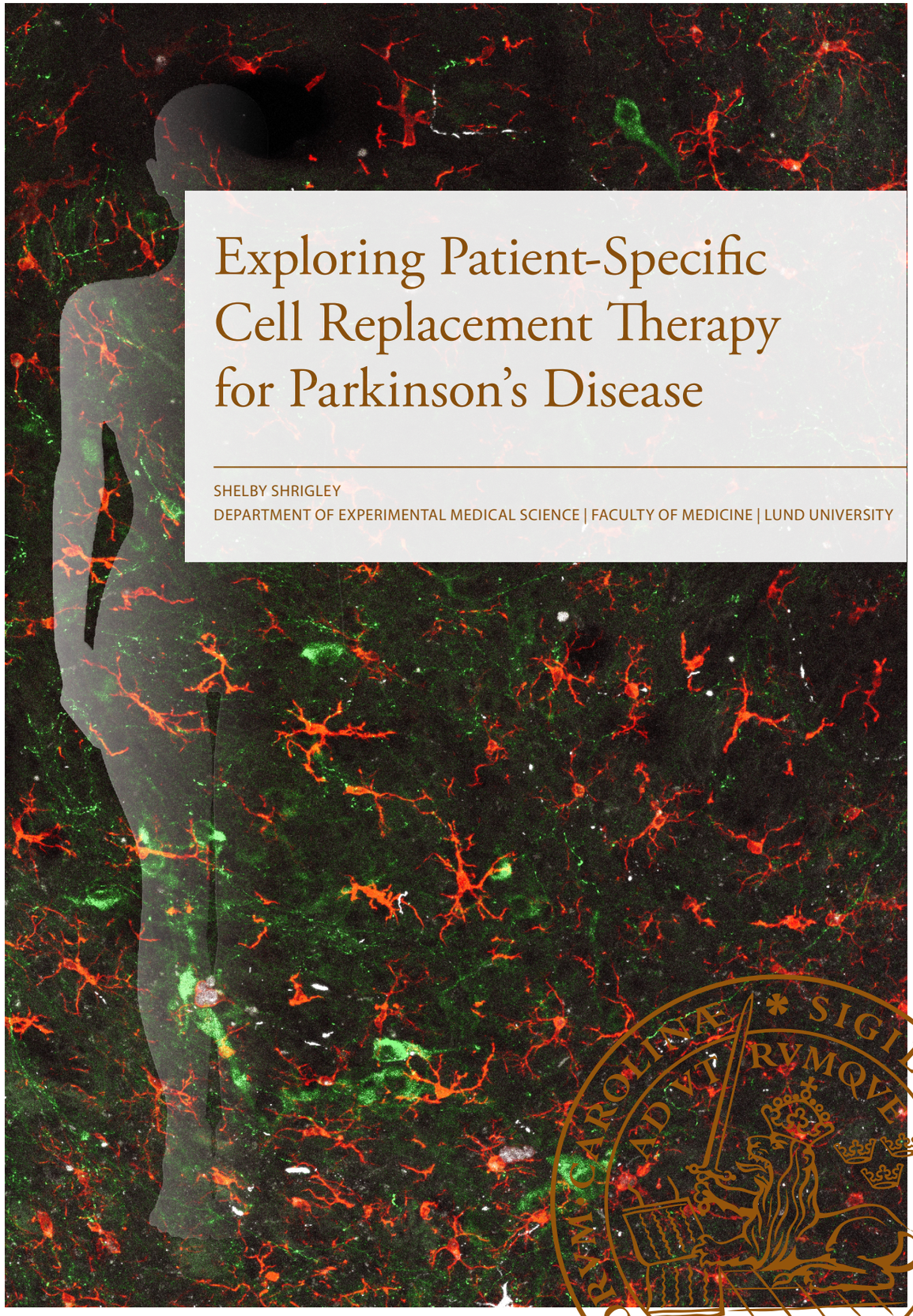

Exploring Patient-Specific Cell Replacement Therapy for Parkinson's Disease

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DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY

Academic dissertation

Exploring Patient-Specific Cell Replacement Therapy for Parkinson's Disease

Shelby Shrigley

2021

With approval of the Faculty of Medicine of Lund University,
this thesis will be defended
at 09:00 on 12th March, 2021 in Segerfalksalen,
Wallenberg Neuroscience Center, Lund, Sweden.

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Abstract Parkinson's disease (PD) affects over six million people worldwide and is characterised by the progressive loss of dopaminergic (DA) neurons in the substantia nigra, accumulation of pathological alpha-synuclein (αSyn), and inflammation in the brain. This leads to motor impairments including rigidity, akinesia, bradykinesia, resting tremor, and postural instability. Cell replacement therapy aims to replace the midbrain DA neurons which have been lost in the disease to restore normal motor function. Previously, DA cells for transplantation have been derived from fetal mesencephalic tissue or human embryonic stem cells (hESCs). However, an alternative route to generate DA cells is via cellular reprogramming. This is a rapidly emerging field which allows somatic cells to be reprogrammed either into human induced pluripotent stem cells (hiPSCs) or directly into induced neurons (iNs) by forced expression of specific factors. This creates the possibility to use patient-specific cells which could reduce the risk of immune rejection and eliminate ethical concerns.

The overall aim of this thesis is to evaluate if patient-derived cells could be a suitable alternative in cell replacement therapy for PD. Firstly, an efficient protocol to directly reprogram human adult fibroblasts into iNs was developed. Following this, factors that could convert cells specifically towards a DA subtype were investigated and used to examine if cells from healthy donors and PD patients could be reprogrammed with similar efficiency. Next, a new humanized αSyn xenograft model of PD was established to assess the impact of host pathology on grafted cells. Finally, we explored if DA cells derived from a patient hiPSC line harbouring an αSyn triplication mutation could survive intracerebral transplantation and function on par with hESC-derived DA neurons. This patient line was also assessed for the presence of pathological features in different models of PD. These results will help to pave the way for future research assessing the potential of patient-derived cells for brain repair

Key words: Dopaminergic neurons, Parkinson's disease, cell replacement therapy, cellular reprogramming, alpha-synuclein

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View of dopaminergic cells transplanted into the rodent brain with ongoing pathology and inflammation.

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There is no scientific study more vital to man
than the study of his own brain.
Our entire view of the universe depends on it.
- Francis Crick

TABLE OF CONTENTS

ORIGINAL PAPERS AND MANUSCRIPTS INCLUDED IN THE THESIS

Paper I

Simple generation of a high yield culture of induced neurons from human adult skin fibroblasts.

Shrigley S, Pircs K, Barker RA, Parmar M & Drouin-Ouellet J.

Journal of Visualized Experiments, 132, e56904, 2018.

Paper II

Age related pathologically relevant impairments in directly reprogrammed dopaminergic neurons derived from patients with idiopathic Parkinson's disease.

Drouin-Ouellet J, Nilsson F*, Pircs K*, **Shrigley S**, Birtele M, Pereira M, Storm P, Sharma Y, Bruzelius A, Vuono R, Kele M, Stoker TB, Ottosson DR, Falk A, Jakobsson J, Barker RA & Parmar M.

Under peer review in Brain.

Paper III

Impact of α-synuclein pathology on transplanted hESC-derived dopaminergic neurons in a humanized α-synuclein rat model of PD.

Hoban DB, **Shrigley S**, Mattsson B, Breger LS, Jarl U, Cardoso T, Wahlestedt JN, Luk KC, Björklund A & Parmar M.

Proceedings of the National Academy of Sciences, 117(26), 15209-15220, 2020.

Paper IV

Grafts derived from an α-synuclein triplication patient mediate functional recovery but develop disease-associated pathology in the 6-OHDA model of Parkinson's disease.

Shrigley S, Nilsson F, Mattsson B, Fiorenzano A, Mudannayake J, Bruzelius A, Ottosson DR, Björklund A, Hoban DB* & Parmar M*.

Manuscript accepted in Journal of Parkinson's disease.

Paper V

Grafts derived from an α-synuclein triplication patient show accelerated pathological changes in a humanized α-synuclein rat model of PD.

Shrigley S, Mattsson B, Corsi S, Nilsson F, Wahlestedt JN, Fiorenzano A, Luk KC, Björklund A, Hoban DB & Parmar M.

Unpublished manuscript.

TRANSLATIONAL PROJECTS IN CONJUNCTION WITH THE THESIS

In vivo preclinical assessment for the STEM-PD trial

In the STEM-PD clinical trial patients with Parkinson's disease (PD) will be transplanted with stem cell-derived dopaminergic (DA) progenitors. This trial is planned to be a multicenter transplantation trial sponsored by Lund University, Skåne University Hospital, University of Cambridge, and Cambridge University Hospital (Barker, 2019; Barker et al., 2017). The stem cell-derived DA progenitors used in this trial have been GMP manufactured at the Royal Free Hospital in London and must first be approved as an advanced therapy medicinal product in order for the clinical trial to commence.

I have been directly involved in the preclinical efficacy studies, preparing documents and following standard operating procedures for transplantation of these stem cell-derived DA progenitors into a rodent model of PD. These data will be reported to the Medicines and Healthcare products Regulatory Agency (MHRA) and Swedish Medical Products Agency (MPA). The clinical trial is due to begin this year pending approval from the regulatory bodies.

ABSTRACT

Parkinson's disease (PD) affects over six million people worldwide and is characterised by the progressive loss of dopaminergic (DA) neurons in the substantia nigra, accumulation of pathological alpha-synuclein (αSyn), and inflammation in the brain. This leads to motor impairments including rigidity, akinesia, bradykinesia, resting tremor, and postural instability. Cell replacement therapy aims to replace the midbrain DA neurons which have been lost in the disease to restore normal motor function. Previously, DA cells for transplantation have been derived from fetal mesencephalic tissue or human embryonic stem cells (hESCs). However, an alternative route to generate DA cells is via cellular reprogramming. This is a rapidly emerging field which allows somatic cells to be reprogrammed either into human induced pluripotent stem cells (hiPSCs) or directly into induced neurons (iNs) by forced expression of specific factors. This creates the possibility to use patient-specific cells which could reduce the risk of immune rejection and eliminate ethical concerns.

The overall aim of this thesis is to evaluate if patient-derived cells could be a suitable alternative in cell replacement therapy for PD. Firstly, an efficient protocol to directly reprogram human adult fibroblasts into iNs was developed. Following this, factors that could convert cells specifically towards a DA subtype were investigated and used to examine if cells from healthy donors and PD patients could be reprogrammed with similar efficiency. Next, a new humanized αSyn xenograft model of PD was established to assess the impact of host pathology on grafted cells. Finally, we explored if DA cells derived from a patient hiPSC line harbouring an αSyn triplication mutation could survive intracerebral transplantation and function on par with hESC-derived DA neurons. This patient line was also assessed for the presence of pathological features in different models of PD. These results will help to pave the way for future research assessing the potential of patient-derived cells for brain repair.

LAY SUMMARY

Parkinson's disease is a neurodegenerative disorder affecting over six million people worldwide. It is often classified as a motor disorder as the main symptoms include rigidity, loss of movement, slowness of movement, resting tremor, and balance problems. These motor symptoms are due to the loss of dopaminergic neurons in the midbrain. In a healthy person, these neurons release dopamine to specific targets in the brain in order to control movement. However, in Parkinson's disease these dopaminergic neurons are gradually lost causing the symptoms to get worse over time.

Most treatments for Parkinson's disease aim to reduce the motor symptoms by giving patients drugs that increase the level of dopamine in the brain. However, this does not stop the dopaminergic neurons in the brain from dying or the disease from progressing over time. Also, these drugs can have major side effects which can severely affect the patient's quality of life. Cell replacement therapy is an alternative treatment option which aims to replace the dopaminergic neurons which have been lost with new healthy cells. Once transplanted into the brain, these cells can release dopamine and restore normal motor function.

Previously, dopaminergic cells for transplantation have been derived from human fetal tissue or human embryonic stem cells (hESCs). However, there are ethical concerns associated with their use and patients also need to take immunosuppressive drugs to prevent transplant rejection. An alternative option is to use reprogrammed cells. Cellular reprogramming is a process by which a mature cell can be reprogrammed back into a stem cell, called human induced pluripotent stem cells (hiPSCs), or directly into a neuron, called induced neurons (iNs). This creates the possibility to use the patient's own cells for cell replacement therapy, removing the need for immunosuppressive drugs and associated complications.

In this thesis, I have investigated if patient-derived cells could be a potential alternative in cell replacement therapy for Parkinson's disease. Firstly, I have developed protocols for direct reprogramming of adult skin cells into neurons. I have also investigated factors which could specifically generate dopaminergic neurons and applied these to determine if cells from healthy donors and Parkinson's disease patients could be reprogrammed with similar efficiency. Following this, I wanted to examine transplanted cells in an environment that more closely resembles a Parkinson's disease patient brain. Therefore, a new transplantation model was developed to assess the impact of a pathological environment on dopaminergic neuron grafts. Finally, I have explored if dopaminergic cells derived from a patient hiPSC line could survive transplantation into the brain and function on par with hESC-derived dopaminergic cells. I also assessed if this patient line displayed any pathological features across different models of Parkinson's disease. Collectively, these results will contribute to assessing the suitability of patient-specific cells for brain repair.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Parkinsons sjukdom är en neurodegenerativ sjukdom som drabbar över sex miljoner människor världen över. Det klassificeras ofta som en motorisk störning eftersom de viktigaste symptomen inkluderar stelhet, svårigheter att påbörja rörelser, skakningar, och balansproblem. Dessa motoriska symtom beror på förlusten av dopaminerga nervceller i mellanhjärnan. Hos en frisk person frisätter dessa neuroner dopamin till specifika mål i hjärnan för att kontrollera rörelse. Men vid Parkinsons sjukdom går dessa dopaminerga nervceller gradvis förlorade och försämrar symtomen med tiden.

De flesta behandlingar för Parkinsons sjukdom syftar till att minska motoriska symtom genom att ge patienter läkemedel som ökar nivån av dopamin i hjärnan. Detta hindrar dock inte de dopaminerga nervcellerna i hjärnan från att dö eller att sjukdomen utvecklas över tiden. Dessa läkemedel kan också ha stora biverkningar som kan påverka patientens livskvalitet allvarligt. Cellterapi är ett alternativt behandlingsalternativ som syftar till att ersätta de dopaminerga nervcellerna som har gått förlorade med nya friska celler. När de väl har transplanterats i hjärnan kan de frigöra dopamin och återställa normal motorisk funktion.

Tidigare har dopaminerga celler för transplantation tagits från mänsklig fostervävnad eller humana embryonala stamceller (hESCs). Det finns emellertid etiska problem i samband med deras användning och patienter måste också ta immunsuppressiva läkemedel för att förhindra avstötning av transplantat. Ett alternativ är att använda omprogrammerade celler. Cellulär omprogrammering är en process genom vilken att mogna specialiserade celler, kan omprogrammeras tillbaka till en stamcell, som kallas humana inducerade pluripotenta stamceller (hiPSCs), eller direkt till nervceller, kallad inducerade nervceller (iNs). Detta skapar möjligheten att använda patientens egna celler för cellterapi, vilket tar bort behovet av immunsuppressiva läkemedel och tillhörande komplikationer.

I denna avhandling har jag undersökt om celler som tagits från patienter kan vara ett potentiellt alternativ i cellterapi för Parkinsons sjukdom. För det första har jag utvecklat protokoll för direkt omprogrammering av vuxna hudceller till nervceller. Jag har också undersökt faktorer som specifikt kan generera dopaminerga nervceller och använt dessa för att avgöra om celler från friska givare och patienter med Parkinsons sjukdom kan omprogrammeras med en liknande effektivitet. Efter detta ville jag undersöka transplanterade celler i en miljö som mer liknar en hjärna hos Parkinsons sjukdom.

Därför utvecklades en ny transplantationsmodell för att bedöma effekten av en patologisk miljö på dopaminerga celltransplantat. Slutligen har jag undersökt om dopaminerga celler tagits från en patient hiPSC-linje kan överleva transplantation i hjärnan och fungera i nivå med dopaminerga celler från hESCs. Jag bedömde också om denna patientlinje visade några patologiska egenskaper över olika modeller av Parkinsons sjukdom. Sammantaget kommer dessa resultat att bidra till att bedöma lämpligheten hos patientspecifika celler för hjärnreparation.

ABBREVIATIONS

αSyn	alpha-synuclein
AAV	adeno-associated virus
CNS	central nervous system
COMT	catechol-O-methyltransferase
DA	dopamine/dopaminergic
DAB	3,3'-diaminobenzidine
DAT	dopamine transporter
DBS	deep brain stimulation
GMP	good manufacturing practice
GPi	internal globus pallidus
hESCs	human embryonic stem cells
hiPSCs	human induced pluripotent stem cells
HLA	human leukocyte antigen
iNs	induced neurons
iDANs	induced dopaminergic neurons
KPBS	phosphate-buffered saline with potassium
LB	Lewy body
L-DOPA	3,4-dihydroxy-L-phenylalanine
LV	lentiviral vector
MAO-B	monoamine oxidase type B
MFB	medial forebrain bundle
MHC	major histocompatibility complex
MOI	multiplicity of infection
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+	1-methyl-4-phenylpyridinium ion
ORF	open reading frame
PCR	polymerase chain reaction
PD	Parkinson's disease
PFA	paraformaldehyde
PFFs	preformed fibrils
PGK	phosphoglycerate kinase
pSer129	phosphorylated alpha-synuclein at serine 129
pSyn	phosphorylated alpha-synuclein
REST	RE1-silencing transcription factor
RT	room temperature

SD	Sprague-Dawley
SN	substantia nigra
shRNA	short hairpin RNA
TH	tyrosine hydroxylase
VM	ventral mesencephalic
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element
6-OHDA	6-hydroxydopamine

INTRODUCTION

In this chapter, I will provide a general overview of Parkinson's disease (PD) and introduce animal models of PD relevant for xenografting studies. Next, I will discuss cell replacement therapy as a treatment strategy for PD and mention the different cell sources that may be used, with a particular focus on cellular reprogramming. Finally, I will address patient-specific cell replacement therapy and how this may impact future strategies for cell therapies.

