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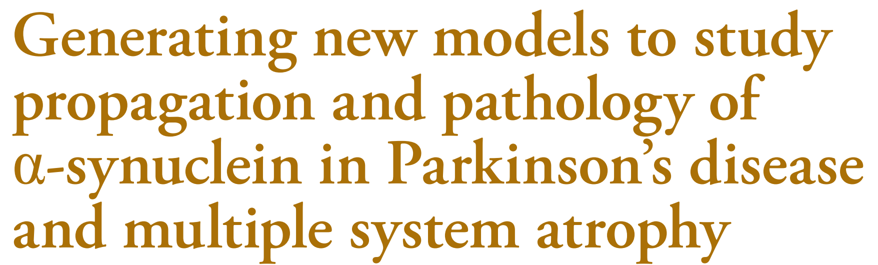

Generating new models to study propagation and pathology of α-synuclein in Parkinson's disease and multiple system atrophy

STAFFAN HOLMQVIST
DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY 2016

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Staffan Holmqvist

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Generating new models to study propagation and pathology of α-synuclein in Parkinson's disease and multiple system atrophy

Abstract

Synucleinopthies are neurodegenerative diseases characterised by the formation of α-synuclein-rich intracellular inclusions in neurons and glia. Traditionally, animal models and immortalized cell lines have been used to investigate why and how these inclusions form, and how they impact on cellular function. Recent advances in cellular reprogramming and directed differentiation have enabled the generation of neurons and glia, providing new models to study alpha-synuclein biology in patient-tailored brain cells.

The work presented in this thesis aimed to establish a platform of novel models to further address questions relevant to α-synucleinopathies. We created a library of human induced pluripotent stem cell lines from patients diagnosed with familial Parkinson's disease (PD) and multiple system atrophy (MSA), as well as healthy controls, which we extensively characterized. Using these new cellular models, we generated defined regionalized cellular subtypes relevant for modelling PD and MSA, such as dopaminergic neurons, oligodendrocytes and astrocytes, using efficient differentiation protocols. In contrast to previous studies, we found that α-synuclein is transiently expressed in oligodendrocytes during development and in the adult human brain. We also devised a transgenic strategy for generating reporter lines, from which pure populations of astrocytes could be obtained. These human astrocytes were capable of releasing cytokines and chemokines in response to stressors, and readily took up α-synuclein from their surroundings, demonstrating their relevance in modelling of synucleinopathies. Braak's hypothesis suggests that the pathology starts in the peripheral nervous system and progresses to the central nervous system, based on clinical observations of Lewy pathology distribution. We found that following injection into the intestinal wall of rats, α-synuclein was transported via the vagal nerve to the brain, thereby strengthening the hypothesis postulated by Braak.

The models and cell systems presented in this thesis have provided unprecedented possibilities to address key questions relevant to the initiation and progression of α-synucleinopathies PD and MSA.

Key words

Stem cells, iPSC, Astrocytes, oligodendrocytes, alpha-synuclein, Parkinsons disease, MSA, Braak

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   Accumulation and spreading of α-synuclein oligomers in iPSC-derived human astrocytes
   Manuscript.

II. Aldrin-Kirk P, Davidsson M, **Holmqvist S**, Li JY, Björklund T.
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Abstract

Synucleinopthies are neurodegenerative diseases characterised by the formation of α-synuclein-rich intracellular inclusions in neurons and glia. Traditionally, animal models and immortalized cell lines have been used to investigate why and how these inclusions form, and how they impact on cellular function. Recent advances in cellular reprogramming and directed differentiation have enabled the generation of neurons and glia, providing new models to study alpha-synuclein biology in patient-tailored brain cells.

The work presented in this thesis aimed to establish a platform of novel models to further address questions relevant to α-synucleinopathies. We created a library of human induced pluripotent stem cell lines from patients diagnosed with familial Parkinson's disease (PD) and multiple system atrophy (MSA), as well as healthy controls, which we extensively characterized. Using these new cellular models, we generated defined regionalized cellular subtypes relevant for modelling PD and MSA, such as dopaminergic neurons, oligodendrocytes and astrocytes, using efficient differentiation protocols. In contrast to previous studies, we found that α-synuclein is transiently expressed in oligodendrocytes during development and in the adult human brain. We also devised a transgenic strategy for generating reporter lines, from which pure populations of astrocytes could be obtained. These human astrocytes were capable of releasing cytokines and chemokines in response to stressors, and readily took up α-synuclein from their surroundings, demonstrating their relevance in modelling of synucleinopathies. Braak's hypothesis suggests that the pathology starts in the peripheral nervous system and progresses to the central nervous system (CNS), based on clinical observations of Lewy pathology distribution. We found that following injection into the intestinal wall of rats, α-synuclein was transported via the vagal nerve to the brain, thereby strengthening the hypothesis postulated by Braak.

The models and cell systems presented in this thesis have provided unprecedented possibilities to address key questions relevant to the initiation and progression of α-synucleinopathies PD and MSA.

Populärvetenskaplig sammanfattning

Parkinsons sjukdom och multipel systematrofi är exempel på alfa-synuklein sjukdomar. Parkinsons sjukdom upptäcks oftast då tidiga symptom som skakningar och muskelstelhet ger den drabbade problem att utföra vardagliga ting som att skriva eller att använda en smartphone. Problemen med rörelser beror på att nervceller dör i delar av hjärnstammen som har stor betydelse för just rörelser.

Man vet inte varför sjukdomsförloppet startar, men vid Parkinsons sjukdom hittar man anhopningar bestående av proteinet alfa-synuklein, inte bara i nervceller utan också i gliaceller, vars funktion är att stödja nervcellerna. Traditionellt har man använt sig av djur och enkla cellmodeller för att ta reda på varför och hur dessa anhopningar bildas, vilken påverkan de har på cellers funktion och om detta bidrar till att nervceller dör. I hjärnan finns tusentals olika nervceller och gliaceller och de är dramatiskt olika. Det är därför viktigt att använda sig av mänskliga celler som verkligen representerar de celler som påverkas vid Parkinsons sjukdom.

I denna avhandling har jag utvecklat nya modeller för att studera alfa-synukleiproteinets påverkan på hjärnceller. Ny teknik gör det möjligt att omvandla patienters hud- celler till, först stamceller och sedan nervceller samt glia celler. På så sätt har vi skapat en stor samling av patient-specifika stamcellslinjer och, utifrån dessa, genererat de celltyper som påverkas vid Parkinsons sjukdom. Cellerna är inte endast av rätt typ, de har även relevant genetisk bakgrund, vilket gör dem lämpliga för studier av de bakomliggande mekanismerna vid Parkinsons sjukdom. Dessutom visar vi att oligodendrocyter, en typ av glia- celler, producerar eget alfa-synuklein, vilket kan visa sig vara av betydelse vid utvecklandet av multipel systematrofi. Vidare har vi utvecklat ett genetiskt verktyg och en strategi för att kunna studera astrocyter, en annan typ av stödjande gliaceller. Vi visar att astrocyter kan ta upp och sedan bryta ner alfa-synuclein i sin omgivning.

Anhopningar av alfa-synuclein, förekommer inte endast i hjärnan, utan hittas vid ett tidigt stadium, även i de nervceller som finns kring tarmarna. Det har därför föreslagits att Parkinsons sjukdom börjar i tarmen och sedan sprids via de sammankopplade nervbanorna till hjärnan. Genom att injicera alfa-synuklein i tarmväggen på råttor och sedan analysera råttornas nervbanor och hjärna, visar vi att alfa-synuklein kan transporteras från tarmen till hjärnan. Dessa resultat indikerar att en möjlig behandlingsstrategi vid utvecklandet av Parkinsons sjukdom, kan vara att bromsa eller förhindra denna spridning av alfa-synuclein.

De modeller och cellodlingsustrategier som presenteras i denna avhandling öppnar för nya möjligheter att studera hur alfa-synucleinsjukdomar som Parkinsons och multipel system atrofi börjar utvecklas.

Abbreviations

AA	Ascorbic acid
ANOVA	Analysis of variance
Atto488	Commercial fluorochrome
BAC	Bacterial artificial chromosome
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BiFC	Bimolecular complentation fluorescence
bp	Base pairs
BSA	Bovine serum albumin
CBD	Cortico basal degeneration
cDNA	Complementary DNA
CDNF	Cerebral dopamine neurotrophic factor
ChAT	Choline acetyltransferas
CHIR	Aminopyrimidine derivative inhibitor of GSK3 (CHIR99021)
CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS	Central Nervous system
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9
CSC	Stem Cell laboratory for CNS disease modelling
DAPI	4',6-diamidino-2-phenylindole
DAT	Dopamine transporter
db-cAMP	N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt
DEAE	Diethylaminoethyl
DLB	Dementia with Lewy bodies
DMEM	Dulbecco's Modified Eagle Medium
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMV	Dorsal motor nucleus of the vagus
EB	Embryoid body
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay

ER	Endoplasmatic reticulum
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF2	Basic fibroblast growth factor
FRAP	Fluorescen recovery after photobleach
GCH1	GTP cyclohydrogenas 1
GCI	Glial cytoplasmic inclusions
GDNF	Glial cell derived neurotrophic factor
GFA_{ABC1D}	GFAP promoter fragment
GFAP	Glial fibrillary acid protein
GPC	Glial progenitors cell
GTPase	Hydrolyze guanosine triphosphate
HBSS	Hanks buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HN	Hypoglossal nuclei
ICC	Immunocytochemistry
IGF1	Insulin-like growth factor
IHC	Immunohistochemistry
iPSC	Induced pluripotent stem cell
L-DOPA	L-3,4-dihydroxyphenylalanine
Lam	Laminin
LDN	Small molecule inhibitor of BMP type I receptors ALK2 and ALK3 (LDN193189)
LRRK2	Leucine-Rich Repeat Kinase 2
MAO-B	Monoamine oxidase B
MBP	Myelin binding protein
MOG	Myelin oligodendrocyte glycoprotein
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Mitochondrial ribonucleic acid

MSA	Multiple system atrophy
MSA	Multiple system atrophy
MSA-C	Multiple system atrophy Cereberal
MSA-P	Multiple system atrophy Parkinsonian
NAC	Non-Abeta component
NDM	Neural induction medium
NEAA	Non essential aminoacids
NEUN	Feminizing Locus on X-3
NFkβ	nuclear factor kapa beta
NIM	Neural induction medium
NPC	Neural progenitor cell
NT3	Neurotrophin 3
o.n.	Over night
OLN-93	Glial cell line
OPC	Oligodendrocyte progenitor cell
P/S	Penicillin Streptomycin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PDGFR	Platelet-derived growth factor receptor, alpha polypeptide
PDL	Poly-D-lysine
PFA	Paraformaldehyde
PLO	Poly-L-ornithine
PrP	Prion protein
PSC	Pluripotent stem cell
PSF	Point-spread functions
PSP	Progressive supranuclear palsy
qPCR	Quantitative polymerase chain reaction
RAB	G-protein
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RT	Room temperature

RT-qPCR	Real-time polymerase chain reaction
SAG	Smoothened agonist
SB	Onhibitor of the transforming growth factor-beta 1 (TGF-β1) activin receptor-like kinases ALK-4, -5 and -7 (SB431542)
SHH	Sonic hedgehog
SNARE	Soluble NSF Attachment Protein receptor
SNCA	Synuclein alpha
SOX10	SRY-related HMG-box 10
T3	Triiodothyronine
TagRFP	Tagged Red fluorescent protein
TH	Tyrosine hydroxylase
TRAP	Telomeric Repeat Amplification Protocol
v/v	Weight per volume
WiCell	Wisconsin Cell (Research Institute in Madison)
Y	Rho kinase inhibitor (Y-27632)

Introduction

I Synucleinopathies: Focus on PD and MSA

Emerging reprograming technologies have allowed the conversion of patient skin cells into pluripotent stem cells that can further be differentiated into patient-tailored cells, for disease modelling e.g. neurons and glia for human brain diseases. The aim of the research presented in this thesis was to establish new rodent *in vivo* models and *in vitro* stem cell-based models for studying onset and progression of synucleinopathies.