Parkinson's disease (PD)

PD, first described in 1817 by James Parkinson in "An assay on the shaking palsy" (Parkinson, 2002), is a prevalent neurodegenerative disorder estimated to affect over six million people globally (Dorsey et al., 2018). PD is a complex and heterogenous disease, typically classified as a movement disorder, although it also presents with non-motor symptoms. The motor symptoms are the main symptoms and include rigidity, akinesia (loss of movement), bradykinesia (slowness of movement), resting tremor, and postural gait impairment (Ross et al., 2004). Non-motor symptoms include olfactory dysfunction, autonomic dysfunction, sleep disturbances, cognitive deficits, and neuropsychiatric manifestations such as depression and anxiety (Chaudhuri et al., 2006; Papagno & Trojano, 2018). Current treatment options for PD are symptomatic in nature and do not offer any form of disease modification in terms of slowing, stopping, or indeed reversing the progressive nature of the disease.

Aetiology

The cause of PD remains unknown but it likely results from a complex interplay of environmental and genetic factors (Kalia & Lang, 2015). The vast majority, approximately 90% of cases are categorised as sporadic PD, however, a number of risk factors have been identified. The greatest risk factor for PD is ageing (Collier et al., 2011; Driver et al., 2009), but other environmental factors include toxins, e.g. pesticides, and traumatic brain injury (Ascherio & Schwarzschild, 2016). The remaining 10% of cases can be linked to genetic causes, also known as familial PD. The first genetic case to be discovered in the late 1990's was due to a mutation in the *SNCA* gene, which encodes for the protein alpha-synuclein (αSyn) (Polymeropoulos, 1997). This was shortly followed by reports of other mutations in the *SNCA* gene that also lead to the development of PD (Appel-Cresswell et al., 2013; Krüger et al., 1998; Lesage et al., 2013; Zarranz et al., 2004). Moreover, genome copy alterations, such as duplication or triplication of the *SNCA* gene, indicate a correlation between gene dos-

age and disease progression (Chartier-Harlin et al., 2004; Singleton, 2003). Mutations in several other genes including *Parkin, LRRK2, GBA,* and *MAPT* have also been implicated in PD (Bandres-Ciga et al., 2020; Lin & Farrer, 2014).

Pathophysiology

PD is characterised by progressive loss of the midbrain dopamine (DA) neurons resulting in a deficiency in DA signalling in the brain. These midbrain DA neurons are located in the substantia nigra (SN) where they send projections to the striatum and release DA to regulate motor output. Another key feature of PD is the presence of intracellular Lewy bodies (LBs) which are comprised of misfolded protein aggregates, predominantly αSyn but may also contain small amounts of ubiquitin and neurofilament (Braak et al., 1999; Spillantini et al., 1997). It has been proposed that this LB or synuclein pathology is capable of spreading from cell to cell in a prion-like manner throughout the brain resulting in cell toxicity (Goedert et al., 2013; Olanow & Brundin, 2013). The accumulation of these proteinaceous deposits, while a common feature of many neurodegenerative diseases, is evident in both genetic and sporadic Parkinson's disease patients with very little exception (Poulopoulos et al., 2012), and thus suggests a role for the involvement of synuclein pathology in the development and progression of the disease process itself (Luk & Lee, 2014).

Alongside the neurodegeneration, there is also ongoing inflammation in the brain of PD patients. This is mainly associated with the activation of microglial cells, which are the resident immune cells of the brain (McGeer et al., 1988; Orr et al., 2002; Vila et al., 2001). Microglia can influence the surrounding microenvironment via the release of immunoregulatory molecules (e.g. chemokines, cytokines, neurotrophic factors, and reactive oxygen species), morphological changes, increased motility, and phagocytotic activity (Kim & De Vellis, 2005; Neumann et al., 2009; Perry et al., 2010; Whitton, 2007). Nevertheless, it remains to be elucidated if inflammation is a causative factor of PD or a consequence of the degeneration of DA neurons, with many believing the existence of a self-propagating cycle of degeneration and inflammation (Tansey & Goldberg, 2010).

Current treatments

The current treatments available for PD focus on symptomatic relief by pharmacological or surgical treatment. Pharmacological options focus on increasing the levels of DA in the brain or modulating DA signalling. The first major breakthrough happened in the late 1960's when 3,4-dihydroxy-L-phenylalanine (L-DOPA) was shown to improve the motor symptoms in PD patients (Cotzias, 1968). However, although L-DOPA works efficiently at early stages of the disease, approximately 90% of patients suffer from side effects after 10 years of treatment (Ahlskog & Muenter, 2001). The most common and concerning side effect is the development of motor fluctuations and involuntary movements, or L-DOPA–induced dyskinesias, caused by the non-physiological release of DA and off-target effects (Thanvi & Lo, 2004). Despite the side-effects associated with L-DOPA, and its discovery more than 60 years ago, it remains the 'gold standard' pharmacological therapeutic option for the treatment of PD. In more recent years, other drugs have also become available such as dopamine agonists, monoamine oxidase type B (MAO-B) inhibitors, and catechol-O-methyltransferase (COMT) to treat motor symptoms of PD (Chen et al., 2007; Ferreira et al., 2008). These drugs can be used alone or in combination with L-DOPA to regulate motor fluctuations. However, physicians must be

careful of drug interactions and common side effects include dizziness, headaches, nausea, and compulsive behaviours such as gambling.

An alternative option is deep brain stimulation (DBS) surgery. In this procedure electrodes are surgically implanted into specific locations in the brain, usually the subthalamic nucleus (STN) or the internal segment of the globus pallidus (GPi), and can relieve some of the motor symptoms in patients (Benabid et al., 2009; Fang & Tolleson, 2017; Hartmann et al., 2019). However, this is a difficult and invasive surgery, and it is only able to treat a subset of the motor symptoms in PD (Lozano et al., 2019). Therefore, new and more effective treatments are urgently needed that have the ability to halt disease progression and/or regenerate or replace the DA neurons lost as part of the disease process.

Animal models of PD

In this section, I have focused on the most relevant models used for xenografting studies. The ideal model of PD should recapitulate all of the pathophysiological features of the disease including progressive DA degeneration, αSyn accumulation, and inflammation and should do so in a progressive but timely manner. It should also represent the clinical features of the disease including both motor and non-motor impairments. However, no model system is perfect and current models only recapitulate some of the characteristics of PD, therefore this must be taken into consideration prior to clinical translation.

Toxin models

Classic models of PD are based on delivery of neurotoxins that lead to rapid DA degeneration and motor impairments, e.g. 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

6-OHDA

This model was the first neurotoxin-based animal model of PD, which causes DA cell death via oxidative damage and mitochondrial dysfunction (Blum et al., 2001; Ungerstedt, 1968). This is most commonly used in rodents with a unilateral injection of 6-OHDA into the medial forebrain bundle (MFB), leaving the opposite hemisphere largely intact and spared of degeneration. This model is highly reproducible, causes robust DA neuron degeneration, and DA loss can easily be quantified via behavioural testing e.g. amphetamine rotations (Björklund & Dunnett, 2019; Ungerstedt & Arbuthnott, 1970). However, this neurodegeneration occurs rapidly, not progressively, and there is no evidence of synuclein pathology.

MPTP

MPTP was first discovered when a number of drug users were admitted to hospital after taking contaminated heroin in the early 1980's (Langston et al., 1983). These patients presented with Parkinsonian-like symptoms and it was later discovered that the heroin had been contaminated with MPTP.

This is a precursor to the neurotoxin MPP^+ which causes mitochondrial dysfunction and oxidative damage leading to DA cell death (Blum et al., 2001). This model is most commonly used in primates via systemic delivery of MPTP which affects both brain hemispheres. This model does not reflect the progressive time course of PD and despite claims that continuous infusion can instigate synuclein pathology (Fornai et al., 2005), this has been disputed in the field (Alvarez-Fischer et al., 2008).

Alpha-synuclein (αSyn) models

Since the discovery that mutations in the *SNCA* gene, encoding for αSyn, can cause PD (Chartier-Harlin et al., 2004; Krüger et al., 1998; Polymeropoulos, 1997; Singleton, 2003), researchers have tried to create model systems focusing on this protein. In addition, αSyn is a particularly interesting candidate due to it being the major component in LBs, the pathological aggregates observed in PD patient brains (Spillantini et al., 1997). αSyn is a small homologous protein of 140 amino acids with multiple well characterised sub-domains. αSyn in predominantly expressed in the CNS and although its function is not yet fully understood, it is believed to be involved in synaptic plasticity and synaptic transmission (Burré et al., 2010; Chandra et al., 2005; Murphy et al., 2000; Watson et al., 2009). αSyn is unfolded in its native state, however, under certain conditions and/or post-translational modifications, it has an increased tendency to change conformation or misfold and subsequently form aggregates which are associated with cytotoxicity (Conway et al., 1998; Fujiwara et al., 2002; Giasson et al., 1999). Phosphorylation at serine 129 (pSer129) is the most common post-translational modification and may exacerbate αSyn toxicity (Anderson et al., 2006; Fujiwara et al., 2002; Tenreiro et al., 2014).

Viral αSyn overexpression models

Viral vectors have been utilised to overexpress the human αSyn transgene in rodents by stereotactic injection. This leads to progressive loss of TH^+ neurons and a reduction of striatal DA innervation, as well as microglial activation (Decressac et al., 2012; Kirik et al., 2002; Lo Bianco et al., 2002; Sanchez-Guajardo et al., 2010). If more than 50% of the nigral DA neurons are lost it is also possible to observe motor impairments (Decressac et al., 2012; Kirik et al., 2002). However, these models are known for their inconsistency in degeneration and behavioural phenotypes and the often-prolonged timeframe in which these degenerative and behavioural characteristics occur. This could be due to a number of factors associated to vector design including viral serotype, the number of viable viral particles injected, and the efficiency of gene expression. Moreover, the expression levels of αSyn in these overexpression models is far above the physiological level that would be seen in a PD patient.

αSyn seeding and propagation models

These models are based on the discovery that short αSyn fibrils, also known as preformed fibrils (PFFs), can act as "seeds" for the formation of αSyn aggregates by recruiting endogenous monomeric αSyn and starting a pathogenic cascade (Luk et al., 2009; Volpicelli-Daley et al., 2011). Following this, later studies investigated this phenomenon by intracerebral injection of pathological forms of αSyn from different sources these included: brain lysates from patients with PD or other synucleinopathies (Recasens et al., 2014; Shimozawa et al., 2017); brain tissue from transgenic αSyn models with pathology (Mougenot et al., 2012); and αSyn PFFs (Duffy et al., 2018; Luk et al., 2012). These studies demonstrated that αSyn pathology can propagate over long distances within the brain

and lead to progressive DA degeneration and inflammation in both rodent and primate systems. The main advantage of these models is that they develop phosphorylated αSyn (pSyn) inclusions that closely resemble those observed in PD patients' brains. Moreover, this process is considerably more efficient if both synuclein strains are generated from the same species (Peelaerts et al., 2015; Thakur et al., 2017). However, these models can take long time to develop significant DA degeneration, and αSyn PFFs do not spread far from the injection site making injection accuracy crucial, especially in the larger brain of rats (Duffy et al., 2018; Paumier et al., 2015; Peelaerts et al., 2015). This led to the development of a new model system that combined both viral overexpression of human αSyn and the delivery of human αSyn PFFs. This viral overexpression provides a pool of αSyn to hasten the seeding and propagation process. This more closely recapitulates the pathophysiological hallmarks observed in human PD including progressive loss of DA neurons in the SN, pathological inclusions of pSyn, and activated microglia (Thakur et al., 2017). However, this model does not demonstrate reproducible behavioural impairments.

Cell replacement therapy

Cell replacement therapy is the concept of replacing degenerated cells with new healthy cells, via intracerebral transplantation, and is currently being investigated for a number of neurodegenerative diseases. PD is an ideal candidate due to the focal degeneration in the midbrain and the loss of one specific cell type, the DA neurons (Lindvall, 2016). Replacing DA neurons, rather than replacing DA, would release DA in a more physiological manner avoiding many of the side effects associated with current treatments. A vital step in the clinical translation of cell replacement therapy was the discovery that transplantation of human fetal ventral mesencephalic (VM) tissue into the 6-OHDA model of PD yielded surviving DA grafts which innervated the surrounding striatum and provided motor recovery (Brundin et al., 1986, 1988). This highlighted human fetal VM tissue as an appropriate cell source that could give rise to authentic DA neurons *in vivo* and inspired a new era of human clinical trials to assess cell replacement therapy.

Proof-of-concept and early trials

The first intrastriatal transplantations of human fetal VM tissue into PD patients began in Sweden in the late 1980's, to investigate whether these cells could survive in the brain and produce a measurable functional improvement (Lindvall et al., 1990). These first patients provided proof-of-principle for cell replacement therapy and lead to the initiation of many larger trials (Barker et al., 2013). Open-label studies showed that transplantation of human fetal VM tissue led to functional graft integration, restoration of DA levels in the striatum, and a marked clinical recovery (Brundin et al., 2000; Piccini et al., 1999, 2000), however, two double-blind trials in the United States failed to find significant improvements in patients (Freed et al., 2001; Olanow et al., 2003). This large variation in clinical outcome alongside the discovery of severe side-effects called graft-induced dyskinesias halted progress of cell replacement therapy for many years. However, there were multiple inconsistencies between the design of these trials including patient selection, tissue preparation, and the immunosuppressive regime have since been identified (Barker et al., 2013). European researchers continued to investigate

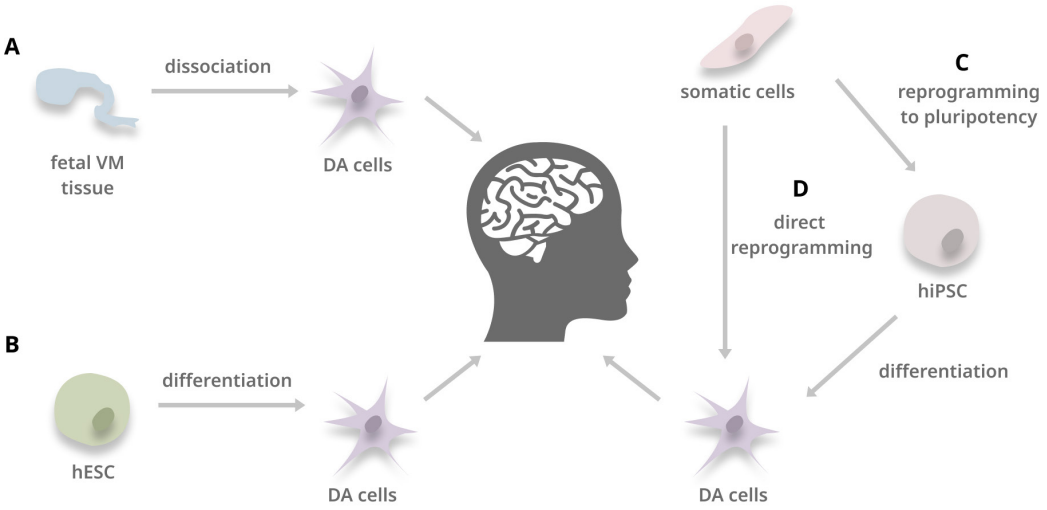


Fig. 1 – Potential cell sources to generate DA cells for cell replacement therapy in PD.

these factors in order to determine which significantly affected patient outcome, which lead to the initiation of a new trial, TRANSEURO, in 2012 (Barker, 2019). The results from the TRANSEURO trial will be reported during 2021. However, logistical issues including the limited availability of fetal VM tissue and lack of standardisation, and ethical concerns of using tissue from aborted fetuses, ultimately prevent this from ever being a competitive therapeutic strategy. Therefore, a new alternative source of DA cells for transplantation is urgently needed (for an overview of potential cell sources see Fig. 1).

Potential cell sources to generate authentic dopamine (DA) neurons

Embryonic stem cells

In 1998, a major breakthrough occurred in the field when the first human embryonic stem cell (hESC) was successfully derived from the inner cell mass of an early blastocyst (Thomson, 1998). This opened up the possibility to study human development and disease, as well as presenting a potential new cell source for cell replacement therapy. hESCs can be identified by the expression of key factors such as Oct4, NANOG, and Sox2, which maintain their pluripotent state (Boyer et al., 2005; Nichols et al., 1998). They can be maintained and expanded *in vitro* and have the potential to form any cell type of the three germ layers (endoderm, ectoderm & mesoderm). However, there was still much to learn about which signals hESCs needed to receive in order to differentiate into a specific cell lineage.

DA neuron differentiation

In the first attempts to differentiate these cells into DA neurons, hESCs had to be sub-cultured on another cell type, e.g. stromal feeder cells or astrocytes. Signalling factors such as SHH and FGF8b were used to try to instruct the fate of a cell towards a DA phenotype (Park et al., 2005; Perrier et al., 2004; Zeng, 2004). The resulting cells could survive intrastriatal transplantation into a rat model of PD, however, only a very low number of TH^+ cells could be found upon post-mortem histological examination. A major concern was that some of the transplanted cells remained undifferentiated giving them the potential for tumorigenesis (Roy et al., 2006; Sonntag et al., 2007). Nevertheless, these early studies showed proof-of-principle for survival of hESC-derived DA neurons in the rodent brain.

When the discovery was made that midbrain DA neurons originate from the floor-plate of the developing neural tube, and not the neuroepithelium as previously believed (Bonilla et al., 2008; Ono et al., 2007), differentiation protocols were refined and subsequently were able to more closely recapitulate human DA neuron development *in vitro* (Fasano et al., 2010). Simultaneously, a new dual SMAD inhibition protocol was published, showing that inhibiting both BMP and TGFß signalling would give rise to a highly enriched population of neural progenitor cells (Chambers et al., 2009). Moreover, it was found that GSK3 inhibition could instruct the cells to the appropriate anterior/posterior fate, while the addition of SHH could ventralise the cells (Fasano et al., 2010; Kirkeby et al., 2012; Kriks et al., 2011). With these new protocols in place, it was now possible to generate human DA neurons via a floor-plate progenitor with high efficiency. When these improved protocols were used to generate DA progenitor cells for transplantation into animal models of PD they showed improved survival, expression of markers of authentic DA neurons, graft connectivity and DA release, and functional recovery in animal models of PD (Grealish et al., 2014; Kriks et al., 2011; Nolbrant et al., 2017).

Reprogrammed cells

Cell differentiation has long been considered a one-way process in which a cell goes from an undifferentiated or pluripotent state to a specialised or physiologically mature state (Waddington, 1957). This dogma was first challenged in experiments whereby the somatic cell nucleus was transferred into an enucleated oocyte in frogs (Gurdon et al., 1958). This was the first evidence that mature cells can be reprogrammed back to an undifferentiated state and become pluripotent. It was many years before this was investigated in mammalian cells, which lead to the successful cloning of a sheep named Dolly (Campbell et al., 1996; Wilmut et al., 1997). These pioneering studies suggested that there are certain reprogramming factors in the oocyte which have the ability to erase the memory of a somatic cell and rejuvenate it to a pluripotent stem cell. However, the factors which enable cell reprogramming in mature cells remained largely elusive until Takahashi and Yamanaka's discovery in 2006.

Induced pluripotent stem cells

Drawing inspiration from the factors which are known to maintain pluripotency in hESCs, the pioneering work of Takahashi & Yamanaka identified four key transcription factors for cellular reprogramming, *Oct4, Sox2, c-Myc,* and *Klf4* (Takahashi & Yamanaka, 2006). Results showed that viral delivery of these factors is sufficient to reprogram mice fibroblasts into induced pluripotent stem cells. The following year this was also shown to be possible with human cells (Takahashi et al., 2007).

Human induced pluripotent stem cells (hiPSCs) are rejuvenated in a manner that erases the ageing signature of the somatic cell so that the cell no longer retains the vulnerabilities associated to ageing (Lapasset et al., 2011; Maherali et al., 2007; Mertens et al., 2015; Miller et al., 2013). To date, the reprogramming process to hiPSCs is slow and inefficient, however, after induction you can have indefinite clonal expansion of the line. hiPSCs have great potential as cells can be obtained from adults avoiding many of the ethical concerns of hESC-derived therapies. Moreover, this allows for the generation of patient-specific cells which could overcome problems with graft rejection and remove the need for immunosuppressive drugs in cell replacement therapies (Emborg et al., 2013; Hallett et al., 2015; Morizane et al., 2013, 2017).

hiPSCs respond to patterning cues in a similar way to hESCs, therefore they can be differentiated using the same protocols for DA neuron generation. Studies of transplantation of hiPSC-derived DA neurons into both rodent and primate models of PD have shown that the cells functionally integrate and give rise to motor recovery (Doi et al., 2014; Kikuchi, Morizane, Doi, Magotani, et al., 2017; Kikuchi, Morizane, Doi, Okita, et al., 2017; Rhee et al., 2011; Swistowski et al., 2010).

Induced neurons

After the pivotal discovery of hiPSCs, scientists began to wonder if it was possible to reprogram directly from one mature cell type to another. Direct reprogramming, also known as transdifferentiation, is the conversion of one somatic cell fate to another specific cell fate without passing through a pluripotent intermediate state. This process requires a transition between epigenetic states and can be mediated by delivery of specific transcription factors, microRNAs, and/or small molecules. In direct reprogramming, as opposed to reprogramming to pluripotency, the cells have been shown to retain many important aspects of the somatic starting cell, including vulnerabilities associated to ageing, e.g. the transcriptome, DNA damage, and telomere length (Drouin-Ouellet et al., 2017; Huh et al., 2016; Kim et al., 2018; Mertens et al., 2015). This may have potential implications for cell replacement therapy if these vulnerabilities associated to ageing could also alter their susceptibility to pathological changes, thereby causing damage and degeneration to the transplanted cells.

This concept was first demonstrated by the conversion of fibroblasts to myoblasts with the overexpression of the transcription factor MyoD (Davis et al., 1987). However, direct reprogramming to a neuronal lineage was not reported until much later. The first study screened a library of transcription factors and identified 3 key transcription factors, *Ascl1, Brn2,* and *Myt1l*, which could directly reprogram mouse postnatal fibroblasts into induced neurons (iNs) (Vierbuchen et al., 2010). These same three factors could also generate functional iNs from human fetal fibroblasts (Pang et al., 2011; Pfisterer, Kirkeby, et al., 2011).

Using these first combinations, iNs were often a mixed population of subtypes, primarily GABAergic and glutamatergic neurons. Therefore, other transcription factors needed to be identified that could direct the cells towards a DA phenotype. The first study to report the generation of induced DA neurons (iDANs), used transcription factors known to play an important role in midbrain DA development, including *Ascl1, Nurr1, Lmx1a, FoxA2* and *Brn2* (Pfisterer, Kirkeby, et al., 2011). Other studies have also included DA fate determinants such as *Ngn2, Sox2, Nurr1,* and *Pitx3* (Addis et al., 2011; Caiazzo et al., 2011; Dell'Anno et al., 2014; Kim et al., 2011; Liu et al., 2012). However, direct reprogramming is quite inefficient for generation of DA neurons, with an approximate 5-10% con-

version efficiency rate for fetal fibroblasts, and an even lower rate for adult fibroblasts. Moreover, in contrast to hiPSC reprogramming, direct conversion does not allow for further expansion of the cells after conversion although a certain degree of expansion is possible at the fibroblast phase.

More recent studies have focused on trying to improve conversion efficiency by the addition of small molecules to the conversion media (Ladewig et al., 2012; Rivetti di Val Cervo et al., 2017) and, for adult cells in particular, the inhibition of the REST protein (Drouin-Ouellet et al., 2017; Masserdotti et al., 2015). REST (also known as NRSF) is a protein that can bind to the DNA and act as a neuronal repressor, which is highly expressed in the human brain as it ages (Ballas et al., 2005; Lu et al., 2014), therefore it was proposed that this protein could act as a roadblock to prevent adult cells from reprogramming.

Human iNs and iDANs survive extremely poorly following intracerebral transplantation (Caiazzo et al., 2011; Dell'Anno et al., 2014; Pereira et al., 2014), making it difficult to study their function or assess their therapeutic potential. Therefore, further improvement is needed both to improve conversion protocols, specifically in order to increase the DA yield and to reprogram cells from adult donors, as well as optimising the transplantation process.

Patient-specific cell replacement therapy

Preclinical studies using DA neurons derived from pluripotent stem cells have shown promising results and have led to the initiation of several new clinical trials across Asia, the United States, and Europe (Barker, 2019; Doi et al., 2020; Kirkeby et al., 2017; Studer, 2017). All of these trials have been designed to transplant patients with DA progenitors derived from healthy donors (with no known disease background) and patients will receive 1 year of immunosuppression. The dawn of cellular reprogramming has opened up the possibility to use a patient's own cells (autologous) or cells from an immunologically-matched donor (allogeneic) for cell replacement therapy (see Fig. 2) (Osborn et al., 2020; Parmar, 2018; Takahashi, 2017). The main advantage of an autologous approach is that it would significantly reduce the risk of graft rejection and remove the need for patient immunosuppression (Emborg et al., 2013; Hallett et al., 2015; Morizane et al., 2013, 2017). This is particularly important given that immunosuppression may not be feasible for elderly or immune-compromised patients and withdrawal of immunosuppression could compromise long-term graft survival (Barker et al., 2013; Piquet et al., 2012).

At present, there have been very few preclinical studies investigating the long-term integrity and function of grafts derived from patients with PD. Moreover, it remains unclear if patient-derived DA neurons will be more vulnerable to dysfunction if they have disease-specific backgrounds, or whether using cells from an immunologically matched donor may be more appropriate.

Preclinical studies of patient-derived cells in models of PD

Transplantation of hiPSC-derived DA neurons from sporadic PD patients, has shown that these cells survive and form large DA-rich grafts, capable of producing functional recovery in both rodent

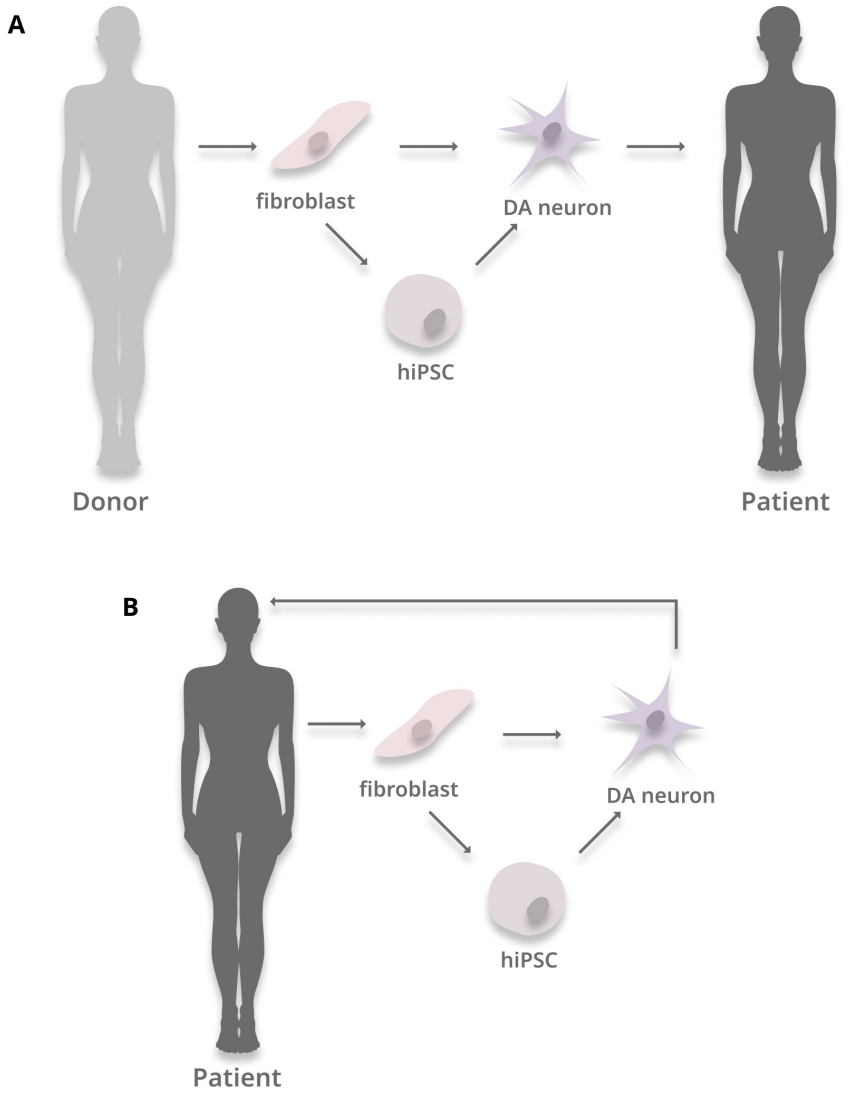


Fig. 2 – Schematic of (A) allogenic grafting strategies and (B) autologous grafting strategies for cell replacement therapy in PD.

and primate models of PD (Hargus et al., 2010; Kikuchi, Morizane, Doi, Magotani, et al., 2017; Kikuchi, Morizane, Doi, Okita, et al., 2017; Song et al., 2020). These grafts behaved on par with grafts derived from healthy donors and did not display any evidence of synuclein pathology. In 2020, the first human patient with PD to receive an autologous hiPSC graft, in a compassionate use treatment was reported (Schweitzer et al., 2020). The patient was a 69-year-old male with a 10-year history of

progressive sporadic PD without dyskinesias. The patient received a bilateral transplant of DA progenitors without any immunosuppressive treatment. Early results seem positive in that no adverse events were reported; however, the functional efficacy is unclear and long-term follow-ups are needed.

The majority of these studies have focused on toxin based models and only one has examined the perspective of transplanting into an αSyn model of PD (Kikuchi, Morizane, Doi, Okita, et al., 2017). This may have significant consequences for patients as cells will be transplanted into a brain with ongoing pathological processes. Therefore, assessments in animal models with relevant synuclein pathology is urgently needed to assess the suitability of autologous cell therapy for PD.

Host-to-graft transfer of pathology

Long-term studies evaluating transplantation of human fetal VM tissue have shown surviving and functional grafts up to 24 years post-transplantation (Barker et al., 2013; Kefalopoulou et al., 2014; Li et al., 2016). However, this brought to light the presence of LB or synuclein pathology within some of the transplanted cells (Kordower et al. 2008; Li et al. 2008, 2010; Li et al. 2016). This unexpected finding suggests that the host brain can impact the graft, and that this pathology can possibly spread from the host brain to the grafted neurons via a prion-like mechanism (host-to-graft) (Brundin & Kordower, 2012; Olanow & Brundin, 2013). On the other hand, the percentage of neurons containing αSyn pathology reported was low (2-12%) (Li et al. 2010; Li et al. 2016), did not affect graft function, and has not been observed in all patients (Hallett et al., 2014; Mendez et al., 2008).

Advocates for autologous cell replacement therapy argue that the patients' resident DA cells do not become dysfunctional and exhibit pathology for around six decades in the majority of cases (Osborn et al., 2020) and therefore patient-derived cells should be functional for the same amount of time post-transplantation. However, a number of studies have indicated that this may not the case (Kordower et al., 2008; Kurowska et al., 2011; Li et al., 2016). In fact, pathophysiological processes may be accelerated when cells are grafted into an already diseased environment in which degenerative changes have been ongoing for decades.

In the first wave of stem cell-based trials, where cells from healthy donors are used (allogenic strategy), pathology in the grafted cells is not a major concern. Although some patients with human fetal VM grafts have shown pathological inclusions, they have also shown clinical benefit for 18 years in some cases. This would significantly improve the quality of life for many PD patients and remains a competitive therapeutic strategy. However, the potential host-to-graft transfer of pathology will be a major concern for autologous cell replacement therapy using patient-derived cells that may inherently be more vulnerable to the disease. Therefore, detailed *in vivo* studies are needed to understand the process of pathology transfer and identify possible cellular characteristics which may be unfavourable for use in cell replacement therapy.

AIMS OF THE THESIS

The general aim of this thesis has been to evaluate patient-derived cells as a source for transplantation in cell replacement therapy for PD. The specific aims of my thesis were to:

1. Develop protocols for direct reprogramming of human adult fibroblasts into induced neurons (Paper I)

2. Explore the possibility to directly reprogram healthy and patient-derived cells specifically towards a DA phenotype (Paper II)

3. Develop a new humanized αSyn xenograft rat model of PD where the impact of host pathology on grafted cells can be assessed (Paper III)

4. Assess if patient-derived DA neurons can survive intracerebral transplantation and function on par with stem cell-derived neurons (Paper IV)

5. Investigate if patient-derived DA neurons display pathological features in rodent models of PD (Paper IV and Paper V)

SUMMARY OF RESULTS & DISCUSSION

In **paper I**, a protocol to directly convert human adult fibroblasts into iNs *in vitro* with high efficiency was developed. This protocol results in mixed neuronal population with different identities. For more targeted PD studies, we therefore adapted this protocol in **paper II**, in order to generate subtype-specific iDANs from both healthy and PD donors. In **paper III**, a new xenograft model was established to assess the impact of host pathology in a disease relevant animal model of PD. In **paper IV**, a hiPSC line derived from a familial PD patient was transplanted into the 6-OHDA model of PD to assess survival, functionality, and if pathology develops over time. In order to assess how patient-derived cells would survive long-term after transplantation into a pathological environment, that same line was then transplanted in our newly developed SynFib model in **paper V**.

A protocol for efficient reprogramming of adult human fibroblasts into induced neurons (iNs) (Paper I)

Direct reprogramming of human adult cells into iNs is of particular interest for patient-specific cell therapy. However, most existing protocols have focused on the conversion of fetal cells and very low conversion efficiencies have been reported when these protocols were applied to cells from adult donors (Caiazzo et al., 2011; Liu et al., 2013; Pfisterer, Wood, et al., 2011). Since direct reprogramming yields post-mitotic cells that can't be further expanded in culture, generation of a sufficient number of iNs largely depends on the expansion of the initial fibroblasts and the subsequent conversion efficiency. In this study, we have developed a protocol using a single lentiviral vector (LV) system expressing *Ascl1* and *Brn2* under the human PGK promoter, and shRNA to inhibit REST to efficiently reprogram human adult fibroblasts into iNs (Fig. 3A).