Synucleinopathies is an umbrella term covering three main disorders, Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), but also including rare forms of neuroaxonal dystrophy. PD, DLB and MSA are clinically different but share their pathology is associated with abnormal cytoplasmic accumulation of protein-rich inclusions in neurons and/or glial cells. These inclusions are mainly composed of a protein called α-synuclein (Spillantini et al., 1997), and are known as Lewy bodies when located in cell bodies and Lewy neurites when located in neuronal processes and terminals. Lewy bodies and Lewy neurits will in this thesis be collectively referred to as Lewy pathology. Lewy pathology is mainly found in neurons in the brain of PD and DLB patients. In the brain of MSA patients, however, Lewy pathology is mainly observed in glial cells, more precisely in oligodendrocytes. In MSA, α-synuclein-containing inclusions are referred to as glial cytoplasmic inclusions (GCIs). MSA is comprised of two distinct subtypes, a parkinsonian subtype with nigral degeneration (MSA-P) and a cerebellar subtype with pontocerebellar degeneration (MSA-C) (Ghaemi et al., 2002). The contribution of Lewy bodies and Lewy neurites to synucleinopathies is still unclear, and it is currently unknown if the prevalence of Lewy pathology leads to neuronal loss, has no significant role, or is the result of cells sequestering misfolded protein in a harmless form.

The *SNCA* gene encodes α-synuclein protein, and is one of several genes associated with PD. Genetically associated familial forms of PD account for only a few per cent of all cases, rendering most cases considered idiopathic. Epidemiological studies have found an association between increased risk of PD and exposure to environmental toxicants such as pesticides, solvents, metals, and other pollutants, and many of these compounds recapitulate some aspects of PD pathology in animal models.

The native function of α-synuclein is still largely unknown, but it has been suggested that the protein is involved in synaptic vesicle release and thus neurotransmission. In neurons, α-synuclein is predominantly expressed in presynaptic terminals. The α-synuclein protein structure conveys a tendency to aggregate through a series of intermediates, eventually forming fibrils, the main components of Lewy bodies. Since glial cells are not known to express α-synuclein, the origin of α-synuclein in GCIs in MSA is still elusive, as is that located in oligodendrocytes and astrocytes in the brains of people with PD.

In this thesis, we aimed to develop new models that would allow us, and the scientific community, to investigate α-synuclein pathology, from its expression to its transfer and spread.

Disease Staging

Staging in PD

The neuropathological hallmark of PD is the loss of dopaminergic neurons in a region of the brain called the substantia nigra pars compacta, which originates from the developing ventral midbrain. Lewy pathology is observed in the remaining surviving dopamine neurons, and other neuronal cells, depending on the stage of the disease (Lebouvier et al., 2009). Indeed, evidence suggests that these neuropathological features are not only restricted to dopaminergic neurons, but are also widely exhibited in non-dopaminergic systems. The progressive spread of Lewy pathology, characterised by α-synuclein immunopositive inclusions, marks the core of the neuropathological staging in most cases of PD.

Staging is based on clinical observations of the topographic and temporal distribution of Lewy bodies in post-mortem tissue (Figure 1). The inclusions first appear in the peripheral nervous system (e.g. enteric neurons in the gut, stage 1), then in lower brain regions, such as the dorsal motor nucleus of the vagus (DMV, stage 2) and to the substantia nigra (where the primary lesion of dopaminergic neurons locates in PD, stage 3). Eventually, inclusions can be detected in the cerebral cortex (associated with dementia, stage 4-6). These findings give rise to the notion that a neurotropic pathogen could penetrate the gut epithelium and enter axons of the enteric neurons in the myenteric plexus (Auerbach's plexus) - these neurons control the activity of gut smooth muscles, and/or axons of the submucosal plexus (Meissner's plexus), in which regulates mucosal secretion and blood flow. Braak and colleagues hypothesized that this pathogen might spread via retrograde transport to different interconnected brain regions (Braak et al., 2006; Braak & Del Tredici, 2009; Hawkes et al., 2009). In Braak's observations, Lewy pathology first manifests in enteric neurons of the gut (stage 1) long before it is present in dopaminergic neurons of the midbrain and PD symptoms are evident (stage 3) (Braak, Del Tredici, et al., 2003). The gap between stages 1 and 3 may take many years. From the periphery, the

pathology gains access to the lower brainstem via the vagal nerve and then follows an ascending pathway reaching the substantia nigra and subsequently, the cerebral cortex (Lerner & Bagic, 2008; Phillips et al., 2008).

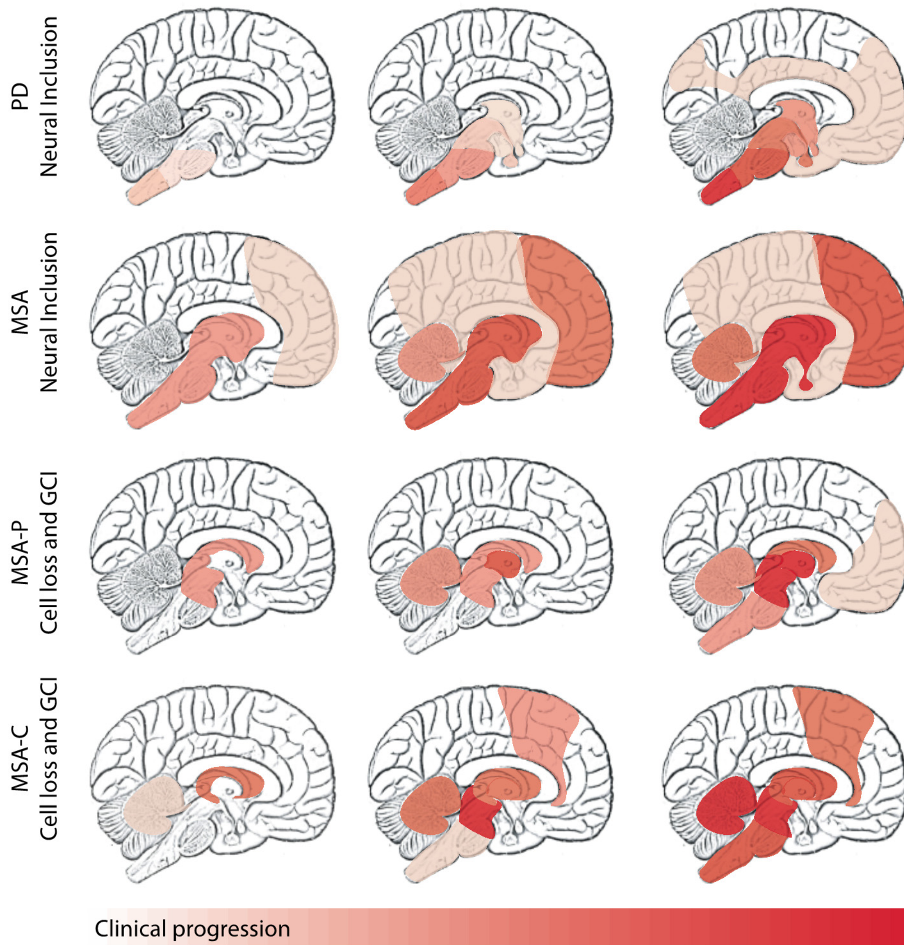

Figure 1.
Distribution of inclusions in PD and MSA brains during disease progression. Adopted from Braak et al., 2006 and Halliday et al., 2015.

Staging in MSA

Disease progression in MSA patients has of late become a topic of growing interest. MSA affects different types of brain cells and inclusions are found in multiple neural systems of the brain, hence the name MSA (Dickson et al., 1999; Papp & Lantos, 1994). Traditionally, neuronal inclusions in MSA were considered to occur in low

numbers, and their topographic distribution is not as well defined as that of the prominent GCIs found in oligodendrocytes. Remarkably, the few studies that revisited this topic were limited to selected brain regions displaying GCIs (Arai et al., 1994; Arima et al., 1992; Benarroch, 2007; Braak, Rub, et al., 2003; Yoshida, 2007). However, α-synuclein containing neuronal inclusions present in other brain regions, were identified in people with MSA using modern immunohistochemistry (IHC) methods (Cykowski et al., 2015; Yoshida, 2007). Similar to the distribution of Lewy pathology in PD, it was shown that neuronal cellular inclusions are topographically and progressively distributed in a hierarchal pattern. Neuronal inclusions are also observed in regions previously not implicated in MSA and in the absence of GCIs. If validated, these findings could provide a basis for disease staging in MSA similar to that of PD. Furthermore, these findings challenge the current view that MSA primarily is a glial disorder with only secondary involvement of neurons, and may suggest the α-synuclein pathology could originate in neurons, with glial pathology being secondary (Halliday, 2015). However, neuronal inclusions correlate neither to cell loss, nor to clinical phenotype. To clarify the implication of neuronal inclusions in MSA, further studies are required. These should focus on, but not restrict to, 1) primary tissue changes occurring at various stages, 2) how connected neural pathways and networks are involved at earlier time points, and 3) studies of genetic and epigenetic contribution to disease initiation. To address these points, relevant human models are necessary.

Genetics of Parkinson's disease

Parkinson's disease was long considered a non-genetic and purely sporadic disorder, but over the course of time, high prevalence of the disease in certain families attracted the attention of researchers. These familial forms of PD accounted only for a minority of the total number of cases but suggested a genetic, and thus, hereditary aspect in the development of PD. Consequently, in 1997 the *SNCA* gene (OMIM-163890) encoding for the α-synuclein protein was the first gene, identified to be associated with PD, further indicating that PD might be hereditary (Polymeropoulos et al., 1996; Polymeropoulos et al., 1997). Soon after these landmark studies, several reports on families with PD, lacking a link to *SNCA* gene appeared, revealing PD to be genetically heterogeneous. Since then, a large number of monogenic PD-loci have been described (Table 1). Mutations in *SNCA* (*PARK1*, *PARK4*), *LRRK2* (*PARK8*) and VPS35 (*PARK17*) cause autosomal dominantly inherited PD, while mutations in *PINK1* (*PARK6*), *DJ-1* (*PARK7*), *PARKIN* (*PARK2*), *ATP12A2* (*PARK9*), *PLA2GB* (*PARK14*) and *FBXO7 (PARK15*) cause autosomal recessive PD and parkinsonism (Hernandez et al., 2016).

Collectively, monogenic forms of PD explain aproximately 30% of familial cases and only a limited number (less than 5%) of all cases (Kumar et al., 2011), suggesting

that PD is a multifactorial complex disease caused by an elaborate interplay between mostly unknown genetic and environmental factors, and these factors impact on the developing and aging brain, therefore leading to disease onset. Additional genes have been associated with PD, but are less studied (Hernandez et al., 2016).

In recent years, genome-wide association studies have examined large cohorts of PD subjects and identified a great number of risk loci (Satake et al., 2009; Simon-Sanchez et al., 2009). Notably, a recent study identified or confirmed 28 different risk loci, one of them being GTP cyclohydrogenase 1 (GCH1), an enzyme involved in the synthesis of a co-factor for tyrosine hydroxylase (TH), the enzyme responsible for the conversion of L-tyrosine into levodopa (L-DOPA), the direct precursor of dopamine (Nalls et al., 2014)

Table 1.
Loci involved in monogenic forms of PD (Hernandez et al., 2016)

Locus	Gene	Protein	Mode	OMIM®
4q22.1	*SNCA, PARK1,4* [1]	α-synuclein [2]	Autosomal dominant	163890
6q26	*PARK2*	Parkin	Autosomal dominant	600116
1p36.12	*PINK1, PARK6*	Pten-induced putative kinase 1	Autosomal recessive	605909
1p36.23	*PARK7*	DJ-1	Autosomal recessive	606324
12q12	*LRRK2, PARK8*	Leucine-rich repeat kinase 2	Autosomal dominant	607060
1p36.13	*ATP12A2, PARK9*	Lysosomal type 5 ATPase	Autosomal recessive	606693
22q13.1	*PLA2GS, PARK14*	Phospholipase A2	Autosomal recessive	603604
22q12.3	*FBXO7, PARK15*	F-box only protein 7	Autosomal recessive	605648
16q11.2	*VPS35, PARK17*	Vacuolar protein sorting 35	Autosomal dominant	601501

[1] NACP, PD1 [2] alpha-synuclein, non-A-beta component of AD amyloid,

The *SNCA* gene

Initially, a guanine-to-adenine mutation (A53T, exon 4) was found consecutively in one Italian and two Greek PD families. Soon after this discovery, α-synuclein was revealed as the main proteinous component of Lewy bodies (Spillantini et al., 1997), linking the rare familial forms of PD to idiopathic PD cases. To date, five mutations in the *SNCA* gene (A53T, A30P, E46K, G51D, and H50Q), as well as multiplications (duplication and triplication) of the full locus, have been identified (Chartier-Harlin et al., 2004; Ibanez et al., 2004; Singleton et al., 2003). A53T point mutation and multiplications have been found in several families while the remaining mis-sense mutations have only been found in one family each (Kruger et al., 1998;

Zarranz et al., 2004). Clinically, mutations in *SNCA* are generally associated with early disease onset, as well as L-dopa responsive Parkinsonism, autonomic dysfunction and cognitive decline; moreover, it was identified that disease penetrance varies between mutation (Kruger et al., 1998; Papapetropoulos et al., 2001; Zarranz et al., 2004). Furthermore, triplications and duplications of the full gene region have been identified in a handful of families developing early onset PD. Notably, multiplications of the *SNCA* locus cause an increase in *SNCA* mRNA levels and protein in brain tissue, and triplications of the *SNCA* locus lead to full disease penetrance and early and rapid progression, while duplications lead to delayed disease onset, with a phenotype that in general is more benign. It is interesting to note that both the severity of the PD phenotype and the age of onset correlate with the number of *SNCA* copies, suggesting contribution of a gene-dose effect in PD (Chartier-Harlin et al., 2004; Farrer et al., 2004). This supports the finding that specific polymorphisms in the *SNCA* promoter experimentally can lead to higher expression of a target gene. Although the these polymorphisms have not been linked to PD, it has been suggested that *SNCA* promoter polymorphisms may lead to altered expression levels and contribute to PD.

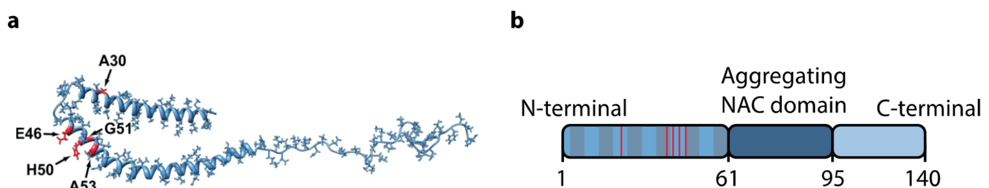

Figure 2.
a) 3D model of α-synuclein cystal structure as bound to lipid vesicle b) Schematic of protein domains of α-synuclein protein. N-terminal membrane interacting region, central aggregation-prone NAC region and unstructured C-terminal region implicated with nuclear localization.

II Biology of α-synuclein.

Protein Structure

Mutations in the *SNCA* gene have established α-synuclein as being critical in PD. Considering this, remarkably little is known about the normal function of α-synuclein. *SNCA* encodes a 140 amino acid protein that is abundantly expressed in the central and peripheral nervous systems (Braak & Del Tredici, 2009; Iwai et al., 1995) (Figure 2). The protein lacks a well-defined structure in aqueous solution but exists in several quaternary states, from unfolded monomers to low molecular weight

oligomers to higher order amyloid fibrils, the latter being detected in Lewy bodies. α-Synuclein is composed of three distinct regions: a) an amino-terminus region (residues 1-60), crucial for mediating α-helical conformation shift upon binding to membranes (George et al., 1995), b) an unstructured c-terminus region, consisting of an acidic carboxyl terminal tail (96-140) implicated in nuclear localization and interaction of the protein with metals and small proteins (Ulmer et al., 2005), and c) a central region, containing a highly hydrophobic motif known as non-amyloid-beta component of amyloid AD plaques (NAC, 61-95) (Ueda et al., 1993). Studies of protein dynamics have shown that the NAC-region is essential for the formation of amyloid beta-sheets and α-synuclein aggregation (El-Agnaf et al., 1998; Giasson et al., 2001; Luk et al., 2009). The sequence of this region separates α-synuclein from other members of the synuclein protein family, since it is not conserved between the paralogues alpha-, beta- and gamma-synuclein. Interestingly, three alternatively spliced α-synuclein transcript isoforms containing the NAC region exist, but their physiological role is not well characterized (Cardo et al., 2014; Maroteaux & Scheller, 1991; Ueda et al., 1993).

Cellular localization, function and propagation

α-Synuclein in neurons

Synucleins were first identified using antisera created by immunizing rabbits with cholinergic synaptic vesicles purified from electric ray (*Narcine brasiliensis*). Immunolabelling using rodent tissue suggested a subcellular localization of synucleins associated with the nuclear envelope and the presynaptic terminal structures (Iwai et al., 1995; Maroteaux et al., 1988; Maroteaux & Scheller, 1991), a finding that coined the name synucleins. It was also proposed that these proteins were specifically expressed in neurons; this was later proven to be false (Barbour et al., 2008), as synucleins were identified in platelets and erythrocytes. Since these initial findings, α-synuclein has been shown to be abundantly expressed in neurons and is found not only in presynaptic terminals, but also within nuclei (Goers et al., 2003; Goncalves & Outeiro, 2013; Mori et al., 2002b), as well as in association with mitochondria (Cole et al., 2008; Devi et al., 2008; Shavali et al., 2008) and lipid rafts of mitochondria-associated ER membranes (Fortin et al., 2004; Guardia-Laguarta et al., 2014). Furthermore, most *SNCA* mutations associated with PD are located in the membrane interacting region. α-Synuclein preferably binds to high curvature membranes enriched in cholesterol, sphingolipids, and acidic phospholipids (Jensen et al., 2011) with greater affinity for high lipid:protein ratio membranes (De Franceschi et al., 2011).

In 1995, a study on song learning in juvenile Zebra finches reported on changed levels of mRNA transcripts in regions involved with control of song-learning. These regions showed sustained reductions in *Snca* expression during the period of song

learning. These results were supported by other studies demonstrating that α-synuclein levels are modulated in conditions that confer injury (George et al., 1995; Kholodilov et al., 1999; Vila et al., 2000) or affect neuronal plasticity, showing that α-synuclein could be a modulator of neurotransmission. A large number of studies have been oriented towards the presynaptic function of synucleins. Notably, studies using *SNCA* null mice or cellular systems where wildtype α-synuclein is overexpressed, have shown, although not conclusively, that α-synuclein acts at the synapse as a break for neurotransmitter release (Fortin et al., 2005; Murphy et al., 2000). Other work indicates a regulatory function of the protein by interaction with synaptic proteins such as phospholipase D2 (Jenco et al., 1998; Payton et al., 2004) and the family of RAB small GTPases (Dalfo & Ferrer, 2005), and acting as chaperon for the SNARE protein complex (Burre et al., 2010; Chandra et al., 2005). Previous studies also implicate α-synuclein in mitochondrial activity (Dauer et al., 2002), chaperon activity (Bennett, 2005; Chandra et al., 2005) and microtubule stabilisation (Alim et al., 2002).

In summary, α-synuclein is not essential for synaptic transmission in general, but may provide long-term regulation and maintenance of the synaptic terminals (Chandra et al., 2004).