A single-vector system with REST inhibition gives a high yield of iNs in 25 days *in vitro*

Typically, if multiple factors are used in direct reprogramming, these are delivered by separate LVs. However, given that cell transduction of is not 100% efficient, and that each fibroblast must be transduced by multiple LVs in order to be successfully reprogrammed, a single vector system eliminates the need for co-infection and thereby increases conversion efficiency (Herdy et al., 2019). In addition, the REST protein has been shown to act as a reprogramming block in adult cells; therefore, inhibiting this protein allows for the expression of epigenetically silenced pro-neural genes (Ballas et al., 2005), thus dramatically increasing neural conversion of adult cells.

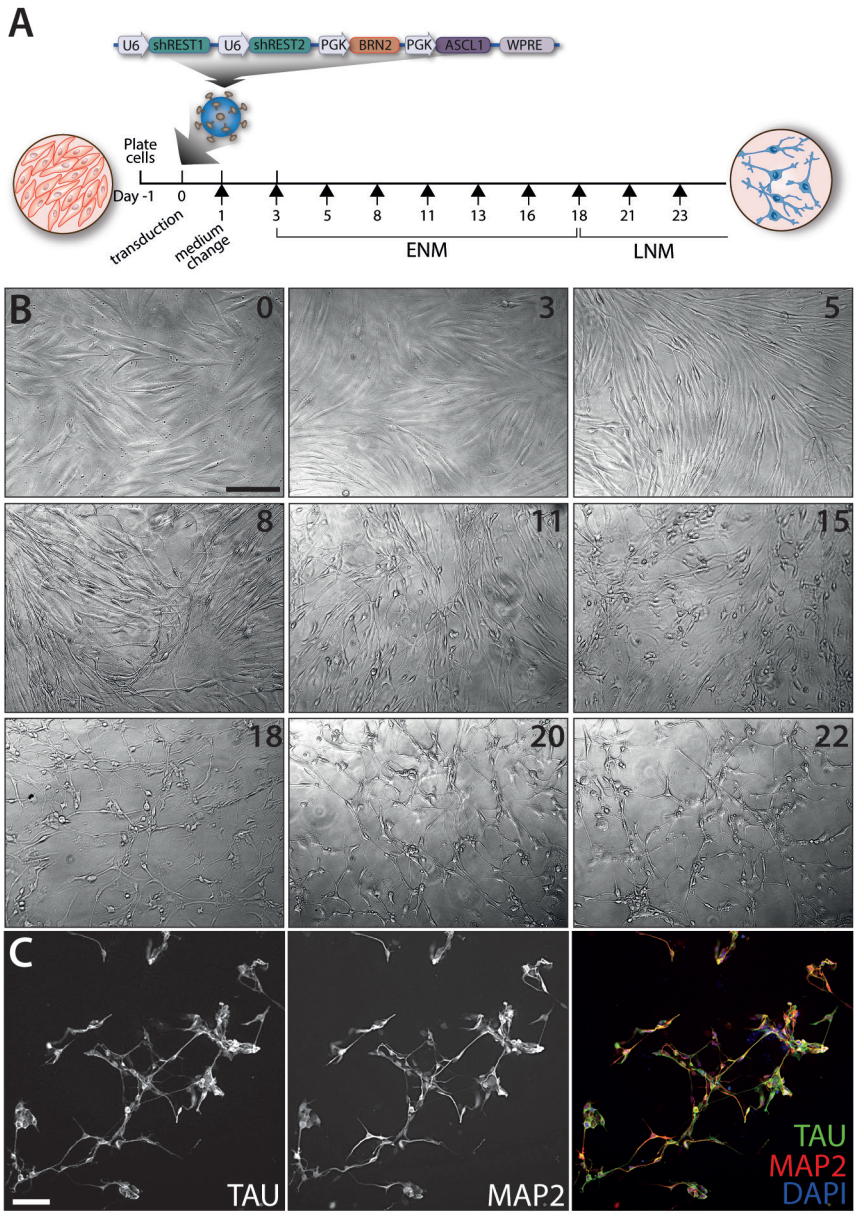


Fig. 3 - Conversion of induced neurons (iNs). (A) Schematic of the lentiviral construct and direct reprogramming protocol. (B) Representative images taken on a brightfield microscope to show morphological changes between day 0 and day 22. (C) Representative images of iNs at day 35 post-transduction. Immunofluorescence of neuronal markers TAU (green) and MAP2 (red), as well as DAPI (blue). Scale bars 100µm. Abbreviations; ENM: early neuronal medium; LNM: late neuronal medium.

Small molecules have previously been shown to increase conversion efficiency (Ladewig et al., 2012; Rivetti di Val Cervo et al., 2017). In this protocol we have used small molecules in addition to LV delivery, taking inspiration from factors known to play a role in midbrain DA neuron differentiation. We have applied a dual SMAD inhibition approach (SB, LDN & Noggin) to enrich for neuronal cells, as well as GSK3b inhibition (CHIR99021). Other factors to promote neuron differentiation, survival, and maturation including cAMP activation (db-cAMP), chromatin remodelling agents (VPA), and growth factors including glial cell line-derived neurotrophic factor (GDNF), a brain-derived neurotrophic factor (BDNF) mimetic (LM22A4), and neurotrophin-3 (NT3) were applied during this conversion protocol (Gascón et al., 2016; Hu et al., 2015; Huangfu et al., 2008; Ladewig et al., 2012; Liu et al., 2013).

When this protocol was applied to human adult fibroblasts, we observed a clear change in cell morphology after just 5 days *in vitro*. The cells continued to develop mature neuronal morphology over 25 days (Fig. 3B). In addition to this, immunocytochemistry showed that approximately 50% of cells converted to iNs as identified by the expression of the neuronal markers MAP2 and TAU (Fig. 3C). This one-step reprogramming method demonstrates a standardised and efficient protocol for use on human adult fibroblasts, providing the opportunity for a wide range of studies in a patient-specific system.

Generating subtype-specific induced dopaminergic neurons (iDANS) via direct reprogramming of human adult skin fibroblasts from healthy donors and PD patients (Paper II)

Next, we sought to generate induced DA neurons (iDANs) as this is the subtype specifically implicated in PD. Previous studies have reported successful reprogramming of mouse and human fetal fibroblasts, as well as astrocytes, by delivering 'pioneer' factors for chromatin remodelling and neuronal maturation (e.g. *Ascl1, Brn2, Myt1l, Ngn2*), and adding specific factors known to play a role in DA neuron patterning (*Lmx1a, Foxa2, Ngn2, Sox2, Nurr1,* and *Pitx3*) (Addis et al., 2011; Caiazzo et al., 2011; Dell'Anno et al., 2014; Kim et al., 2011; Liu et al., 2012; Pfisterer, Kirkeby, et al., 2011). However, successful reprogramming of adult human dermal fibroblasts into iDANs has only been reported at low efficiency (Caiazzo et al., 2011). In this study, our focus was to efficiently generate iDANs from adult cells and to assess whether patient-derived cells could convert as efficiently as cells derived from healthy donors.

A combination of DA specific factors and REST inhibition dramatically increase iDAN conversion

We wanted to investigate the factors controlling conversion to iDANs by using different combinations of transcription factors known to influence DA cell fate. We screened multiple combinations of 10 different factors including: *Ascl1, Lmx1a, Lmx1b, FoxA2, Otx2, Nurr1, Smarca1, CNPY1, EN1,* and *PAX8*. These factors were selected based on their expression in midbrain DA neurons, role in

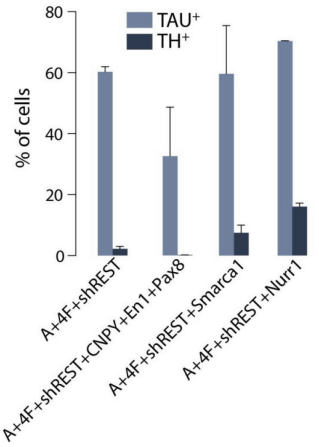


Fig. 4 - Quantification of TAU$^+$ and TH$^+$ cells after conversion using different combinations of reprogramming factors. Abbreviations; A: *Ascl1*; 4F: *Lmx1a, Lmx1b, FoxA2*, and *Otx2*.

DA neuron differentiation, or chromatin remodelling abilities. In all of the combinations we also used REST inhibition according to the published protocol in paper I for highly efficient reprograming of adult fibroblasts. The five best combinations to generate both the highest yield of iNs (TAU$^+$ cells) and highest proportion of iDANs (TH$^+$ cells) are shown in Fig. 4. The combination which generated the best yield of iDANs was found using the combination *Ascl1* + 4F *(Lmx1a, Lmx1b, FoxA2, Otx2)* + *Nurr1* + shREST. This gave rise to approximately 70% neuronal cells, of which 16% also expressed the DA marker TH.

Characterisation of iDANs shows they express key DA makers and are electrophysiologically functional *in vitro*

All subsequent analysis was performed on iDANs converted using the *Ascl1* + 4F + *Nurr1* + shREST combination (schematic in Fig. 5A). Gene expression analysis showed that a number of key DA markers were upregulated in the converted cells including genes related to DA patterning including *TH, Pitx3,* and *GIRK2*, as well as genes related to DA synaptic function including *DRD2, DAT*, and *AADC* (Fig. 5B). Immunocytochemistry also demonstrated that these iDANs co-expressed markers such as VMAT2 and ALDH1A1 (Fig. 5C). In agreement with the gene expression, patch-clamp electrophysiology confirmed that these cells were functionally mature (Fig. 5D,E).

Conversion to iDANs is equally efficient with fibroblasts derived from healthy donors and sporadic PD patients

In order to assess whether healthy and patient-derived cells could be converted with equal efficiency, we reprogrammed dermal fibroblasts using the new iDAN reprogramming method above and analysed the cells *in vitro*. We found a similar conversion efficiency for both healthy and sporadic PD

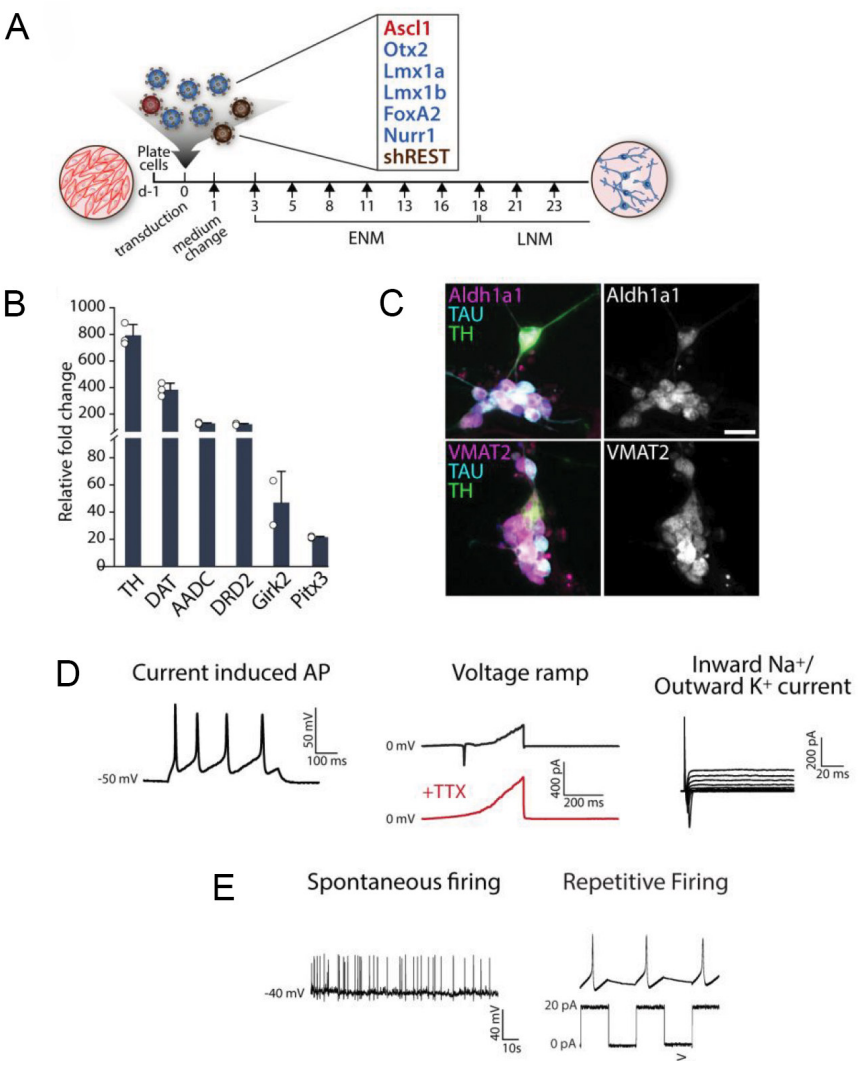


Fig. 5 – Successful generation of iDANs from human adult fibroblasts. (A) Schematic of the lentiviral construct and direct reprogramming protocol. (B) Quantification of gene expression relative to levels in unconverted fibroblasts, white circle indicating replicates. (C) Immunofluorescence of TAU$^+$ (cyan) and TH$^+$ (green) iDANs co-expressing the DA markers ALDH1A1 and VMAT2 (magenta), scale bar 25µm. (D+E) Electrophysiological recordings of iDANs at day 65. Abbreviations; ENM: early neuronal medium; LNM: late neuronal medium; AP: action potential, TTX: tetrodotoxin.

patient lines, as demonstrated by the number of TAU$^+$ and TH$^+$ cells (3 replicates for each line; Fig. 6A-C). They showed a comparable morphological profile, with no significant differences found in neurite width, branch points, neurite count, or neurite length (Fig. 6D). Moreover, they had a similar functional profile using electrophysiological assessment.

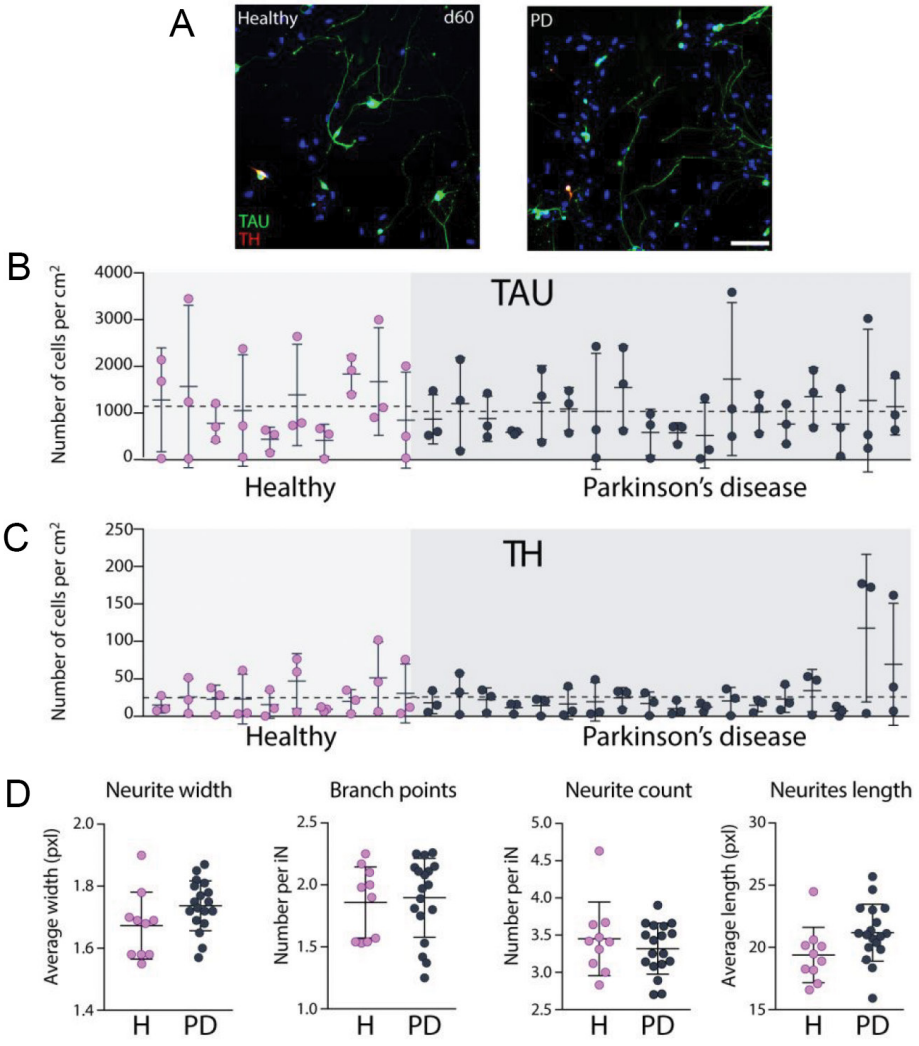


Fig. 6 - Generation of iDANs from heathy donor and sporadic PD patient lines. (A) Immunofluorescence staining showing double TAU$^+$ (green) and TH$^+$ (red) iDANs at day 60, scale bar 100µm. Quantification of (B) TAU$^+$ cells and (C) TH$^+$ cells from each line, each circle indicates replicates and dashed lines represent the mean. (D) Quantification of the neurite profile in TAU$^+$ cells from heathy donor (H) and sporadic PD patient lines (PD).

iDANs from sporadic PD patients show age- and disease-related pathological impairments

Autophagy is the degradation of cytoplasmic organelles and proteins within lysosomes and is important to maintain homeostasis of the cell (Mizushima, 2007). Autophagy is most commonly triggered by cell starvation, including conditions such as nutrient deprivation and low oxygen levels (Banerjee et al., 2010; Lynch-day et al., 2012). Studies have shown that this process becomes less ef-

ficient with age (Rubinsztein et al., 2011) and impairments in autophagy can lead to accumulation of misfolded proteins, such as pSyn as observed in Parkinson's disease (Banerjee et al., 2010).

In this study, we investigated 18 sporadic PD patient and 10 healthy donor cell lines. We assessed the cells at baseline or induced autophagy by starving the cells for 4 hours (culture medium was replaced with HBSS and $Ca2^{+}/Mg2^{+}$). We found that iDANs from sporadic PD patients have an altered autophagic response, both and baseline and following starvation. Moreover, gene expression analysis highlighted significant changes to genes linked to lysosomal function and early autophagic processes in iNs generated from sporadic PD patients when compared to healthy donors.

Next, we combined cell starvation with an agent to block autophagic flux (Bafilomycin A1), in order to examine if this would lead to development of pathological protein inclusions, such as pSyn. While this did not induce any significant changes in iNs from healthy donors, it did increase the number of cytoplasmic pSyn ($pSer129^{+}$) inclusions in iNs from sporadic PD patients. Interestingly, we did not observe the same phenotype in neurons derived from hiPSCs from a subset of the same patient cell lines. This suggests that iN reprogramming may lead to specific impairments which could be due to the fact that they retain characteristics associated to ageing (Drouin-Ouellet et al., 2017; Huh et al., 2016; Kim et al., 2018; Mertens et al., 2015) whereas hiPSCs are rejuvenated in a manner that erases the ageing signature of the somatic cell (Lapasset et al., 2011; Maherali et al., 2007; Mertens et al., 2015; Miller et al., 2013).

Establishing a new humanized αSyn xenograft rat model of PD to assess the impact of host pathology on grafted cells (Paper III)

In order to assess the impact of pathology on grafted cells, we first needed to establish a xenograft model of PD that could closely mimic the synuclein pathology observed in PD patient's brains. Thakur et al. (2017) previously developed a rodent model of PD using viral overexpression of human αSyn followed by the delivery of human αSyn PFFs into the SN. This triggered DA neuron death, inflammation and pathological inclusions of pSyn. However, delivering these components at separate times requires separate surgeries, which is more time-consuming and could also increase variability in the model considering that PFFs require precise injection accuracy. Therefore, to allow for more efficient seeding of pathology we have combined AAV overexpression of human αSyn (which does not cause significant degenerative effects alone) simultaneously injected with human αSyn PFFs in the SN, termed the 'SynFib' model.

The SynFib model shows behavioural impairments and progressive DA neuron cell death

When establishing the model, animals were tested in a battery of behavioural tests, including the stepping test, cylinder test, and amphetamine rotation test, in order to assess the degree of motor impairment. Approximately half of the SynFib animals showed impairments in all of the tests at 4 weeks post-injection (purple bars in Fig. 7A,B,C). These impairments were sustained at 16 weeks post-injection, signifying a stable motor deficit in the SynFib animals which was not observed in the AAV-αSyn alone group (red bars in Fig. 7A,B,C).

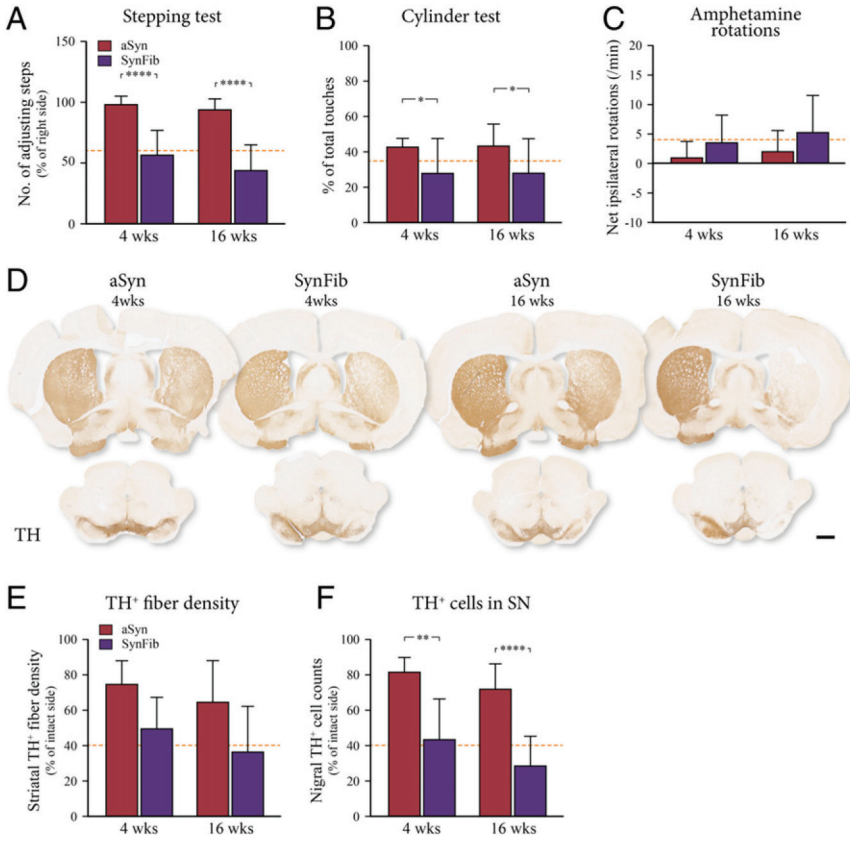
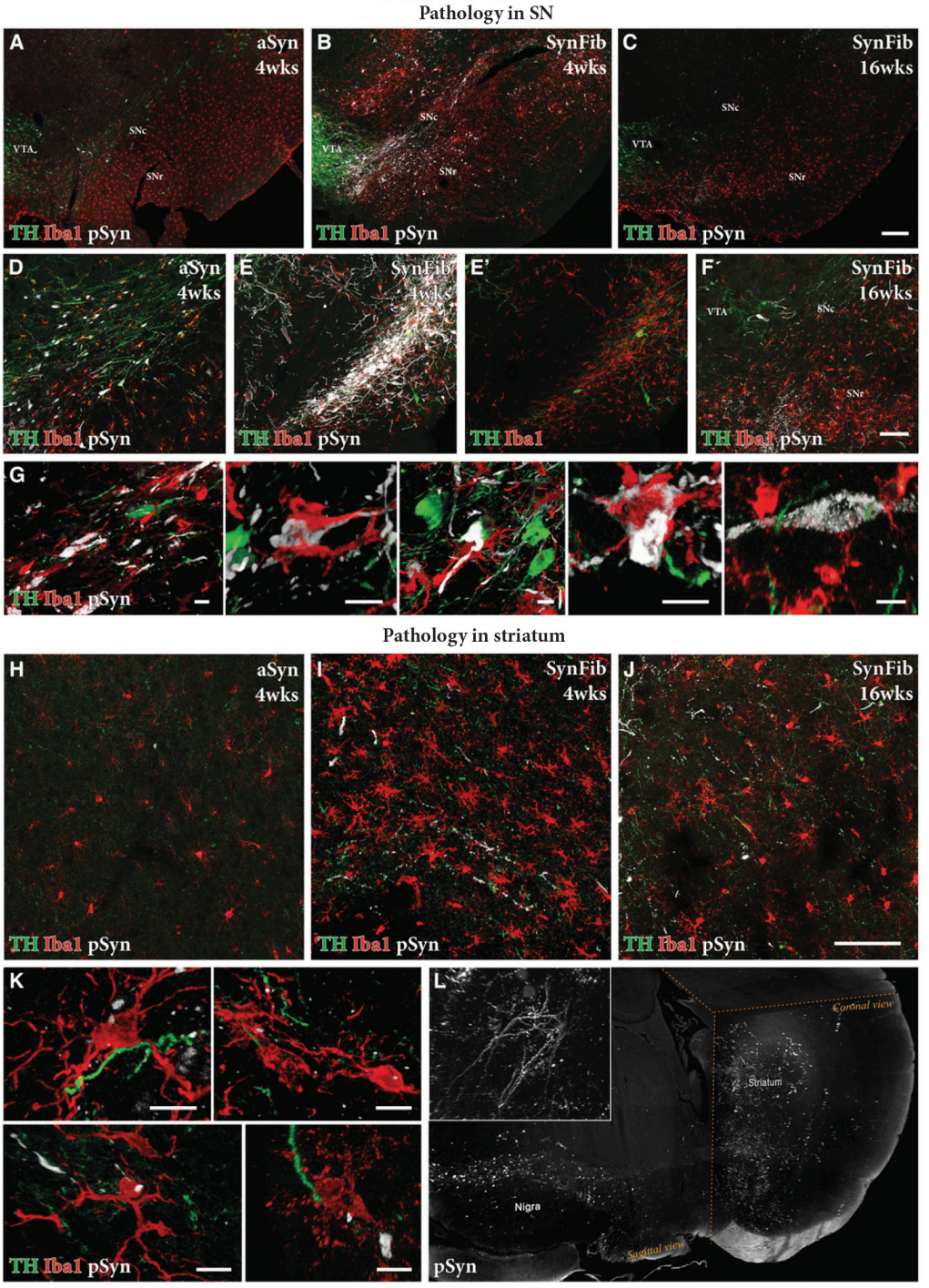


Fig. 7 - Assessment of DA degeneration in AAV-αSyn alone (αSyn) and SynFib animals at 4- and 16-weeks post-injection. Behavioural impairments assessed using (A) the stepping test, (B) cylinder test, and (C) amphetamine rotations. (D) DAB staining for TH showing representative sections of the striatum and SN, scale bar 1 mm. Quantification of (E) TH^+ fiber density in the striatum and (F) TH^+ cells in the SN expressed as a percentage of the contralateral side.

Moreover, animals in the SynFib group displayed a significant loss of TH^+ neurons in the SN on the injected hemisphere, combined with a reduction of TH^+ striatal fibers (Fig. 7D,E) which progressed over time. The DA loss was much greater in the SynFib group compared to the AAV-αSyn alone group, demonstrating the additive degenerative effect of simultaneous injection of αSyn AAV and PFFs.

Fig. 8 – Assessment of the pathological environment in AAV-αSyn alone (αSyn) and SynFib animals at 4- and 16-weeks post-injection. (A-F) Representative images showing pSyn pathology (white), $IBA1^+$ microglia (red), and TH^+ neurons (green) in the substantia nigra (SN). (G) Examples of microglia surrounding and engulfing cells containing $pSyn^+$ inclusions in the SN. (H-J) Representative images of the same staining in the striatum, with (K) microglia containing $pSyn^+$ inclusions. (L) An iDISCO cleared brain showing the dense network of $pSyn^+$ pathology in SynFib animals. Abbreviations: SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area. (Scale bars, 200 μm [A–C], 100 μm [D–F and H–J], and 10 μm [G and K].)


Pathology in SN
A
aSyn
4wks
SNc
VTA
SNr
TH Iba1 pSyn
B
SynFib
4wks
SNc
VTA
SNr
TH Iba1 pSyn
C
SynFib
16wks
SNc
VTA
SNr
TH Iba1 pSyn
D
aSyn
4wks
TH Iba1 pSyn
E
SynFib
4wks
TH Iba1 pSyn
E'
TH Iba1
F
SynFib
16wks
VTA
SNc
SNr
TH Iba1 pSyn
G
TH Iba1 pSyn
Pathology in striatum
H
aSyn
4wks
TH Iba1 pSyn
I
SynFib
4wks
TH Iba1 pSyn
J
SynFib
16wks
TH Iba1 pSyn
K
TH Iba1 pSyn
L
Coronal view
Striatum
Nigra
Sagittal view
pSyn

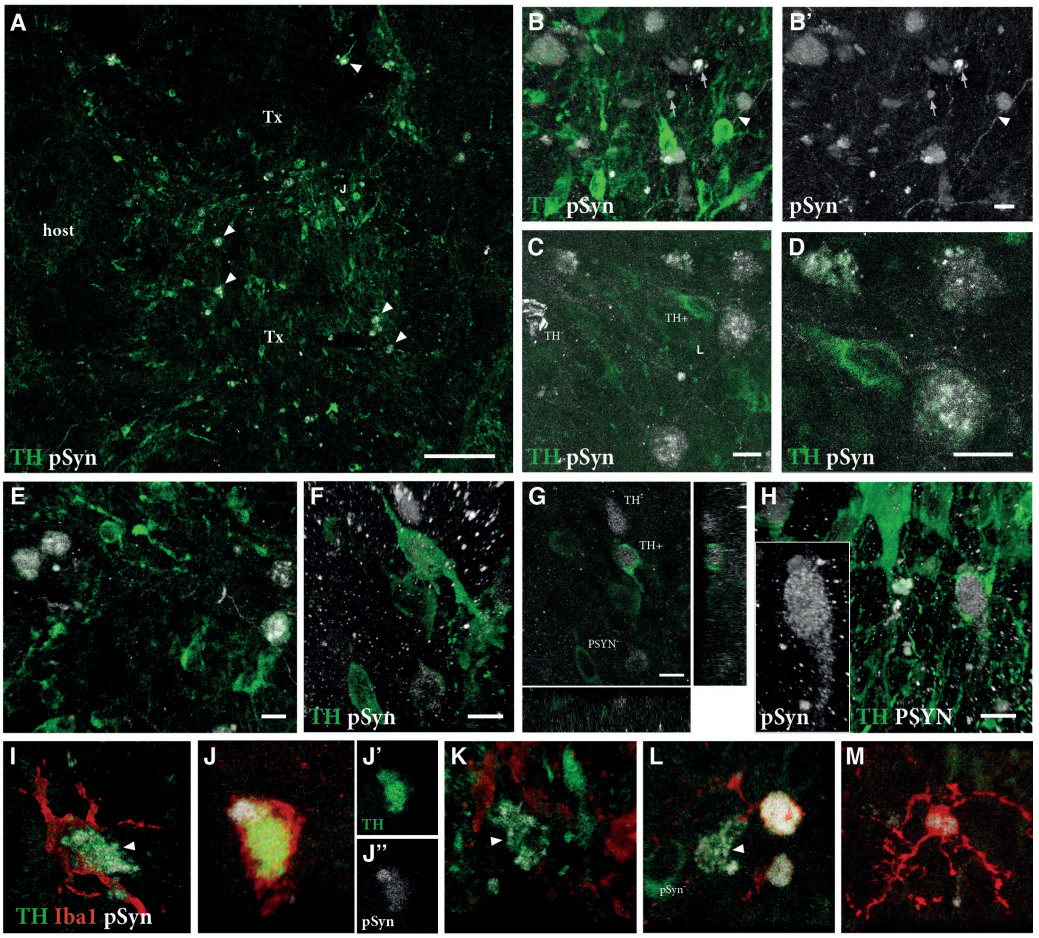


Fig. 9 – Assessment of environment in the grafted striatum. (A-H) Shows immunofluorescent labelling of TH (green) and pSyn (white) with arrowheads in indicating double-positive cells. (B and B') arrowheads showing $pSyn^+$ deposits in (mostly TH^-) fibers, and arrows showing extracellular $pSyn^+$ deposits. (C-H) Double staining of TH and pSyn within grafted neurons and confirmed with an orthogonal view in (G). (I-M) Triple staining of TH (green), IBA1 (red), and pSyn (white) showing the colocalization of activated microglia around $TH^+/pSyn^+$ cells in the graft core. Scale bars, A 100μm and B-M 10μm.

Extensive proteinopathy and inflammation in the SynFib model

To perform a detailed characterisation of pathology in the model we stained for pSyn, the pathological form of αSyn which is the main component of Lewy bodies. Minimal pSyn pathology was found in the SN and the striatum in the AAV-αSyn alone group (Fig. 8A,D,H), in stark contrast to the extensive pSyn pathology found in the SynFib animals already at 4 weeks post-injection (Fig. 8B,D,I). Moreover, the large majority of $pSyn^+$ cells in the SN at this timepoint were TH^- (Fig. 8E'), indicative of TH down-regulation. At 16 weeks post-injection, there was a reduction in the number of neurons expressing pSyn pathology and almost complete loss of TH^+ neurons in the SN (Fig. 8C,F) indicating

that the affected neurons have died (Fig. 8E versus Fig. 8F), although pSyn pathology could still be observed at the level of the striatum in the SynFib group at this timepoint (Fig. 8J).

Next, to assess the inflammatory response, we stained for the microglial marker IBA1. SynFib animals showed an increase in microglial activation throughout the nigrostriatal pathway at 4 weeks post-injection (Fig. 8B,I). At 16 weeks post-injection this inflammation returned to a level comparable to the AAV-αSyn alone group at 4 weeks post-injection (Fig. 8C,J vs 8A,H), suggesting that the active reaction to pathology was finished. We found microglia localised to areas of ongoing pathology (Fig. 8E, E'), appearing to surround and engulf cells containing $pSyn^+$ inclusions (Fig. 8G). We also observed examples of microglia containing these pathological inclusions (Fig. 8K), suggesting either phagocytotic activity of degenerating cells/axons and debris in the striatum, or transfer of pathology from nearby affected cells.