α-Synuclein in oligodendrocytes

Glial cytoplasmic inclusions, distinctive for MSA, are found in oligodendrocytes. These are characterized by their main component, α-synuclein protein (Kato & Nakamura, 1990; Papp et al., 1989; Tu et al., 1998; Wakabayashi et al., 1998). The distribution and occurrence of α-synuclein rich GCIs in oligodendrocytes indicates that these may play a pivotal role at disease onset and during its progression (Ozawa et al., 2001; Wenning et al., 1994; Wenning et al., 2008). The source of α-synuclein and how GCIs are formed is however still debated. Earlier studies suggested that α-synuclein present in GCIs has an exclusively neuronal origin since *SNCA* is expressed in neurons, and not in oligodendrocytes (Miller et al., 2005; Ozawa et al., 2001; Solano et al., 2000). Thus, it was suggested that α-synuclein might be released by injured neurons and actively taken up and accumulated by oligodendrocytes. However, since it was difficult to know whether neuronal injury preceded GCIs formation, it has also been postulated that an increase of *SNCA* expression could, over time, lead to increased α-synuclein cellular accumulation and consequent aggregation. This aggregation and formation of GCIs may lead to oligodendrocyte dysfunction and subsequent neuronal injury (Fellner et al., 2011; Ubhi et al., 2011).

The hypothesis of oligodendroglial uptake is supported by several studies demonstrating neuronal release (Emmanouilidou et al., 2010; Hansen et al., 2011) and cell-to-cell transfer (Hansen et al., 2011; Luk et al., 2012) of α-synuclein. Furthermore, mechanisms of oligodendrocyte incorporation of α-synuclein have been described *in vitro* (Kisos et al., 2012). However, none of the studies observed the formation of GCI-like structures, including those employing inoculation of MSA

brain lysates into the brains of healthy mice (Ozawa et al., 2004; Prusiner et al., 2015). Perhaps healthy oligodendrocytes can handle and degrade exogenous α-synuclein and GCI formation is dependent on functionally impaired oligodendrocytes (Wenning et al., 2008).

In 2000, Richter-Landsberg and colleagues, reported on the transient expression of *Snca* in rodent glial cultures enriched in oligodendrocytes (Richter-Landsberg et al., 2000). In contrast, several studies showed a lack of *SNCA* mRNA in oligodendroglial cells (Miller et al., 2005; Ozawa et al., 2001; Solano et al., 2000). Interestingly, the finding by Richter-Landsberg and colleagues was neither confirmed nor further explored until Asi and colleagues, 14 years later, reported the detection of *SNCA* transcripts in post mortem brains by expression analysis of laser-capture dissected oligodendrocytes from the brain of control and MSA patients (Asi et al., 2014). Although not statistically significant, there was a trend towards increased *SNCA* levels in the MSA samples, demonstrating that *SNCA* is expressed in human oligodendrocytes, and supporting the hypothesis that a slight increase, over decades, might contribute to endogenous α-synuclein accumulation and GCI formation in MSA oligodendrocytes. The cellular mechanisms of GCI formation and altered oligodendrocyte function remain unknown. Patient-derived oligodendrocytes generated through reprogramming technologies (induced pluripotent stem cells and directed reprogramming) may help us identify the first steps leading to α-synuclein accumulation and GCI formation, and provide new insights into the mechanisms behind MSA.

α-Synuclein in Astrocytes

Astrocytes are involved in a wide range of brain functions and are no longer considered merely passive homeostasis-maintaining cells of the brain. Their role in maintaining brain homeostasis in terms of pH (Obara et al., 2008), ion concentration, fluids (Simard & Nedergaard, 2004), and neurotransmitters (Sattler & Rothstein, 2006) is well established. During the last two decades, further functional roles have emerged, including formation and maintenance of the blood-brain barrier (BBB) (Abbott et al., 2010), regulation of blood flow (Carmignoto & Gomez-Gonzalo, 2010; Koehler et al., 2009), metabolic regulation (A. M. Brown & Ransom, 2007), formation and pruning of synapses, co-ordination of synaptic and non-synaptic signaling for surrounding cells (Hirrlinger et al., 2004; Volterra & Meldolesi, 2005) and facilitating neurotransmission in what has become known as the tripartite synapse (Halassa & Haydon, 2010).

The most direct evidence of astrocyte involvement in dopaminergic cell loss in the substantia nigra is that the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) cause L-DOPA responsive parkinsonism (Langston et al., 1983). MPTP is lipophilic and readily crosses the BBB. In astrocytes, MPTP is metabolized by monoamine oxidase B (MAO-B) to toxic MPP^+. After release from the astrocytes, MPP^+ is taken up by dopaminergic neurons via the dopamine

transporter (DAT), where it induces neurotoxicity by inhibiting complex I of the mitochondrial respiratory chain. Since the discovery that MPTP causes Parkinsonism, this neurotoxin has been used for creating animal models of PD. An increase in MAO-B expression has not been observed in PD, but enzyme levels increase with age, which is a risk factor. MAO-B activity also correlates with cell loss in the substantia nigra (Fowler et al., 1980; Mahy et al., 2000). Furthermore overexpression of MAO-B under the glial fibrillary acidic protein (GFAP) promoter in mice causes age-related motor dysfunction and degeneration of neurons in the substantia nigra, suggesting that increased MAO-B expression and activity is sufficient to induce Parkinsonism (Mallajosyula et al., 2008). In mice, MPTP toxicity and loss of TH fibres is to some extent dependent on astrocytic expression of myeloperoxidase (MPO), an oxidant-producing enzyme that is up-regulated in the ventral midbrain of PD patients, suggesting that its inhibitors might be of therapeutic value.

All astrocytes express the PD associated genes *PINK1*, *DJ1* and *PARKIN*. Protoplasmic astrocytes also express *PACRG*, in contrast to fibrous astrocytes (Song et al., 2009). The supportive function that astrocytes have on neurons has been directly addressed using a *DJ1* knockdown model, as astrocyte specific *DJ1* knockdown was found to impair their neuroprotective properties. These findings support that astrocytic dysfunction may contribute to the progression of neurodegenerative disorders in general, and of PD in particular (Larsen et al., 2011; Mullett & Hinkle, 2009). There is no clear evidence that α-synuclein is expressed in astrocytes (Gu et al., 2010); still, α-synuclein immunoreactive astrocytes are observed in cases of PD (Hishikawa et al., 2001; Shoji et al., 2000; Takeda et al., 2000; Terada et al., 2003; Wakabayashi et al., 2000), raising the question of the origin of α-synuclein located in the astrocytic inclusions in PD? Abnormal accumulation of α-synuclein in astrocytes correlates with neuronal degeneration in the substantia nigra (Mori et al., 2002a; Wakabayashi et al., 2000), as well as the distribution of neural inclusions and disease severity (Braak et al., 2007). Reports of astrogliosis observed in post mortem PD brains are inconclusive and astrocyte activation has been described as pronounced (Hirsch et al., 2005), mild (Vila et al., 2001) or minimal (Mirza et al., 2000), suggesting large variability in astrocytic activation among PD patients. Interestingly, Song and colleagues compared the prevalence of α-synuclein and astrogliosis between PD, MSA, progressive supranuclear palsy (PSP), and cortico basal degeneration (CBD) patients (Song et al., 2009). They found that astrocytic inclusions paralleled the spread of neuronal pathology, regardless of the disease. However, in contrast to PSP and -, protoplasmic astrocytes in PD and MSA did not display upregulation of GFAP, suggesting an attenuated protoplasmic astrocyte response in PD and MSA patient brains. It has also been experimentally demonstrated in cell systems that α-synuclein can propagate from neurons to astrocytes (H. J. Lee et al., 2010). Astrocytes also readily incorporate α-synuclein from their surrounding environment (Fellner et al., 2013).

In summary, astrocytes might have a central role in both initiation and progression of PD. Astrocytic release of neurotrophic (Saavedra et al., 2006; Sandhu et al., 2009), as well as pro- and anti-inflammatory factors, likely play a regulatory role in the potential accumulation of α-synuclein and the progression of PD. Whether α-synuclein can induce activation of astrocytes, and thus contribute to its regulatory role and disease initiation remains unknown.

α-Synuclein aggregation and propagation

Majority of brain regions affected in PD display inclusions consisting of aggregated α-synuclein protein. These inclusions are made up of many different "clumped-together" proteins that are involved in different aspects of cellular function: molecular chaperones that fold and re-fold proteins and ubiquitins that tag proteins for degradation by the proteasome system (Halliday et al., 2005; Uryu et al., 2006; Uversky, 2007). However, the main proteinaceous component of Lewy bodies is α-synuclein, specifically in its aggregated fibrillar form. α-Synuclein is considered to natively be of unfolded monomeric form. This assumption has recently been challenged by reports of a tetrameric natively folded form, and proposed that a shift in the balance between tetramers and monomers may play a role in the onset of PD (Bartels et al., 2011; Burre et al., 2013; Dettmer et al., 2015).

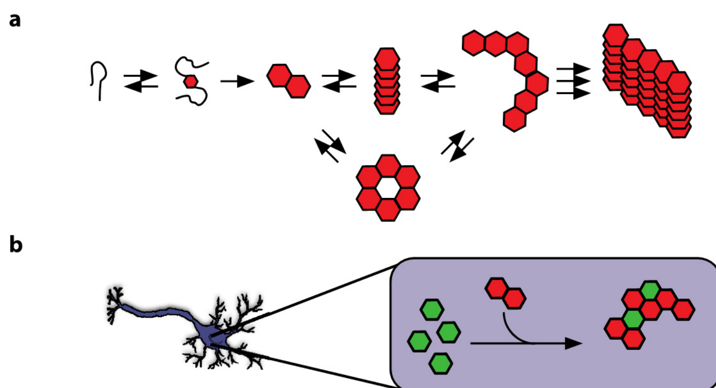

Figure 3.
a) Schematic of the aggregation process of α-synuclein in the formation of inclusions through a series of alternative intermediate forms. Adpated from Oueslati et al,. 2010 b) The pion-propagation hypthosis suggests misfolded α-synuclein acts as template for the conversion of native α-synuclein into misfolded form. α-synuclein translocated from one cell to next propagates the inclusion spread.

Experimentally, in physiological solution, α-synuclein exists as a mixture of disordered conformations of monomers (Weinreb et al., 1996). Many different factors have been reported to induce or modify α-synuclein fibril formation including, mutations and post-translational modifications such as phosphorylation, truncation, oxidation, cross-linking and ubiquitination (D. R. Brown, 2007; Hoyer et

al., 2004; Oueslati et al., 2010; Paik et al., 1999). It is interesting to note that many disease-associated mutations are not located in the β-sheet conveying NAC region, but in the membrane interacting N-terminal. Fibrils are produced through the formation of a series of oligomeric intermediates. Artificially, many different oligomeric intermediates can be generated, depending on formation conditions, generated oligomeric intermediates can be of varying sizes and stability, adopting spherical, ring-like or chain-like structures (Figure 3a) (Ding et al., 2002). Each of these oligomeric forms are accompanied by specific traits in terms of toxicity, membrane interaction, protein interaction and propensity to seed further aggregation (Danzer et al., 2009; H. Y. Kim et al., 2009; Lashuel et al., 2013).