Pathological inclusions detectable in a small number of grafted DA neurons in the SynFib model

The pathological environment in the striatum of the SynFib animals is of particular interest to investigate cell transplantation, given that this is the site for cell transplantation in PD patients. hESC-derived DA progenitors were transplanted into the striatum of SynFib animals and assessed 12 to 18 weeks post-transplantation. When we stained for TH and pSyn to examine co-labelling of DA neurons with pathology we found numerous examples in the SynFib animals (Fig. 9A-H). At first, these pathological inclusions appeared to be extracellular and looked to be within structures that could resemble degenerating neurites or cellular debris (arrowheads in Fig. 9B and B′). However, orthogonal projections on the confocal microscope confirmed the presence of $pSyn^+$ inclusions within the cytoplasm (Fig. 9G) and nucleus (Fig. 9H) of grafted DA neurons. Some inclusions were also found in cells that did not express TH (Fig. 9G), although it is possible that this could also be due to TH down-regulation. Previously, it has been shown that pathological αSyn aggregates can lead to the activation of microglial cells (Lee et al., 2008; Sanchez-Guajardo et al., 2015; Zhang et al., 2005), therefore we next wanted to examine if this was the case in the SynFib model. There were numerous examples of activated microglia surrounding the DA neurons with $pSyn^+$ inclusions (Fig. 9 I-M), suggesting that microglia may play a role in eliminating pathological inclusions (Fig. 9 L,M).

Patient-derived DA cells can survive intracerebral transplantation and provide motor recovery in the 6-OHDA model of PD (Paper IV)

To date, primarily cells derived from sporadic PD donors have been investigated *in vivo* and no phenotype has been observed in these reports (Hargus et al., 2010; Kikuchi, Morizane, Doi, Magotani, et al., 2017; Kikuchi, Morizane, Doi, Okita, et al., 2017; Song et al., 2020). However, given that αSyn is heavily implicated in the pathophysiology of PD we decided to investigate a familial PD patient line caused by a mutation in the *SNCA* gene. αSyn triplication leads to 4 copies of the *SNCA* gene, resulting in increased αSyn protein expression, causing early onset and rapidly progressing PD (Devine et al., 2011; Singleton, 2003). We therefore postulated that pathological changes may present

earlier in this line compared to lines derived from sporadic PD patients. In this study, we used a hiPSC line derived from a patient with an Alpha Synuclein Triplication mutation, AST18. As a point of reference, we also used a clinical grade hESC line, RC17. Both lines were differentiated into DA progenitors and transplanted into the striatum of 6-OHDA-lesioned rats.

αSyn triplication DA progenitor cells survive transplantation into the rodent brain and generate DA-rich grafts

This patient line (AST18) has not previously been studied *in vivo*, so we first tested the ability of the cells to survive and mature after grafting into the rodent brain. We examined the grafts at early time points, 7-8 weeks post-transplantation, and found that all animals had surviving grafts. These grafts were rich in neurons as detected by hNCAM staining, and a large proportion of the grafted cells also expressed the DA neuron marker TH. These results showed that DA progenitors differentiated from the AST18 patient line could survive and mature into DA-rich grafts similar to hESCs following transplantation.

Grafts derived from an αSyn triplication hiPSC line survive long-term and are able to mediate functional recovery

After establishing that the AST18-derived DA progenitors could form large grafts in the rodent brain, we next wanted to assess their functionality. The amphetamine rotation test was used to evaluate graft-induced motor recovery in a subset of animals confirmed to have successful lesions at time of transplant (representative images demonstrating TH^+ cell loss in SN; Fig. 10A). All animals were rotated prior to transplantation, followed by repeated rotations at 16, 18, 20, 22, and 24 weeks post-transplantation. These results demonstrated that all of the animals (5/5) with hESC-derived grafts had full functional recovery by 24 weeks post-transplantation (Fig. 10B) and 4/5 of the animals with grafts derived from the patient AST18 line showed full functional recovery at this timepoint (Fig. 10C). Histology was performed at 24 weeks post-transplantation and, as above, hNCAM and TH immunostaining showed that all animals had surviving grafts containing DA neurons (representative images shown in 10 D,E). Graft quantifications confirmed that both RC17- and AST18-derived grafts had a similar DA content (Figure 10F). Also, when quantifying the animal in the AST18 group which did not show full functional recovery, we found that this animal had a particularly low DA neuron count, which was at the threshold for recovery in the amphetamine rotation test. Therefore, this study provides evidence that a hiPSC line derived from a patient with αSyn triplication can generate functional grafts rich in DA neurons capable of innervating the surrounding host striatum and mediating functional recovery.

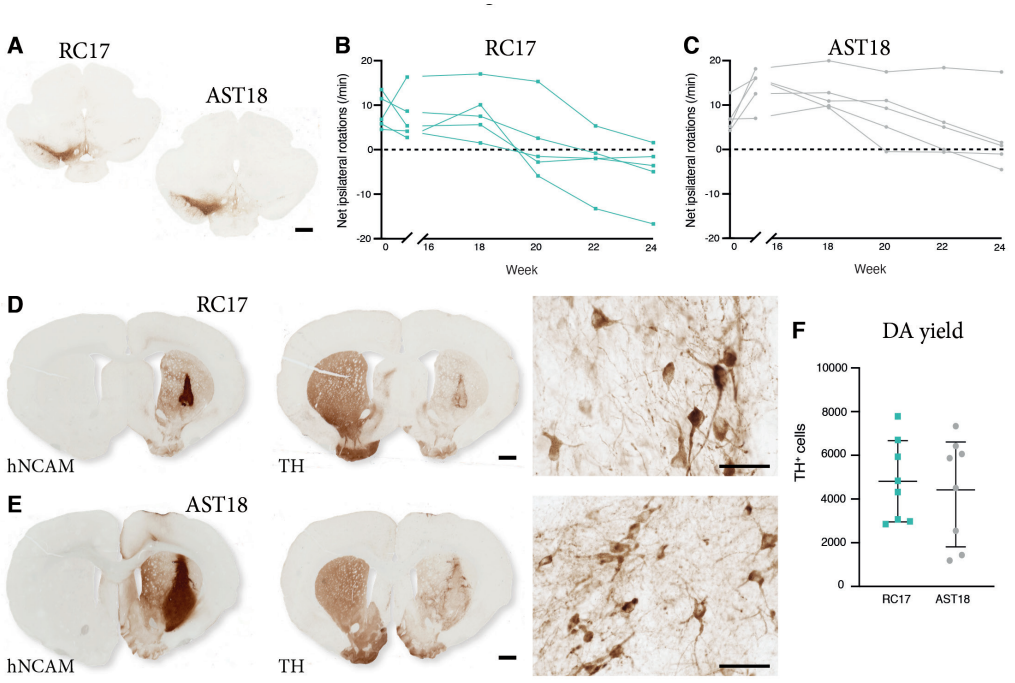


Fig. 10 – Long-term graft analysis. (A) Representative images showing loss of TH^+ cells in the SN on the lesioned hemisphere, scale bar 1 mm. Net ipsilateral rotation scores showing functional recovery in (B) 5/5 animals in the RC17 group and in (C) 4/5 animals in the AST18 group. Immunohistochemistry showing surviving $hNCAM^+$ and TH^+ grafts at 24 weeks post-transplantation from (D) RC17 VM progenitor cells and (E) AST18 VM progenitor cells, scale bar 1 mm. High magnification images of grafted TH^+ cells, scale bar 50µm. (F) No major difference was found in the DA content of the grafts when TH^+ cells were quantified.

Patient-derived grafts develop disease-associated pathology (Paper IV and V)

Finally, we sought assess if patient-derived cells display any pathological features in rodent models of PD. We used the same cell lines as previously mentioned, the patient-derived line AST18 with RC17 as a reference. These cells were used to generate DA progenitors which were then transplanted into the 6-OHDA model of PD (paper IV) or the SynFib model of PD (paper V). Since it has previously been shown that the pathological environment of the patient's brain can impact grafted cells, we wanted to compare the grafts in these two different models to evaluate the extent of a host pathological environment in a preclinical study.

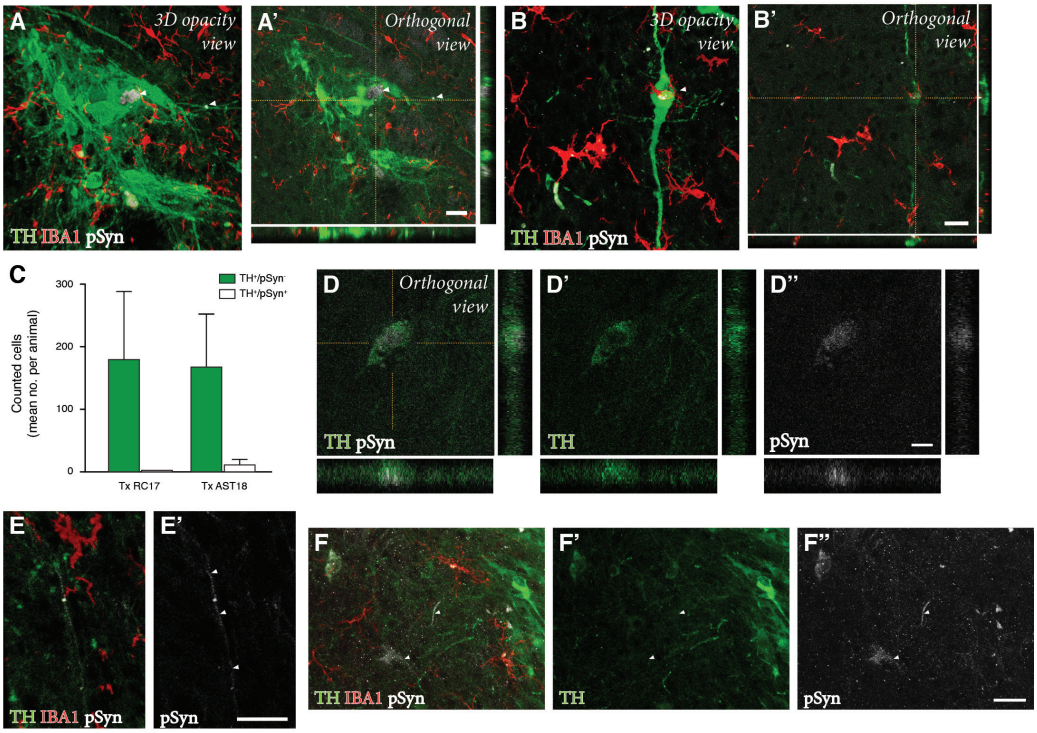
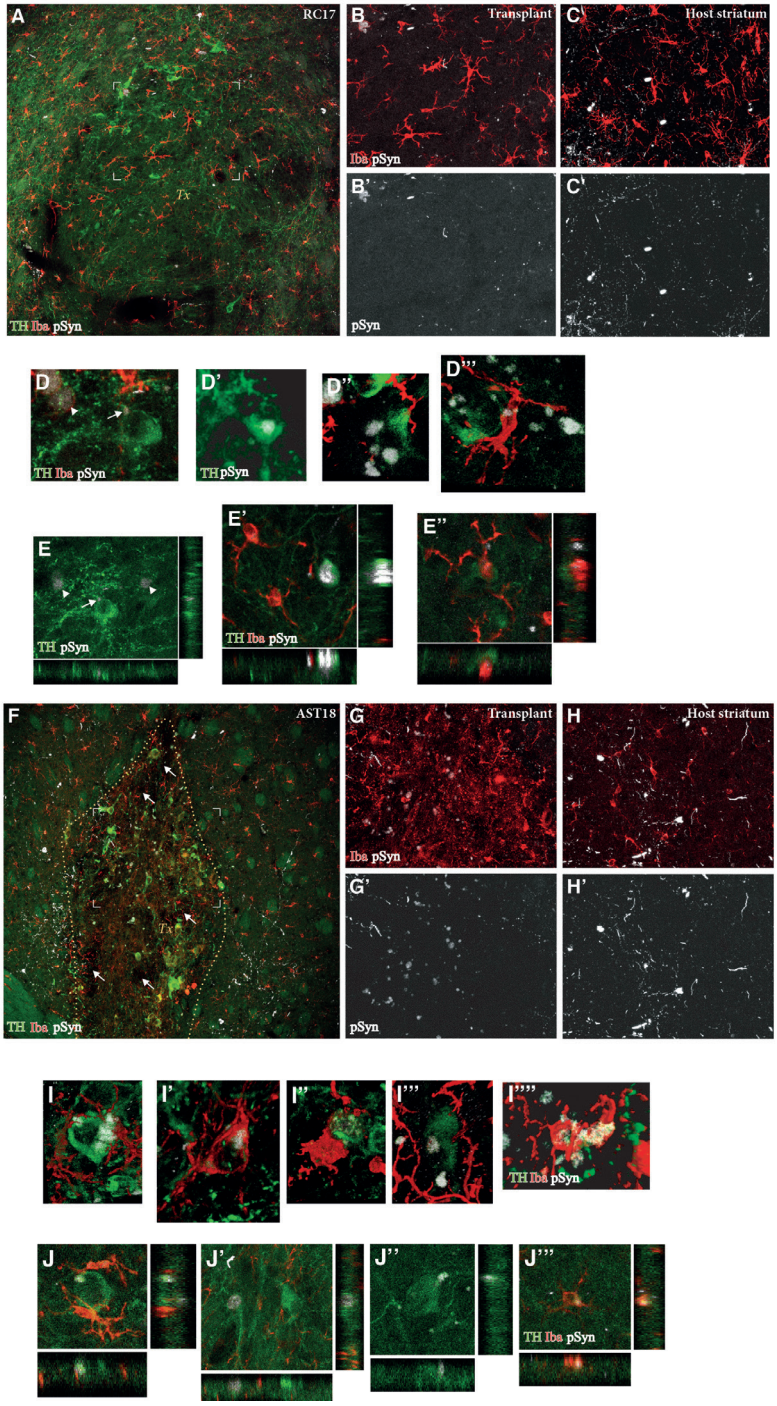


Fig. 11 – Assessment of pathology within grafts at 24 weeks post-transplantation. (A, A', B, B') Immunofluorescent staining for TH (green), IBA1 (red), and pSyn (white) within the graft showing $TH^+/pSyn^+$ cells, scale bar 20 μm. (C) $pSyn^+$ inclusions were quantified in grafted TH^+ cells. TH and pSyn double staining showing (D, D',D") a TH^+ neuron with small granular aggregates of pSyn, scale bar 10um, and (E, E') a $TH^+/pSyn^+$ neurite (indicated by arrowheads), scale bar 20 μm. (F) Possible down-regulation of TH (shown by arrowheads) in a weakly stained TH^+ neurons containing granular pSyn, scale bar 20 μm.

Cell intrinsic factors can lead to pathological changes in the 6-OHDA model of PD

We found low levels of microglial activation in animals grafted with both cell lines, which is to be expected in a xenograft setting. The overall density of microglia in the grafts was similar to levels in the host striatum, and there was no difference between RC17- and AST18- derived grafts in the

Fig. 12 - (A-E) A representative animal from the RC17 group at 24 weeks post-transplantation. (D) Overview of the transplants showing good-sized grafts containing large numbers of apparently healthy TH^+ neurons (green). These transplants showed only minor signs of $pSyn^+$ pathology (white), as shown in B,B', which is in contrast to the prominent expression of pSyn in axons and terminals in the surrounding host striatum (C,C´). The $Iba1^+$ microglia (red) in the transplants, as illustrated in B, tended to be smaller in size, but present in higher density than in the surrounding host striatum (C). $pSyn^+$ inclusions were seen in single TH^+ neurons and $Iba1^+$ microglia, as shown in D and E. (F-J) A representative animal from the AST18 group at 24 weeks post-transplantation. (F) The grafts in this group were notably small in size and the TH^+ neurons exhibited often a shrunken and distorted morphology. These transplants showed more extensive $pSyn^+$ pathology, as shown in G,G´, similar to the surrounding host striatum (H,H´). The $Iba1^+$ microglia in the transplants, as illustrated in G, tended to be smaller in size, but present in higher density than in the surrounding host striatum (H). (I-J) TH^+ neurons (green) containing $pSyn^+$ inclusions and granular aggregates were much more common in the AST18-derived grafts, often surrounded, or engulfed, by microglia some of which contained pSyn.


A
RC17
Tx
TH Iba pSyn
B
Transplant
Iba pSyn
B'
pSyn
C
Host striatum
C'
D
TH Iba pSyn
D'
TH pSyn
D''
D'''
E
TH pSyn
E'
TH Iba pSyn
E''
F
AST18
Tx
TH Iba pSyn
G
Transplant
Iba pSyn
G'
pSyn
H
Host striatum
H'
I
I'
I''
I'''
I''''
TH Iba pSyn
J
J'
J''
J'''
TH Iba pSyn

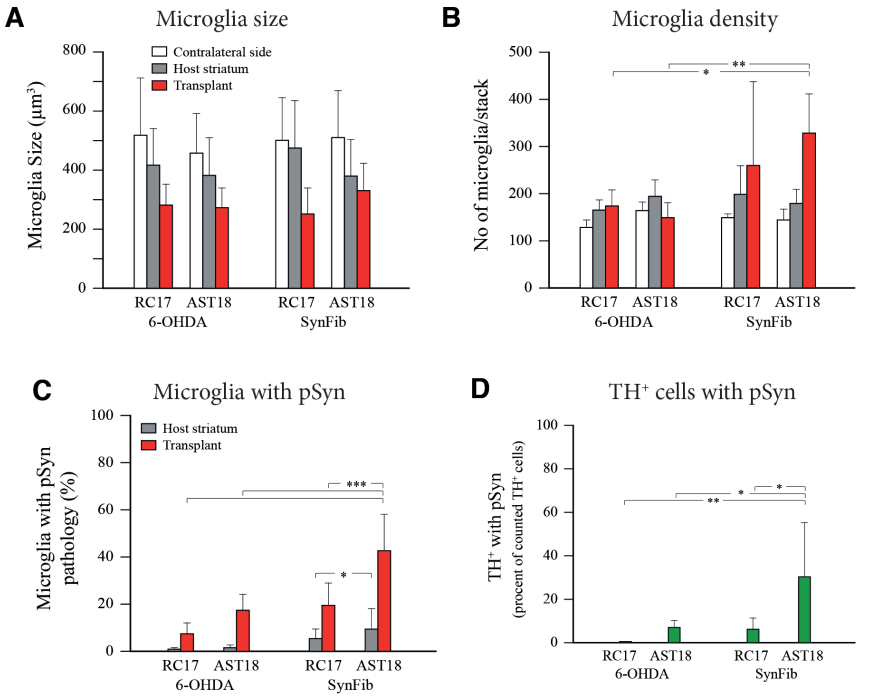


Fig. 13 - Pathological changes seen in RC17- and AST18-derived grafts transplanted in the 6-OHDA (Shrigley et al., in press) and SynFib model of PD. (A) The average size of the Iba1 microglia in the graft did not differ between graft type or lesion model, but they tended to be overall smaller than those seen in the host striatum and the contralateral intact side. (B) Iba1$^+$ microglia were significantly more abundant in the AST18-derived grafts when transplanted in the SynFib model. pSyn$^+$ inclusions in (C) Iba1$^+$ microglia and (D) TH$^+$ neurons were significantly more abundant in the AST18-derived grafts than in the RC17-derived grafts, and they were also significantly more abundant in AST18 grafts transplanted in the SynFib model than in the 6-OHDA lesion model.

6-OHDA model. However, we did observe significantly more microglia containing pSyn$^+$ inclusions inside the AST18-derived grafts. Confirming previous reports in the 6-OHDA model, we did not find any pSyn$^+$ inclusions in DA neurons in RC17-derived grafts. In contrast, we did see pSyn$^+$ inclusions in a small number, around 7%, of grafted DA neurons in AST18 animals (Fig. 11 A,B,C). These inclusions were typically found in the cytoplasm (Fig. 11D) or along the neurites (arrowheads in Fig. 11E), and we also observed some evidence of TH down-regulation (Fig. 11F). Given that the 6-OHDA model does not induce pSyn pathology, it is possible these changes are linked to the endogenous overexpression of αSyn in the AST18 line.

αSyn triplication grafts exhibit significant pathology in the SynFib model of PD

Next, we assessed the impact of the pathological host environment of grafts between 18- and 24-weeks post-transplantation in the SynFib model of PD. In spite of the ongoing pathology in the

host brain, animals grafted with RC17-derived DA progenitors showed only minor signs of $pSyn^+$ pathology in the transplant (Fig. 12A-E). In contrast, the patient-derived grafts showed more pronounced signs of pathology and degeneration. Moreover, the AST18-derived grafts were smaller (Fig. 12F vs 12A) with more sparsely distributed DA neurons (Fig. 12I,J). We also observed small areas or 'pockets' of degeneration and possible cell death in the AST18-derived grafts (Fig. 12F arrowheads), and found $pSyn^+$ inclusions localised within DA neurons (Fig. 12 I,J) and microglia (Fig. 12 I). The number of DA neurons and microglia containing pathological inclusions were increased approximately 3-fold in AST18- vs RC17-derived grafts (Fig. 12D). In addition, there was an aggravated microglial response in the patient-derived grafts. As previously observed, microglial cells were positioned close to cells with pathological inclusions (Fig. 12I, J), suggesting their involvement in the pathogenic cascade.

Pathological changes are accelerated in the SynFib model of PD

Finally, we compared the grafts in the SynFib model with the same cell lines transplanted in the 6-OHDA model. When examining the inflammatory response, we found no significant difference in the size of the microglia between the SynFib and 6-OHDA models (Fig. 13A). However, the microglial density was increased in transplants in the SynFib model (Fig. 13B). The most noticeable difference was the increase of microglia with pathological inclusions in the SynFib model, which was further escalated in AST18-derived grafts in this model (Fig. 13C). When examining pathology specifically in the grafted DA neurons, we found no evidence of $pSyn^+$ inclusions in RC17-derived grafts, whereas around 7% of DA neurons in AST18-derived grafts contained $pSyn^+$ inclusions in 6-OHDA lesioned animals. The expression of pathological inclusions increased in both groups when the same cell lines were transplanted into the SynFib model. Approximately 5% of the grafted DA neurons in the RC17 animals were found to contain pathological inclusions, whereas this dramatically increased to 30% in the AST18 animals. (Fig. 13D). Taken together, these findings suggest an effect of both cell line and model in the development of pathology within transplants.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Allogeneic trials using healthy pluripotent stem cell lines (without any known disease background) for PD have begun in Japan (CiRA trial; Doi et al. 2020) and others are due to commence in the coming year in both the United States (NYSTEM-PD) and Europe (STEM-PD) (Barker, 2019; Barker et al., 2017). Much of current research is focused on investigating the use of patient-derived cells, i.e. an autologous grafting strategy, as a future source of DA neurons for use in cell replacement therapy for PD, with the first study using an autologous grafting strategy in a PD patient reported last year (Schweitzer et al., 2020).

Cellular reprogramming provides the unique opportunity to generate DA neurons that are patient-specific. Throughout this thesis, two strategies for the generation of patient-specific cells have been investigated, both direct reprogramming to iDANs, and reprogramming to pluripotency (hiPSCs), with further differentiation into DA cells. However, many questions remain unanswered, including whether patient-derived grafts can function on par with those derived from healthy donors and if they are more susceptible to pathological changes.

In **paper I**, we aimed to improve the conversion efficiency of direct reprogramming of adult human fibroblasts from previous reports (Caiazzo et al., 2011; Liu et al., 2013; Pfisterer, Wood, et al., 2011). Inhibition of the REST protein allowed for the expression of epigenetically silenced pro-neural genes in fibroblasts from adult donors and significantly increased conversion efficiency (Ballas et al., 2005; Drouin-Ouellet et al., 2017). Moreover, small molecules were added to promote conversion, survival, and maturation of iNs (Gascón et al., 2016; Hu et al., 2015; Huangfu et al., 2008; Ladewig et al., 2012; Liu et al., 2013). Using this protocol approximately half of adult fibroblasts convert into iNs over 25 days *in vitro*. This allows for the generation of iNs in a standardised and efficient manner for experimental assays.

This protocol was modified in **paper II** in order to generate subtype-specific iDANs. In this study, we wanted to investigate the factors controlling conversion specifically to a DA subtype by using different combinations of transcription factors known to influence DA cell fate. After screening multiple factors, we identified the optimal combination; *Ascl1, Lmx1a, Lmx1b, FoxA2, Otx2, Nurr1* and shREST. Moreover, gene expression analysis showed an upregulation of key DA markers and electrophysiology confirmed that these neurons were also functionally mature. Following this, we established that healthy and sporadic PD patient-derived fibroblasts could be converted with similar efficiency. However, patient-derived iDANs exhibited an altered autophagic response and an increase in the number of cytoplasmic pSyn inclusions following starvation when compared to healthy donors.

Direct reprogramming is an attractive approach for patient-specific cell replacement therapy as conversion is considerably quicker and cheaper than hiPSC generation and differentiation. Moreover,

during direct reprogramming cells do not pass through a pluripotent stage therefore eliminating the risk of tumorigenesis after transplantation. However, previous studies have reported that the process of direct reprograming preserves many key aspects of the starting cell, including their vulnerability to ageing (Drouin-Ouellet et al., 2017; Huh et al., 2016; Kim et al., 2018; Mertens et al., 2015). This may have potential implications for cell replacement therapy as patient-derived iNs may therefore be more susceptible to pathological changes, causing earlier degenerative changes to the grafted cells which could affect their functional capacity. Therefore, these data combined with our findings in paper II suggest that iDANs may be better suited for disease modelling studies than cell replacement therapy. In contrast, hiPSC reprogramming is perhaps better suited to cell replacement therapy as the cells are 'rejuvenated' during the reprogramming process. Moreover, with the recent improvements to pluripotent stem cell DA differentiation protocols there is a significantly reduced risk of tumorigenesis following transplantation of hiPSC-derived DA progenitors (Doi et al., 2014; Kikuchi, Morizane, Doi, Magotani, et al., 2017; Kikuchi, Morizane, Doi, Okita, et al., 2017; Kriks et al., 2011; Nolbrant et al., 2017).

Long-term studies from the human fetal VM trials have shown the presence of αSyn pathology within some of the grafted cells (Kordower et al. 2008; Li et al. 2008, 2010; Li et al. 2016). This finding could not have been predicted from preclinical studies in standard toxin models of PD, and suggests that the host brain environment can impact the graft. In **paper III**, a new xenograft model was established to assess the impact of host pathology in an animal model of PD. Rats were coinjected with an AAV expressing human αSyn and human αSyn PFFs in the SN, termed the 'SynFib' model. This model induces behavioural impairments and progressive DA neuron cell death, alongside extensive pSyn pathology and inflammation. Following transplantation of into the model, pathological $pSyn^+$ inclusions can be detected in a very small proportion of grafted DA neurons derived from a healthy hESC line. This environment more closely mimics the environment in a PD patient's brain and allows for the first time the replication of what has been reported in patients receiving fetal VM transplants.

To date, predominantly cells derived from sporadic PD donors have been investigated for cell replacement therapy with no pathological phenotypes reported (Hargus et al., 2010; Kikuchi, Morizane, Doi, Magotani, et al., 2017; Kikuchi, Morizane, Doi, Okita, et al., 2017; Song et al., 2020). However, given that αSyn is heavily implicated in the pathophysiology of PD we decided to investigate a familial PD patient line with a triplication of the *SNCA* gene. In **paper IV**, an αSyn triplication patient hiPSC line was transplanted into the 6-OHDA model of PD to assess graft survival and functionality. In this study we found that grafts derived from an αSyn triplication hiPSC line are DA-rich, survive long-term, and are able to mediate functional recovery. However, these grafts showed pathological changes, with a small proportion of DA neurons containing $pSyn^+$ inclusions. As this toxin model does not induce pSyn pathology in itself, it would appear that cell intrinsic factors influenced the observed pathological changes, likely connected to their endogenous overexpression of αSyn. Furthermore, in order to assess how patient-derived cells would survive long-term after transplantation into a pathological environment, similar to the PD patient brain, the same line was transplanted in our newly developed SynFib model in **paper V**. Despite the ongoing host pathology, animals grafted with healthy hESC-derived DA progenitors showed only minor signs of $pSyn^+$ pathology in the transplant, whereas the patient-derived grafts showed more pronounced signs of pathology and degeneration. Finally, we also showed that the level of pathological inclusions increased in transplants derived from both cells lines when transplanted into the SynFib model compared to the 6-OHDA model. These

data suggest that the development of pSyn pathology in grafted cells is driven by both cell intrinsic and extrinsic factors, and that they act to potentiate each-other.

Taken together, these results show that familial PD patient lines with increased levels of αSyn expression, such as αSyn duplication and triplication patients, are not suitable for cell replacement therapy unless they are gene corrected or otherwise made resistant to pathology. In future studies, and prior to use in clinical trials, it will be vital to investigate all patient-derived lines for efficacy, not only in standard toxin models, but also in models that more closely recapitulate the disease environment in a PD patient brain, such as the SynFib model, to evaluate their susceptibility to synuclein pathology.

Furthermore, there are also concerns about the cost of patient-specific therapies, as this requires the generation of a hiPSC line from each patient, differentiation to a DA phenotype, and subsequent quality control checks. In the future, an alternative option would be to transplant 'immune evasive' cells, this could be done either via human leukocyte antigen (HLA) matching or gene editing strategies. HLA matching reduces the risk of transplant rejection and generation of a bank of hiPSC lines for HLA-matching cell therapies could be feasible in some populations. Its estimated that this bank would need to contain between 50-140 hiPSC donor lines to match approximately 90% of the population in regions such as the UK and Japan (Nakatsuji et al., 2008; Taylor et al., 2012). It could also be possible to CRISPR edit cells to delete immune markers such as the major histocompatibility complex (MHC) genes combined with signalling to evade natural killer cells (Deuse et al., 2019). However, these need to be carefully evaluated to be sure there is no increased risk of tumorigenesis.

MATERIALS & METHODS

In this chapter I will summarise the key methods used in the papers included in this thesis. For additional details and other procedures not described here, please refer to the methods section of the respective paper (see appendices).

In vitro studies

Culture of human dermal fibroblasts

Adult human dermal fibroblasts were obtained from the Parkinson's Disease Research and Huntington's disease clinics at the John van Geest Centre for Brain Repair (Cambridge, United Kingdom) and used under ethical approval (REC 09/H0311/88; for skin biopsy procedure see Drouin-Ouellet et al. 2017). Fibroblasts were thawed and plated on an uncoated T75 flask at a density of approximately 2,500 cells/cm^2 in fibroblast medium (DMEM, 10% FBS, and 1% penicillin-streptomycin) at 37°C in 5% CO_2. The medium was changed every 2-3 day until the cells reached approximately 90% confluency. Fibroblasts were then dissociated with trypsin (0.05%) and either frozen down as stock or replated for reprogramming experiments.

Lentiviral vectors (LVs)

The LVs used in these studies were third generation vectors (*cis* and *trans* acting viral sequences were removed or separated into three individual plasmid vectors) in order to prevent replication-competent virus being formed (Dull et al., 1998). LVs were produced by transfection of HEK-293T cells as described in Zufferey et al. (1997). The viral titer was measured by quantitative real-time PCR using primers specific to Albumin (reference gene) and WPRE. All LVs used in these studies contained a ubiquitous PGK promoter except for shRNA targeting the repressor protein REST which had a non-regulated U6 promoter. In paper I the DNA plasmid used expressed the ORF for *Ascl1, Brn2*, and shREST and was used at a MOI of 20. In paper II DNA plasmids expressing ORFs for *Ascl1, Lmx1a, Lmx1b, Otx2, FoxA2, Nurr1*, as well as two shRNAs targeting REST, and transduction was performed at a MOI of 5 for each vector.

Direct neuronal reprogramming

Adult human dermal fibroblasts were plated on gelatin (0.1%) coated 24 well plates (Nunc™ treated) at a density of approximately 25,000 cells/cm^2 in fibroblast medium at 37°C in 5% CO_2. The following day, cells were transduced with fibroblast medium containing the LVs. On day 3, the fibroblast medium was removed and replaced with early neuronal conversion medium (NDiff227

+ penicillin-streptomycin) supplemented with the small molecules CHIR99021 (2μM), SB431542 (10μM), Noggin (0.5μg/mL), LDN1931189 (0.5μM), VPA (1mM), LM22A4 (2μM), GDNF (2ng/mL), NT3 (10ng/μL), and db-cAMP (0.5mM). There was a 50% medium change of early neuronal conversion medium 2-3 times a week until day 18. On day 18, this was replaced with late neuronal conversion medium (NDiff227 + penicillin-streptomycin) supplemented with the small molecules LM22A4 (2μM), GDNF (2ng/mL), NT3 (10ng/μL), and db-cAMP (0.5mM). There was continued 50% medium changed of late conversion medium every 2–3 days until day 25 or the experimental endpoint.

Culture of pluripotent stem cells

hESCs (RC17) and hiPSCs (AST18) were used in these studies. For maintenance of pluripotency, the cells were cultured on laminin 521 (LN521, 0.5μg/cm^2) coated plates at a density of 2,500 cells/cm^2 in iPS brew medium at 37°C in 5% CO_2. Cells were passaged when they reached approximately 90% confluency using EDTA (0.5mM) and replated at a density of 2,500 cells/cm^2. After thawing or replating, ROCK inhibitor (Y27632; 10μM) was added to the iPS brew medium for the first 24 hours (Watanabe et al., 2007).

Differentiation into midbrain DA progenitors

For the initiation of DA differentiation, pluripotent cells were seeded onto Laminin 111 (LN111, 1μg/cm$_2$) coated plates at a density of 10,000 cells/cm^2 in N2 medium (49% DMEM/F12, 49% Neurobasal, 1% N2 supplement, 1% L-Glutamine, and 0.2% penicillin-streptomycin) supplemented with Y27632 (10μM), SB431542 (10μM), Noggin (100ng/mL), SHHC2411 (300ng/mL), CHIR99021 (0.6-1.0μM). The medium was changed every 2-3 days. On day 9, the medium was changed to N2 medium supplemented with FGF8b (100ng/mL). On day 11, the progenitors were dissociated using accutase and replated on LN111 coated plates at a density of 800,000 cells/cm^2 in B27 medium (97% Neurobasal, 2% B27 supplement, 1% L-Glutamine, and 0.2% penicillin-streptomycin) supplemented with Y27632 (10μM), BDNF (20ng/mL), AA (0.2mM), and FGF8b (100ng/mL). Fresh B27 medium + BDNF, AA, and FGF8b were added to the cells on day 14. On day 16, patterning of midbrain DA progenitors was complete and cells underwent quality control (QC). In order to pass QC, immunostaining must demonstrating between 80-95% co-expression of Lmx1a/FoxA2 and Lmx1a/Otx2 and gene expression levels must fall within the defined limits in Nolbrant et al. (2017). Following this DA progenitors were cryopreserved, prepared for transplantation, or terminally differentiated.