PD is considered a protein misfolding disease, sharing similar traits with other neurodegenerative diseases including Alzheimer's disease and the motor neuron disease, amyotrophic lateral sclerosis, where protein aggregation is also identified. Braak and colleagues, to explain the spread of α-synuclein pathology, first suggested the hypothesis of a prion-like form of Lewy pathology propagation (Braak, Del Tredici, et al., 2003; Hardy, 2005). This hypothesis implied that misfolded α-synuclein would act as a template for the conversion of native α-synuclein and in the end, the formation of inclusions (Figure 3b). Indeed, experimental *in vitro* investigations have shown that α-synuclein transfers from neuron-neuron and can display such seeding-nucleation potential (Danzer et al., 2009; Iljina et al., 2016). Importantly, it was found that healthy dopamine transplanted into the brains of patients with PD, had developed Lewy pathology 20 years later (Kordower et al., 2008; Li et al., 2008). It is speculated that the transplanted cells were too young to have developed inclusions on their own, since they were derived from the foetal midbrain region of aborted human embryos. These findings suggested a transfer of α-synuclein from the host tissue to the grafted cells leading to inclusion formation, and supportive of the prion-like hypothesis.

However, there are important differences between PD and prion disorders, and it remains to be determined whether the aggregation of α-synuclein observed in the grafts was the result of a lack of proper regulation of dopamine release via the synapse and consequent aggregation of α-synuclein or a prion-like aggregation after seeding, since many of the grafts and most of the cells transplanted did not display any signs of α-synuclein accumulation; this is contrasted by the relatively rapid progression observed in patients suffering from prion disorders (Mendez et al., 2008). There is also a clear gene-dosage effect of *SNCA* in PD, as evident in cases of *SNCA* triplication; while over-expression of the prion protein (PrP) protein might be detrimental to cell function, it does not result in the development of prion disease (Bendor et al., 2013). Furthermore, PD develops over several decades while prion disorders usually manifest within weeks or even days.

III Emerging models to study α-synuclein pathology

Emerging stem cell Models

Numerous animal and cell line models have been developed and used in investigations of the disease mechanisms behind PD. However, cell lines used are usually immortalized and do not well represent human dopaminergic neurons and other cell types, affected in PD, animals require a lot of work and might show different vulnerability to disease-causing factors. Therefore many aspects of the underlying disease mechanisms behind PD are at the risk of being overlooked.

Induced pluripotent stem cells are a group of pluripotent stem cells that can be generated by over-expression of certain pluripotency transcription factors in somatic, terminally differentiated cells, such as skin fibroblasts. Less than a decade ago, it was reported that human 293T cells expressed T-cell gene functions following reprogramming using primary human T-cell extract (Hakelien et al., 2002). Soon after, Yamanaka and colleagues presented the "now routinely used" strategy of production of induced pluripotent stem cells (iPSC)s, where overexpression of rodent pluripotency genes *Sox2*, *Oct4*, *Klf4* and *c-Myc*, allows large-scale generation of iPSCs (Takahashi et al., 2007; Takahashi & Yamanaka, 2006).

Cell-reprogramming technologies have provided researchers with an unlimited access to patient derived cells, previously only accessible through post-mortem tissue. Each iPSC harbours a full representation of the patient's genetic landscape, which enables investigations of patient-specific models of genetic and sporadic diseases. Importantly, hopes are that iPSCs will provide terminally differentiated cells that can be used to develop completely humanized assays for high-content screens of neuroprotective compounds (Lamas et al., 2014; Y. M. Yang et al., 2013) and alternative therapeutic options. Cell reprogramming has also proven a useful resource for investigations of human cellular development (Figure 4).

To successfully apply cell-reprogramming technologies to the modelling of human brain diseases, it is essential to generate defined populations of human neurons and glia. Seminal work of directing pluripotent cells towards neural lineage by recapitulating embryonic development (Chambers et al., 2009; Munoz-Sanjuan et al., 2002; Ying et al., 2003; S. C. Zhang et al., 2001), has allowed efficient generation of neural progenitors.

Recent advances in culturing strategies allow generation of specific cell types of the midbrain identity required for modelling synucleinopathies *in vitro*. Naturally, most efforts have been directed towards generating functional dopaminergic neurons, capable of dopamine release and displaying expected electrophysiological characteristics (Chambers et al., 2009; Fasano et al., 2010; Kirkeby et al., 2012; Nat et al., 2007; Perrier et al., 2004; S. C. Zhang et al., 2001).

. Although protocols for generating bona fide astrocytes and oligodendrocytes exists (Jiang et al., 2013; Juopperi et al., 2012; Kondo et al., 2013; Krencik et al., 2011; Roybon et al., 2013; Serio et al., 2013; Shaltouki et al., 2013; Yuan et al., 2011), (Czepiel et al., 2011; Hu et al., 2009; Kang et al., 2007; Nistor et al., 2005; Stacpoole et al., 2013; Wang et al., 2013), few describe the generation of astrocytes specifically of midbrain identity, and none exist to generate midbrain oligodendrocytes. There is currently a lack of reliable markers or functional properties that can be used to distinguish between glial cells from different regions of the brain, unless positional identity markers such as *HOXB4* or *OTX2* are used. However, since glial cells and neurons share the same developmental origin in neural progenitor cells (NPC) and radial glia, protocols describing the generation of glia from regionally defined NPCs can be implemented. So far, no protocols have been presented for the differentiation of iPSC lines into microglia, however there have been reports of direct conversion of monocytes into immunologically functional microglia (Ohgidani et al., 2015).

These new human cellular models have provided an unprecedented venue for modelling the initiation and the progression of multifactorial and complex diseases like synucleinopathies.

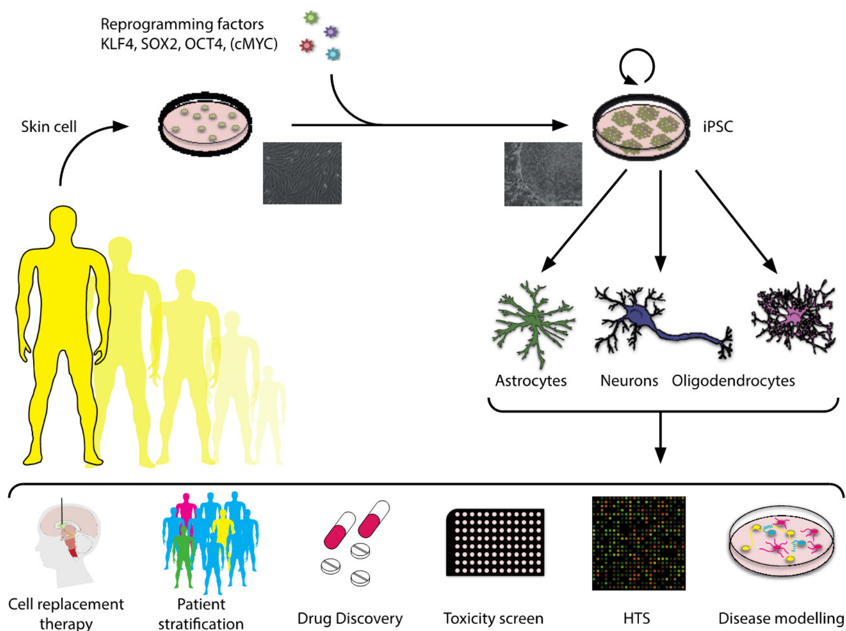

Figure 4.
Research strategies using induced pluriptotent stem cells. Skin biopsies from healthy or patient people are reprogrammed into stem cells by introducing stem cell transcription factors. The self-renewing iPSC cell lines are characterised and used to generate cell subtypes; astrocytes, neurons and oligodendrocytes for use in cell replacement therapy, patient stratification, drug discovery, hight content screens and disease modelling

Aims

The overarching goal of this thesis was to establish a platform of models to further address questions relevant to the initiation and progression of α-synucleinopathies, PD and MSA. We wanted to initiate our investigations, while developing models, by addressing a) whether α-synuclein could propagate from the periphery to the brain, b) whether it could be taken up by brains cells, mainly glia, and c) the extent by which brains cells in general do express α-synuclein.

Paper I:

- Address Braak's hypothesis and examine whether α-synuclein pathology can propagate from the gut to the brain
- Investigate the mechanism of transport of aggregating forms of α-synuclein.

Paper II:

- Generate a library of iPSC lines with genetics relevant to PD and MSA.
- Generate neural subtypes of cells for prospective modelling of synucleinopathies in a dish

Paper III:

- Critically review whether *SNCA* is expressed in oligodendrocytes
- Investigate whether *SNCA* is expressed during oligodendrocyte development and in oligodendrocytes in the adult brain.
- Examine *SNCA* expression in oligodendrocytes from the brains of MSA patients.

Paper IV:

- Generate astrocytes from embryonic stem cell (ESC) and iPSC lines
- Create stable astrocyte reporter lines for enrichment of and reporting on GFAP expressing astrocytes
- Evaluate the relevance of astrocyte reporter lines in *in vitro* modeling of PD

Results and Comments

Lewy pathological features of PD are not restricted to dopaminergic neurons; they are also widely distributed in non-dopaminergic systems correlating with disease severity. With increased clinical severity, α-synuclein containing inclusions progressively appear in anatomically interconnected brain regions, including cortical regions (Braak et al., 2006; Braak, Rub, et al., 2003; Phillips et al., 2008; Wakabayashi et al., 1990). During stage 1, Lewy pathology is identified in the peripheral nervous system (e.g. enteric neurons of the gut). In stage 2 and 3, it is observed in lower brain regions, such as the DMV and the substantia nigra (where the primary lesions of dopaminergic neurons locates). Eventually, in stages 4-6, inclusions can be found in the cerebral cortex.

Reprogramming of human somatic cells by over-expression of "stemness" transcription factors, into pluripotent iPSC has offered unlimited access to patient cells for the study of human diseases (Takahashi et al., 2007; Takahashi & Yamanaka, 2006). Seminal work where pluripotent cells are efficiently directed towards neural lineage progenitors, by recapitulating embryonic development (Chambers et al., 2009; Munoz-Sanjuan et al., 2002; Ying et al., 2003; S. C. Zhang et al., 2001), has enabled efficient generation of specific cell neural subtypes. Subsequent advances in culturing strategies have allowed the generation of specific cell types of the brain such as dopaminergic neurons (Chambers et al., 2009; Fasano et al., 2010; Kirkeby et al., 2012; Nat et al., 2007; Perrier et al., 2004; S. C. Zhang et al., 2001), oligodendrocytes (Czepiel et al., 2011; Hu et al., 2009; Kang et al., 2007; Nistor et al., 2005; Stacpoole et al., 2013; Wang et al., 2013) and astrocytes (Jiang et al., 2013; Juopperi et al., 2012; Kondo et al., 2013; Krencik et al., 2011; Roybon et al., 2013; Serio et al., 2013; Shaltouki et al., 2013; Yuan et al., 2011), from these neural progenitors. These findings have provided an unprecedented venue for modelling initiation and progression of multifactorial diseases like PD and MSA.

As previously mentioned, this thesis work aimed to address a) whether α-synuclein could propagate from the periphery to the brain, b) whether it could be taken up by brain cells, mainly glia, and c) evaluate by which extent brains cells in general express α-synuclein, having in mind the idea that exogenous α-synuclein could possibly seed aggregation of endogenous synuclein, therefore leading to its aggregation in a prion-like manner.

Paper I - α-Synuclein transport from the gut to the brain

α -Synuclein transport from the enteric nervous system to the brain

Lewy pathology is not restricted to dopaminergic neurons in the substantia nigra. A progressive topographical distribution of inclusions from the peripheral nervous system to the CNS is observed in the brain of people diagnosed with PD (Kosaka et al., 1984). Braak and colleagues hypothesised that a neurotropic pathogen that penetrates the gut epithelium and induces pathology, retrogradely spreads to interconnected brain regions (Braak & Del Tredici, 2009; Braak, Del Tredici, et al., 2003). α-Synuclein is propagated from neuron-to-neuron in experimental models (Desplats et al., 2009; Freundt et al., 2012; Hansen et al., 2011; Luk et al., 2009), including transplantation studies (Hansen et al., 2011). Experimentally, α-synuclein spreads along projection tracts after injection into the brains of mice (Luk et al., 2012; Peelaerts et al., 2015), leading to loss of dopaminerigic neurons, reduced dopamine levels and motor deficits.

Paper I aimed to determine whether α-synuclein protein could propagate from the enteric nervous system to the CNS. Wild type rats were injected with lysates containing human α-synuclein of mixed molecular weights, prepared from the brain of a patient with confirmed PD (Figure 5). Within 48 hours, α-synuclein was observed within axons of the vagal nerve, demonstrating that enteric neurons could take up α-synuclein and transport the protein via the vagal nerve to the brain. When recombinant labelled aggregated α-synuclein was injected, a similar time-dependent pattern of transport was observed.

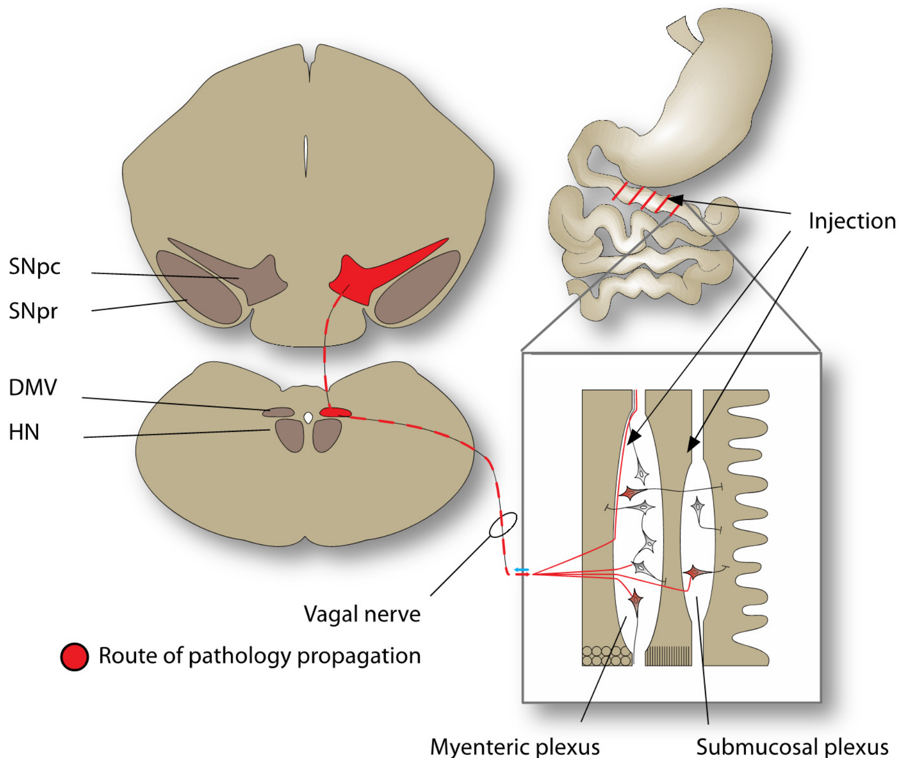

Figure 5.
Schematic of α-synuclein injection into rodent gut. Overview of the injection sites and pathways interconnecting the enteric nervous system and the CNS. DMV dorsal motor nucleus of the vagus, SNpc substantia nigra pars compacta, SNpr substantia nigra pars reticulata, HN hypoglossal nucleus.

α-Synuclein was not detected in the vagal nerve 12 hours after injection. Interestingly, in a parallel recently published study, where investigators injected α-synuclein into the olfactory bulb of mice, it was found that α-synuclein protein could spread to distant interconnected brain regions within few hours of injection (Reyes et al., 2014). This discrepancy in transport rate could be explained by the relative immune privilege of the brain compared to the gut. Here, most of the injected α-synuclein may be readily cleared away, leaving only a fraction of possibly aggregated synuclein subject to uptake and subsequently transport. Another explanation could be that the gut-to-brain propagation, in contrast to injections into the olfactory bulb, is mediated by a cell-cell and/or trans-synaptic dependent route of transport.

In order to verify that different forms of α-synuclein are transported, animals were injected with defined recombinant forms of α-synuclein (monomers, oligomers or fibrils). We found that 48 hours later, α-synuclein was transported to neurons of the DMV. After 6 days, α-synuclein was detected in Choline acetyltransferas ChAT^{+} cholinergic neurons of the DMV (Figure 6), suggesting α-synuclein was transported

to the brain regardless of its form. Notably, we did not observe any neuronal loss, astrogliosis or microglial activation in the midbrain of injected animals (data not shown). Interestingly, it has been shown that primary enteric neurons treated with the pesticide rotenone induce release of α-synuclein and formation of inclusions in surrounding cells (F. J. Pan-Montojo & Funk, 2010). A study, from the same group also demonstrated that loss of $ChAT^+$ neurons in the DMV is induced by oral administration of rotenone, which could be rescued by vagotomy (F. Pan-Montojo et al., 2012). In Paper I, we did not observe any cell loss in the DMV, which could indicate an inherent resilience to some levels of potentially pathogenic α-synuclein. Since rotenone is an inhibitor of complex I of the respiratory chain (Sherer et al., 2007), and has been reported to destabilize microtubules (Choi et al., 2008), one explanation for the observed cell loss reported by Pan-Montojo and colleagues could be due to the strong direct effect of rotenone on mitochondria residing in neuronal projections originating from the cell bodies in the DMV.

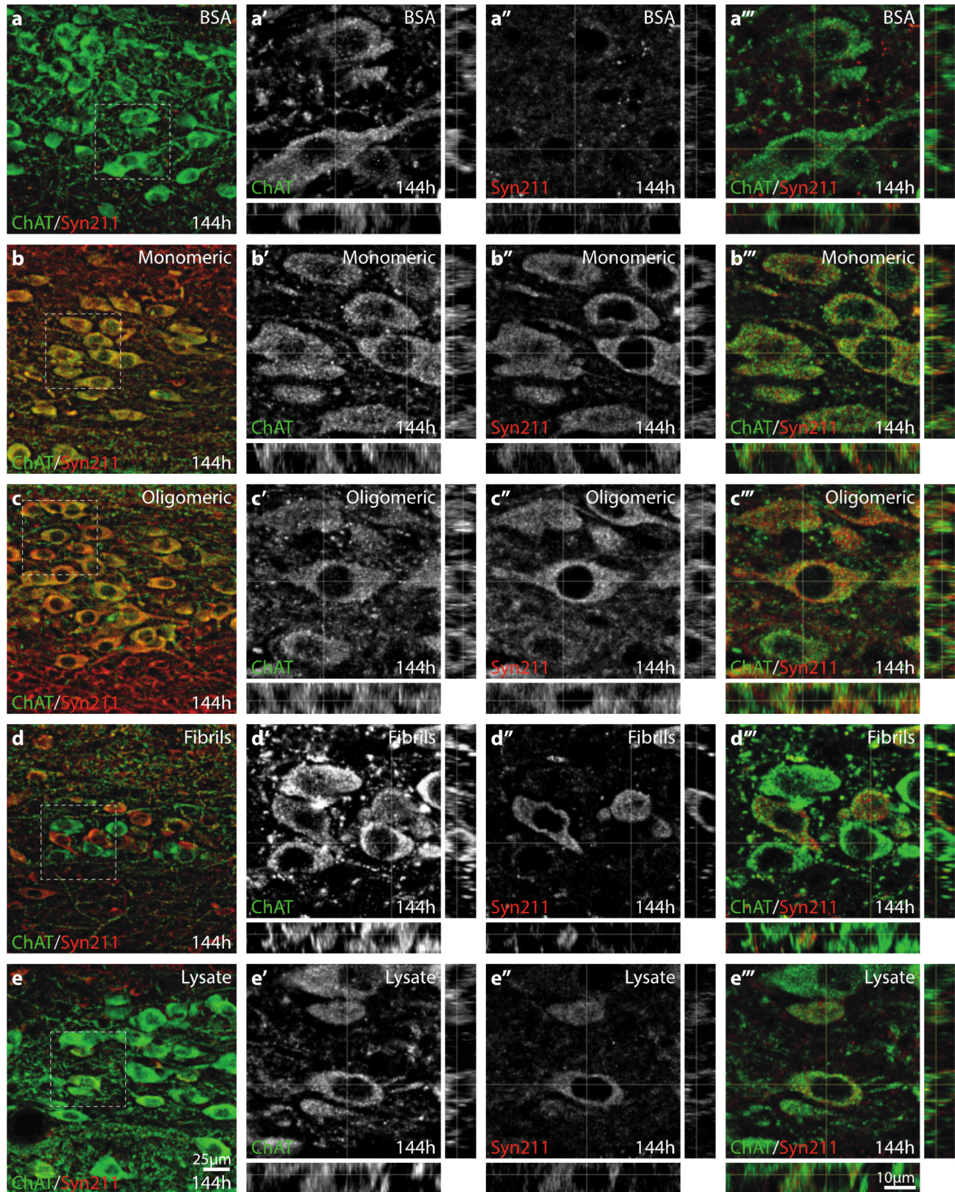

Figure 6.

Atto-550-α-synuclein forms specifically transported into the DMV, 144 h after injection into the wall of the intestine. To enhance the signal, the brain sections were immunostained with antibodies directed specifically against human α-synuclein (Syn211, *red*, **a–e**, **a″–″**, **a‴′–e‴**) and ChAT (*green*, **da–e**, **a′–d′**, **a‴′–e‴**), the phenotypic markers of the DMV. Monomeric (**b**), oligomeric (**c**), fibrillar (**d** α-synuclein and PD patient brain lysate (**e**) injected animals display double-labeled neurons (*arrows*), while BSA injection does not yield any α-synuclein-positive neurons (a″). *Scale bars* 25 μm in **a–e**, and 10 μm in **a‴′–e‴′**.