Immunocytochemistry

Cells were fixed in 4% PFA for 15 minutes and washed twice using 0.1 M KPBS (pH = 7.4). Before staining cells were washed once with KPBS, and then incubated in blocking solution (KPBS containing 0.1% Triton-X and 5% serum specific to the species of the secondary antibody) for 1 hour at RT. Following this, the primary antibody in blocking solution was added overnight at 4°C. The primary antibodies used are listed in table 1. The next day, cells were washed three times and incubated with fluorophore-conjugated secondary antibodies (1:200, Jackson ImmunoResearch Laboratories) and DAPI (1:1,000) in blocking solution for 2 hours at RT. Cells were then washed with KPBS a further three times, and then stored at 4°C until analysis.

Table 1 - Details of antibodies used in vitro.

Antibody	Host	Dilution	Provider
TAU	Rabbit	1 : 500	Agilent Technologies, A0024
TAU(HT7)	Mouse	1 : 500	Thermo Fisher Scientific, MN1000
MAP2	Chicken	1 : 10,000	Abcam, Ab5392
TH	Rabbit	1 : 1,000	Millipore, Ab152
TH	Sheep	1 : 1,000	Millipore, Ab1542
Lmx1a	Rabbit	1 : 1,000	Millipore, Ab10533
Otx2	Goat	1 : 2,000	R&D Systems, AF1979
FoxA2	Mouse	1 : 500	Santa Cruz, sc101060
αSyn	Chicken	1 : 1,000	Abcam, Ab190376
αSyn	Mouse	1 : 250	BD Biosciences, 610787
pSyn(81A)	Mouse	1 : 10,000	Gift from Kelvin Luk, University of Pennsylvania
ALDH1A1	Rabbit	1 : 200	Abcam, Ab23375
VMAT2	Rabbit	1 : 200	Sigma, Ab1598P
GIRK2	Goat	1 : 200	Millipore, Ab65096
CD24	Mouse	1 : 200	Thermo Fisher Scientific, AM5-11828
HSC70	Rat	1 : 2,000	Abcam, Ab19136
LAMP2	Mouse	1 : 100	DSHB, H4B4
LAMP2a	Rabbit	1 : 1,000	Abcam, Ab18528
LC3B	Rabbit	1 : 500	Sigma, L7543
P62	Rabbit	1 : 500	Abcam, Ab91526
NANOG	Rabbit	1 : 200	Cell Signalling, 4903S
Oct4A	Rabbit	1 : 200	Cell Signalling, C30A3

High content screening

The Cellomics™ Array Scan (Array Scan VTI, Thermo Fischer), is an automated process ensuring unbiased measurements between groups. The total number of $DAPI^+$, TAU^+, and TH^+ cells per well, as well as the average fluorescence intensity for αSyn, were quantified using the program "Target Activation" in which fields were acquired in a spiral fashion starting from the center at 10X magnification. For the analysis of the number of neurites per TAU^+ cell, the program "Neuronal Profiling" was used at 20X magnification. Neuronal purity was calculated as the number of TAU^+ or $MAP2^+$ cells over the total number of cells in the well at the end of the experiment. DA subtype purity was calculated as the number of TH^+ or $ALDH1A1^+$ cells over the total number of TAU^+ or $MAP2^+$ cells in the well at the end of the experiment. Average dot number and size (p62, LC3, LAMP2, LAMP2a, HSC70, αSyn, and pSyn) were measured using a "Spot Detection" program in neurons in which the cytoplasm and neurites were defined by TAU or TH staining.

In vivo studies

Animal experiments

All procedures were performed in accordance with the European Union Directive (2010/63/EU) and approved by the local ethical committee at Lund University, as well as the Swedish Department of Agriculture (Jordbruksverket). Female Sprague-Dawley (SD) rats were purchased from Charles River Laboratories and female nude athymic rats (Hsd:RH-*Foxn1*^{rnu}) were purchased from Envigo. All rats weighed at least 225g at the beginning of experiments. Rats were housed in ventilated cages with *ad libitum* access to food and water under a 12-hour light/dark cycle.

Drug preparations

All surgeries were performed under general anaesthesia using a solution of fentanyl citrate (fentanyl; 0.36mg/kg) and medetomidine hydrochloride (Domitor; 0.36mg/kg) injected intraperitoneally. After surgery, anaesthesia was reversed with atipamezole (Antisedan; 0.28mg/kg) and analgesia was administered using buprenorphine (Temgesic; 0.04mg/kg) injected subcutaneously. All drugs were diluted in sterile saline, except for antisedan which was diluted in sterile water.

Stereotactic surgery

All surgeries were performed using aseptic technique. A 10μL Hamilton syringe with a 22-gauge needle fitted with a thin glass capillary was used for all surgical procedures.

Firstly, animals were anaesthetised, the top of the animal's head was shaven and then sterilized with chlorhexidine. Animals were carefully placed into the stereotactic frame and secured with ear and tooth bars so that there was no movement of the head in any dimension. Next, a scalpel was used to make an incision along the midline of the head, cutting through both cutaneous and subcutaneous layers to expose the skull. Bulldog clips were placed on the skin on either side of the incision to ensure a clear field of view. Surgeries were performed under a "flat head" position, in which the dorsal/ventral value of lambda and bregma was the same (± 0.2mm of error). Bregma was used as the reference

to calculate the coordinates for injection, and the skull was carefully drilled at the location of injection in order to avoid excess pressure or damage of brain tissue. A cotton gauze was placed over the drill hole and the Hamilton syringe was flushed with sterile saline to ensure no blockages. Following this, the Hamilton syringe was used to draw up a 1μL air bubble into the glass capillary followed by the volume of solution for injection. The cotton gauze was then removed and the Hamilton was lowered carefully to measure the dorsal/ventral coordinate of the dura mater. The glass capillary was lowered to the injection site and the solution injected at a specific rate until the total volume was injected. Next, the specific diffusion time was waited and the glass capillary slowly retracted from the brain to avoid reflux of solution through the injection tract. The head wound was sutured using a simple interrupted suture pattern (5-0 vicryl suture with a 19mm 3/8c triangle needle). Finally, the animal was removed from the stereotactic frame, the sutured wound cleaned with chlorohexidine, and the animal was marked to ensure identification for future procedures. Reversal of anaesthesia and post-operative analgesia was administered and the animal was placed into a clean cage. A heating pad was utilized to maintain body temperature as anaesthesia alters thermoregulation.

6-OHDA model

Aliquots of 6-OHDA were stored at -20°C in individual Eppendorf tubes, kept in a container with desiccant away from any light exposure. When required, the aliquot was taken from the freezer and diluted in ascorbic saline to avoid rapid oxidation. Each aliquot was kept for a maximum of 2 hours after dilution in order to ensure toxicity. To induce the model, 6-OHDA (3.5μg/μL of free base, dissolved in 0.02% ascorbic saline) was injected unilaterally into the right MFB at a rate of 0.3μL/minute with no diffusion time (see table 2 for volume and coordinates).

SynFib model

An AAV6 vector expressing human wild-type αSyn under the human synapsin-1 promoter was used in this model (for more detail see Decressac et al. 2011). The viral titer was 4.7×10^{14} genome copies/mL, and was used at a working dilution of 20%. Sonicated human αSyn PFFs (5mg/mL) were prepared from full length recombinant human αSyn (for more detail see Volpicelli-Daley, Luk, and Lee 2014). These were combined using a 1:1 ratio to yield a final concentration of 10% AAV-αSyn and 2.5 mg/mL sonicated αSyn PFFs. To induce the SynFib model, rats were injected with the AAV-αSyn and PFF mix in the midbrain at a rate of 0.2μL/minute with a diffusion time of 2 minutes (see table 2 for volume and coordinates).

Cell Transplantation

Cells were washed twice with KPBS and dissociated with accutase. After the cells were washed and centrifuged twice, they were resuspended in HBSS + DNase (1:10) to achieve the desired cell concentration. The cell suspension was kept on ice during the entire duration of the surgeries and transplanted within a 4-hour window of preparation to ensure high cell viability. In order to ensure a homogenous density, cells were resuspended with a pipette prior to loading in the glass capillary. For cell transplantation, the cell suspension was injected into the striatum at a rate of 1μL/minute with a diffusion time of 2 minutes (see table 2 for volume and coordinates).

Table 2 - Coordinates used for surgeries.

	A/P	M/L	D/V	Volume
6-OHDA model (MFB) *SD & Nude athymic rats*	-4.4	-1.1	-7.8	3µL
SynFib model SD rats	-5.3	-2.6	-6.7	2µL
	-5.3	-1.6	-7.2	2µL
SynFib model Nude athymic rats	-5.0	-2.6	-6.1	2µL
	-5.0	-1.6	-6.8	2µL
	-5.0	-0.8	-6.6	1µL
Cell transplantation SD rats	+0.5	-3.0	-4.5	1µL
	+0.5	-3.0	-5.5	1µL
	+1.2	-2.6	-4.5	1µL
	+1.2	-2.6	-5.5	1µL
Cell transplantation Nude athymic rats	+0.9	-3.0	-4.0	1µL
	+0.9	-3.0	-5.0	1µL
	+1.4	-2.6	-4.0	1µL
	+1.4	-2.6	-5.0	1µL

Coordinates in mm relative to bregma and the surface of the dura (Paxinos & Watson, 2005).
A/P: anterior/posterior; M/L: medial/lateral; D/V: dorsal/ventral.

For transplantation studies using SD rats, immunosuppression was given via daily injection of cyclosporine (10 mg/kg) intraperitoneally, starting 2 days pre-transplantation.

Amphetamine rotation test

This behavioural test is designed to assess the imbalance of DA in the right vs. left hemisphere. Amphetamine causes the release of DA from stored vesicles and also prevents DA reuptake by blocking the activity of DAT. This leads to a sustained increase of DA levels at the synapse (Sulzer et al., 1995). In unilateral models of PD, DA degeneration only occurs on one side of the brain, e.g. right hemisphere. Since the left-brain hemisphere controls movement on the right side of the body and vice-versa, this DA imbalance causes motor deficits specifically on the contralateral side to the lesion, leaving the ipsilateral side as a control. If the model is successful, this causes the animal to rotate towards the

lesion side, i.e. clockwise. Animals received an intraperitoneal injection of dexamphetamine solution (3.5mg/kg) and were placed into automated rotometer bowls for 90 minutes (Omnitech Electronics Inc.). Full body turns towards the lesion side were given positive values and turns to the opposite side given negative values, with data expressed as net turns per minute. In addition, this test can be used to assess motor recovery from DA grafts if the balance is restored, i.e. rotation score of 0 or less.

Tissue processing and Immunohistochemistry

Rats were terminally anaesthetised with a lethal dose of sodium pentobarbitone injected intraperitoneally. Rats were transcardially perfused with physiological saline solution followed by ice-cold 4% PFA. Brains were post-fixed for 24 hours in 4% PFA, transferred to 25% sucrose for 48 hours and then sectioned coronally using a freezing microtome at a thickness of 35µm (1:8 series). Immunohistochemistry was performed on free floating sections and all washing steps used 0.1M KPBS.

For DAB staining, sections were washed three times and then incubated in a quench solution (methanol, 30% H_2O_2, and KPBS) for 15 minutes at RT. After washing a further three times, the sections were incubated in blocking solution (KPBS containing 0.25% Triton-X and 5% serum specific to the species of the secondary antibody) for 1 hour at RT. Following this, the primary antibody in blocking solution was added overnight at RT (see table 3). The next day sections were washed twice and incubated in blocking solution for 30 minutes. The sections were incubated with secondary biotinylated antibodies (1:200, Vector Laboratories) for 1 hour at RT. After washing a further three times, sections were incubated with avidin-biotin complex (ABC) for 1 hour at RT for amplification. Next, sections were incubated in 0.05% DAB for 1–2 minutes before addition of 0.01% H_2O_2 for 1–2 minutes. After development, sections were mounted on gelatin-coated slides, dehydrated in an ascending series of alcohols, cleared in xylene, and finally coverslipped with DPX mountant.

For fluorescent immunolabeling, sections were washed three times and then incubated in Tris-EDTA (pH 9.0) for 30 minutes at 80 °C for antigen retrieval. After washing a further three times, the sections were incubated in blocking solution for 1 hour. Following this, the primary antibody in blocking solution was added overnight at RT (see table 3). The next day sections were washed twice and incubated in blocking solution for 30 minutes. The sections were incubated with fluorophore-conjugated secondary antibodies (1:200, Jackson ImmunoResearch Laboratories) for 1 hour at RT. After washing a further three times, sections were mounted on gelatin-coated slides and coverslipped with PVA-DABCO containing DAPI (1:1000).

Graft quantifications

Graft volume

Photomicrographs of hNCAM stained coronal sections were taken at the level of the striatum. To determine graft volume, the area of the graft core in every eighth section through the graft was measured using ImageJ (version: 2.0.0-rc-69/1.52p) and calibrated by associating the number of pixels with a known measurement, obtained from a scale taken as a photomicrograph using the same resolution and settings. The graft volume was calculated according to Cavalieri's principle, given the known distance between each section and the known section thickness.

Table 3 - Details of antibodies used *in vivo*.

Antibody	Host	Dilution	Provider
STEM121	Mouse	1 : 300	Takara, 40410
hNCAM	Mouse	1 : 1,000	Sant Cruz, sc106
TH	Rabbit	1 : 2,000	Millipore, Ab152
TH	Sheep	1 : 1,000	Millipore, Ab1542
αSyn	Rabbit	1 : 1,000	Millipore, Ab5038
αSyn(211)	Mouse	1 : 2,000	Santa Cruz, sc12767
Nurr1	Mouse	1 : 500	Abcam, ab41917
VMAT2	Guinea pig	1 : 2,000	Sigma, V6637
Pitx3	Guinea pig	1 : 500	Gift from Thomas Perlmann, Karolinska Institute
GIRK2	Goat	1 : 500	Abcam, Ab65096
Calbindin	Rabbit	1 : 1,000	Swant, CB38
IBA1	Rabbit	1 : 1,000	Wako, 019-19741
pSyn(129)	Rabbit	1 : 1,000	Abcam, ab51253
pSyn(81A)	Mouse	1 : 10,000	Gift from Kelvin Luk, University of Pennsylvania

Dopaminergic neuron quantification

To determine the DA neuron yield, the number of DAB-stained TH^+ neurons in each section was counted manually using the Olympus AX70 inverted microscope at 20X magnification in brightfield. Final counts were adjusted for the number of series (1:8), and Abercrombie's formula was used for correction of cell counts in histological sections to get an estimate of the total number of TH^+ cells within the graft.

Analysis of pSyn pathology

For the analysis of $pSyn^+$ inclusions within TH^+ and $IBA1^+$ cells, fluorescent images were taken on a Leica TCS SP8 laser scanning confocal microscope at 20X objective magnification, and collected in a 3D stack (Fig. 14). Microglia size was calculated by determining the total volume of stained $IBA1^+$

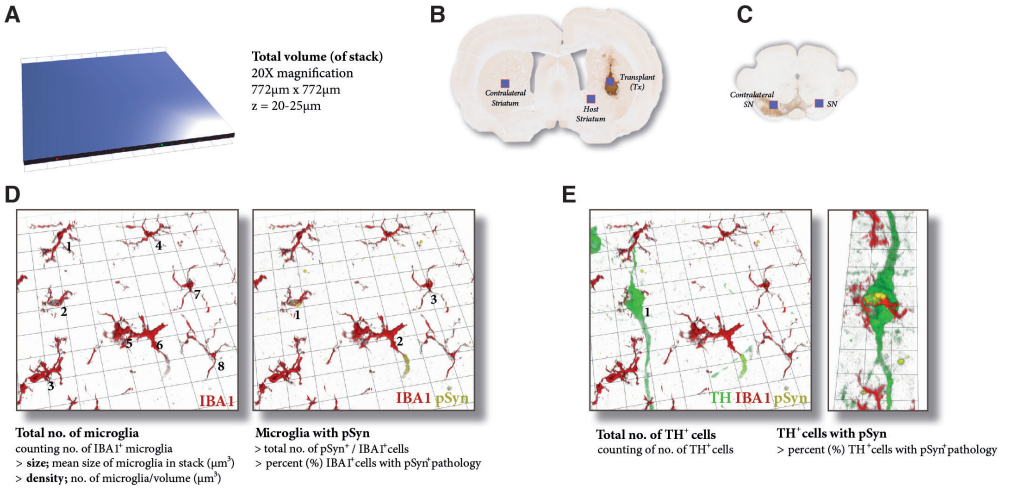


Fig. 14 - Overview of analysis on the confocal microscope. (A) Shows the volume a 3D stack. Images to show the areas that were selected for analysis (indicated by the blue boxes) in the (B) striatum and (C) substantia nigra (SN). Explanation with corresponding images of analysis of (D) IBA1$^+$ microglia and (E) TH$^+$ cells.

cells determined by threshold in the collected 3D stack, divided by the number of IBA1$^+$ cells in each stack. Microglia density was calculated by the number of microglia/stack. TH$^+$ and IBA1$^+$ cells were identified and assessed for co-expression of pSyn$^+$ inclusions using Volocity v.5.4.2 software (PerkinElmer).

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