Moreover, by performing fluorescent recovery after photobleach (FRAP) experiments using a Bimolecular fluorescence complementation (BiFC) strategy, in which fluorescent signal is revealed when complemented, and thus when α-synuclein is aggregated, we demonstrated that aggregated α-synuclein is directly associated with microtubules, possibly involved in active transport (Figure 7). Our unpublished data froma collaboration with the Canelletti laboratory in Milan, support this interaction (data not shown). Likely, the aggregated form of α-synuclein is involved in all components of axonal transport, and associated with fast transporting cargoes just as monomeric α-synuclein is (Jensen et al., 2011; Utton et al., 2005). Given the fact that the full vagal nerve length is about 110-140mm in adult rats, our *in vivo* findings suggest an average translocation rate of dozens of mm per day. This rate is similar to that observed by others (Rey et al., 2013; Utton et al., 2005), and within the reported range for fast microtubule-associated transport (Lasek et al., 1984).

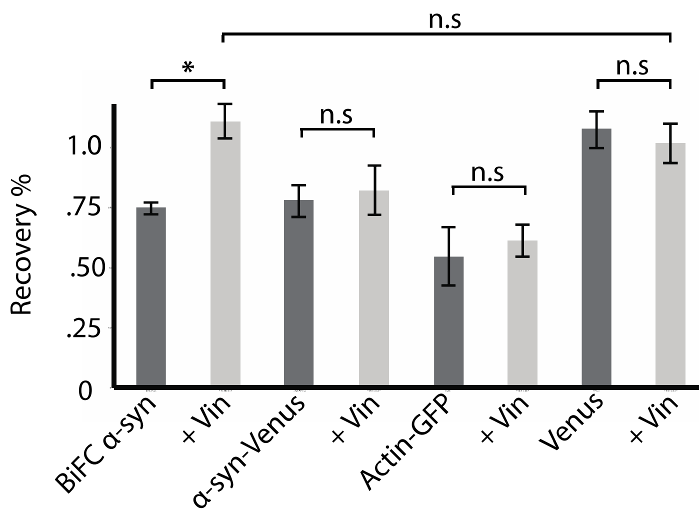

Figure 7.
Normalized and summarized data from fitted curves for mobility of complemented α-synuclein-BiFC-Venus, α-synuclein-Venus, actin-GFP or Venus alone. Data shows increased mobility following destablization of microtubles by treatment with 10 nM vinblastine (Vin). *$p < 0.05$; $p < 0.0002$; $p < 0.0001$.

Taken together, the findings of Paper I provide the first evidence that α-synuclein protein can be retrogradely transported via the vagal nerve from nerves of the gut to the brain. This transport was confirmed for different α-synuclein forms (monomers, oligomers, fibrils). Furthermore, we here provide additional evidence in support of aggregated α-synuclein being subject to slow component a and b as well as the fast component of axonal transport.

Paper II - A library of iPSC lines

Reprograming of PD and MSA patient fibroblasts into induced pluripotent stem cell lines

Several studies have used iPSC based models and reported on neuronal dysfunction reminiscent to mutations in PD-linked genes *SNCA*, *LRRK2*, *PINK*, and *PARK2* as well as in *GBA1*, of which deficiency leads to Gaucher's disease and is associated with increased risk of developing PD. These studies have demonstrated the advantages of iPSC based modeling of PD for mechanistic studies (Byers et al., 2011; Devine et al., 2011; Nguyen et al., 2011; Rhee et al., 2011; Woodard et al., 2014); (Park et al., 2014).

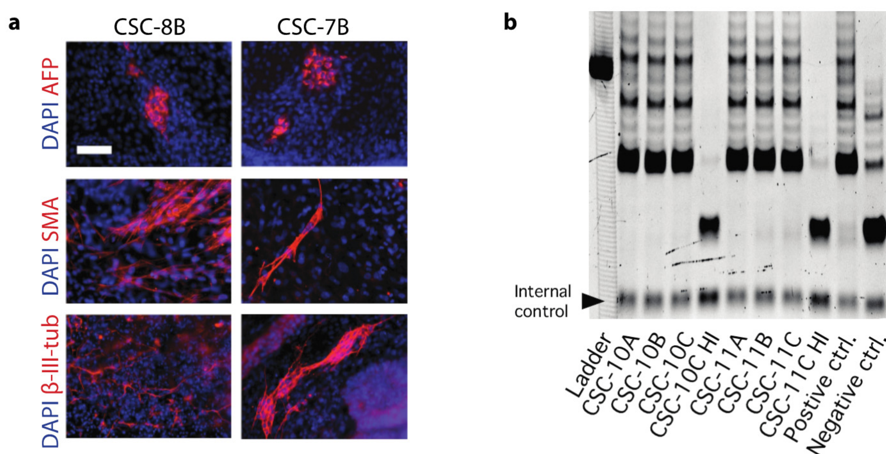

Figure 8.
a) Differentiated embryoid bodies generate cells of the three germ layers, immunopositive for alpha-fetoprotein (endoderm), smooth muscle antibody (mesoderm), and beta IIItubulin (ectoderm); nuclei are counterstained with DAPI. Scale bar represents 100 µm. n=2–3 independent experiments. b) Detection of telomerase activity by the TRAP assay.

The goal of Paper II was to generate a comprehensive library of iPSCs carrying various mutations in PD-associated genes, as well as iPSCs generated from patients diagnosed with MSA, and healthy controls. Various techniques exist to generate iPSC. Vector-facilitated reprogramming can be broadly divided into genome-integrating and genome non-integrating approaches, for example depending on which type of virus is used to introduce the reprogramming factors SOX2, OCT4, KLF4, and c-MYC. We generated iPSCs from patient fibroblasts using two different approaches: retroviral delivery of the reprogramming factors and a non-integrating approach, where factors were delivered using Sendai virus.

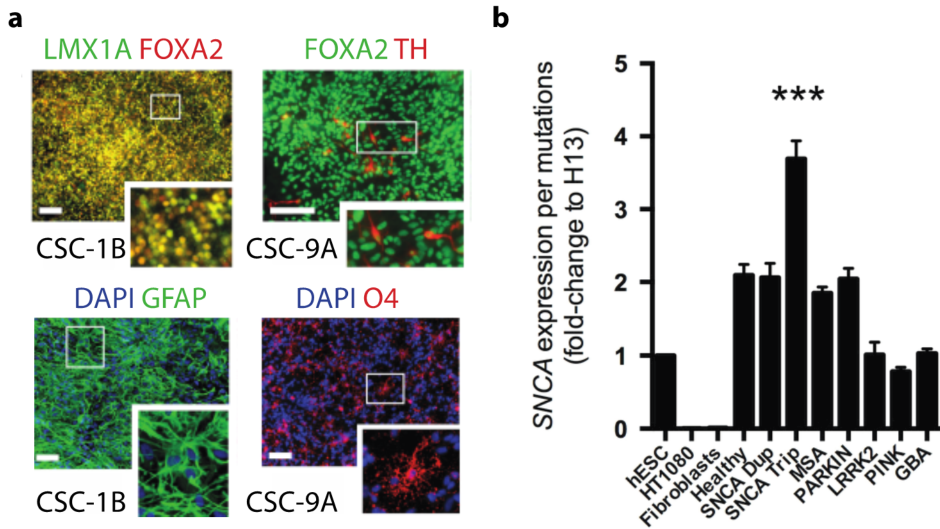

Figure 9.
Examples of pluripotency tests employed at characterization a) Representative images of iPSC lines differentiated towards neural, neuronal, and glial fates. IPSC lines differentiated for 12 days become midbrain neural progenitors co-expressing LMX1A and FOXA2 (shown for iPSC line CSC-1B); when progenitors are kept for 4 additional weeks in culture, they differentiate into FOXA2/tyrosine hydroxylase-expressing neurons (shown for iPSC line CSC-3G; aged 30 days *in vitro*). Culturing of the remaining undifferentiated neural progenitors for 5 additional weeks generates glial fibrillary acidic protein-expressing astrocytes. O4-positive oligodendrocytes can be generated from iPSC using medium devoid of retinoic acid. Nuclei are counterstained with DAPI. Scale bars represent 100 µm. Images are representative of n= 1–2 independent experiments. b) Upregulation of *SNCA* expression in *SNCA* triplication iPSC lines revealed by quantitative real-time PCR. One-way analysis of variance (ANOVA; P40.0001, F(10;49) =30.03) followed by Dunnett's multiple comparisons tests shows SNCA triplication lines have a significantly higher *SNCA* expression over non-*SNCA* triplication lines. Mean ±s.e.m. p<0.001.

Extensive characterization was performed to ensure reprogramming into a pluripotent state (Figure 8a-b). Interestingly, we noticed higher levels of karyotypic abnormalities, as well as higher *SNCA* expression, in the iPSC lines generated using integrating vectors as compared to those generated using non-integrating Sendai virus. However, in order to appropriately answer if these indeed were effects of vector integration, we would need to empirically compare the two approaches using the same parental fibroblasts lines. Nevertheless, this finding was in line with a recent and similar discovery from Daley's laboratory (Schlaeger et al., 2015).

Interestingly, we observed higher *SNCA* expression levels in undifferentiated iPSCs generated from an *SNCA* triplication patient (CSC-3A, B, G, S) compared to other lines lacking the triplication of the *SNCA* locus (Figure 9b), suggesting that pathological changes may be initiated as early as at embryonic stage, although the disease may manifest later during adulthood. Moreover, it was recently reported that *SNCA* triplication impairs the differentiation of iPSC into neurons (Oliveira et al., 2015), in support of *SNCA* affecting early development of the CNS.

In Paper II, we provided the first comprehensive library of iPSC models generated from patients diagnosed with synucleinopathies. These human cellular models will be a valuable resource for identifying common and divergent mechanisms leading to neurodegeneration and glial dysfunction in PD and MSA.

Paper III - *SNCA* expression in Oligodendrocytes

Oligodendrocytes dynamically express SNCA during their development

Evidence of oligodendrocyte expression of *SNCA* has been lacking for more than a decade while much effort has been placed into strengthening the hypothesis that α-synuclein present in GCIs in MSA is of neuronal origin (Kisos et al., 2012; Reyes et al., 2014). It is indeed strongly suggested that neuronal α-synuclein is transferred to oligodendrocytes where it accumulates and potentially leads to oligodendrocyte dysfunction and neuronal injury, similar to the experimentally established transfer of α-synuclein between neurons in pre-clinical experimental PD models (Desplats et al., 2009; Freundt et al., 2012; Hansen et al., 2011; Luk et al., 2009). However, over a decade ago, Richter-Landsberg and colleagues reported on the transient expression of *Snca* in primary cultures enriched in oligodendrocytes (Richter-Landsberg et al., 2000). This finding was neither confirmed nor further explored. Paper III aimed to critically review *SNCA* expression in oligodendrocyte lineage cells.

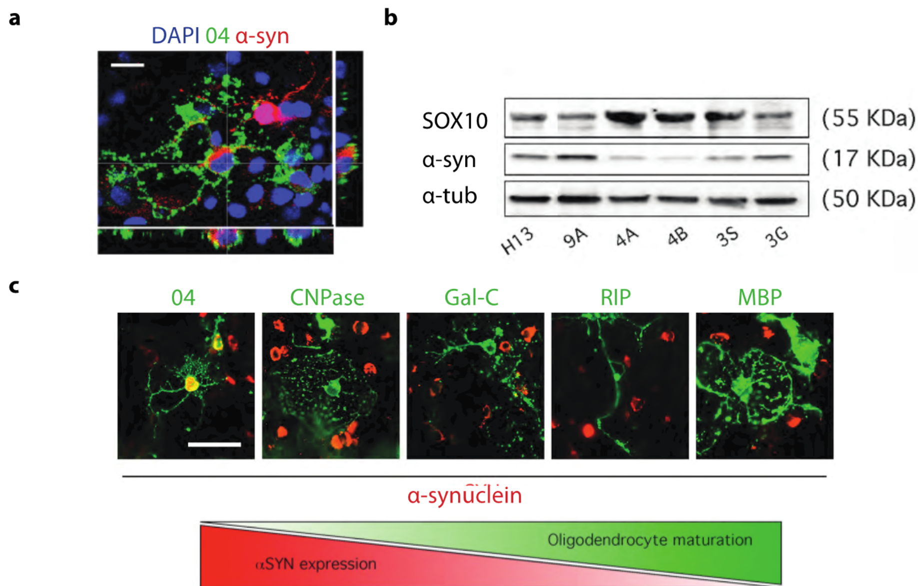

Figure 10.
a) Identification of ASYN in O4+ oligodendrocytes in rodent postnatal primary forebrain culture by confocal microscopy. Nuclei are counterstained with DAPI. Scale bar represents 20 µm b) Western blotting confirms the expression of the oligodendroglial marker SOX10 and α-synuclein in Day 60 iPSCs. c) α-synuclein is detected in O4+ oligodendrocytes, and is lost in mature oligodendrocytesexpressing the markers CNPASE, GAL-C, RIP and MBP. Scale bar represents 50 µm.

Oligodendrocytes are abundantly produced during early post-natal development (Kessaris et al., 2006). In order to evaluate the expression of *Snca* in the major neural cell types, we began to analyse its expression in rodent cells using immunocytochemistry (ICC) and real time quantitative polymerase chain reaction (RT-qPCR). To this aim, we generated cell cultures from the brains of mouse neonates and examined which cell types were immunoreactive for α-synuclein. α-Synuclein protein and transcripts were robustly identified in β-III-tubulin^{+} neurons and in O4^{+} oligodendrocyte progenitor cells (OPCs) Undoubtedly, our results support those made by Richard-Landsberg and colleagues fifteen years ago.

In order to address the dissonance of previous reports on *SNCA* expression in human oligodendrocytes, we wanted to investigate the dynamics of *SNCA* expression during oligodendrocyte maturation. For this purpose, iPSC lines generated from healthy, MSA or PD patients were directed towards an oligodendrocytes lineage using our improved protocol, adapted from the Fossati and Chandra labs (Douvaras et al., 2014; Stacpoole et al., 2013). At day 60, α-synuclein could be detected in most O4^{+} OPCs (Figure 10a-b), depending on the iPSC cell lines employed, in line with what we observed for primary mouse O4^{+} OPCs. In contrast, at day 110, lower levels of α-synuclein where observed in 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase^{+}) and meyline binding protein (MBP^{+}) mature human iPSC-derived oligodendrocytes.

Interestingly, oligodendrocyte maturation was associated with a loss of *SNCA* expression. Importantly, a recent study comparing transcriptome profiles of mouse cortical neurons and glia, found Snca to be highly expressed in immunopanned PDGFR⁺ OPCs but not in myelin oligodendrocyte glycoprotein (MOG) expressing myelinating oligodendrocytes (Y. Zhang et al., 2014), confirming Snca levels are high in immature oligodendrocytes, while they are low in mature ones (Figure 10c). We did not detect α-synuclein protein or transcripts in GFAP⁺ astrocytes and microglia in mixed cultures, which are cell types known to incorporate α-synuclein from their surroundings (Boza-Serrano et al., 2014). This suggested that the α-synuclein detected in oligodendrocytes was not released from neurons and subsequently taken up by the oligodendrocytes, since we also generated pure cultures of O4⁺ oligodendrocytes following fluorescent activated cell sort (FACS)-isolation of PDGFRa progenitors, and could uniformly identify α-synuclein in the cells as they matured into O4⁺ oligodendrocytes. Moreover, our findings suggest that the source of the α-synuclein, present in the inclusions found in astrocytes is still elusive and that α-synuclein present in the CGIs may be come from oligodendrocytes themselves, rather than of neuronal origin.

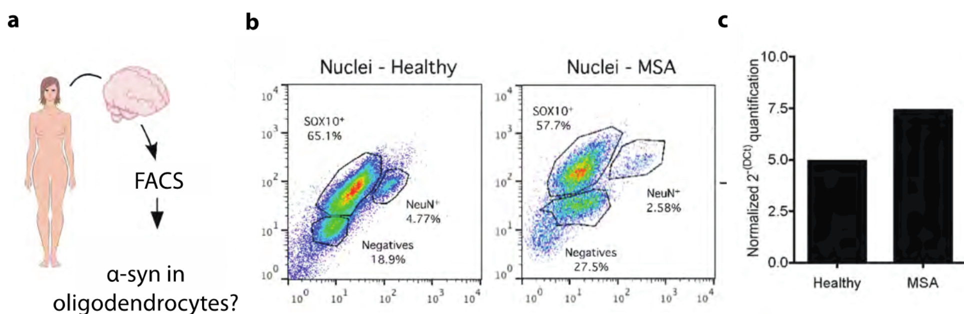

Figure 11.
a) Schematic representation depicting the experimental approach used for assessing ASYN expression in oligodendrocyte lineage nuclei isolated from brains of healthy and MSA patients b) FACS plots representing the gating strategy employed for isolating SOX10+ oligodendrocyte lineage nuclei from the samples c) Quantitative real-time PCR showing SNCA transcripts in SOX10+ oligodendrocyte lineage nuclei samples purified by FACS from patients T172 and T4640.

Reports of absence of *SNCA* transcripts in oligodendrocytes of the adult brain of healthy and MSA patients (Iwai et al., 1995; Miller et al., 2005; Solano et al., 2000), suggest that human oligodendrocytes do not express *SNCA, in vivo*. Based on our results, and conflicting reports, we wanted to revisit this hypothesis and took advantage of a previously developed technique to isolate human oligodendrocyte lineage nuclei by FACS (Ernst et al., 2014; Spalding et al., 2005; Yeung et al., 2014). To assess the presence of *SNCA* transcripts, we prepared and FACS sorted nuclei samples from the pons of post mortem tissue from three healthy individuals and three confirmed MSA patients (Figure 11a-b). We found lower proportions of neurons in

the pons of MSA patients by quantification of neuronal NEUN+ nuclei, confirming neuronal loss in MSA (Cykowski et al., 2015; Papp & Lantos, 1994). In contrast to previous reports, we found that human oligodendrocyte lineage cells do indeed express *SNCA*, as indicated by the presence of *SNCA* transcripts in SOX10+ nuclei isolated from post-mortem brains of healthy and MSA patients (Figure 11c). Interestingly, a parallel study observed *SNCA* expression in laser-capture dissected oligodendrocytes from post mortem brains. No statistically significant difference between MSA and PD brains was found, but this study was in line with our findings of expression of *SNCA* in oligodendrocyte lineage cells in the adult human brain.

Taken together, Paper III bring substantial evidence of *SNCA* expression in oligodendrocytes; thereby challenging the assumption that α-synuclein present in GCIs is of neuronal origin.

Paper IV - Astrocyte reporter lines

Generation and characterization of astrocyte reporter lines for the isolation and identification of human astrocytes

The release of neurotrophic (Saavedra et al., 2006; Sandhu et al., 2009) as well as pro- and anti-inflammatory factors by astrocytes (S. S. Choi et al., 2014; Farina et al., 2007; H. J. Lee et al., 2010), likely plays a regulatory role in neurodegenerative diseases such as PD. Whether α-synuclein can induce the activation of astrocytes, and thus contribute to this regulatory role and prospectively disease initiation, remains unknown. Previous work from the Lee lab (H. J. Lee et al., 2010) showed that α-synuclein, released by *SNCA*-overexpressing neuroblastoma cells, was taken up by primary rodent astrocytes, resulting in an up-regulation of pro-inflammatory mediators e.g. IL-6 and CCL2. This finding was appealing since these factors lead to microglial activation and recruitment (Farina et al., 2007; Gomez-Nicola et al., 2013), which has been postulated to contribute to neuronal loss in PD (Gu et al., 2010; H. J. Lee et al., 2010).

We wanted to investigate if human astrocytes could take up α-synuclein from their surroundings and whether this uptake of α-synuclein would result in a detrimental shift in astrocyte function e.g. release of pro-inflammatory cytokines rather than release of neuronal survival factors. One major concern when analysing whole cultures, is the presence of various cell types, which even in minority, can hinder an assay. Indeed, slight variations in the microenvironment of individual cells differentiated towards a specific lineage, often results in heterogenous cultures. Cellular heterogeneity of stem cell-derived glia includes neuroepithelial cells, neural progenitors cells, oligodendrocytes and few neurons, as well as a minority of cells of another germ layer, which could result in astrocyte specific signatures being concealed

during data analysis. Earlier studies commonly utilised techniques based on rotary-shaking (McCarthy & de Vellis, 1980) or immunopanning (Foo et al., 2011; H. Yang et al., 2016) in order to enrich glial populations. Although these methods work well for primary glial cultures, they are not optimal when working with stem cell-derived glia.

To overcome the issue of heterogeneity, and therefore increase specificity of our read out, one of the goals of Paper IV was to create astrocyte reporter lines that would allow FACS-based purification of astrocytes using a fluorescent tag. Several methods exist to generate fluorescence-based reporter cell-lines. bacterial artificial chromosome (BAC) transgenesis (Placantonakis et al., 2009; Rostovskaya et al., 2012) and gene targeting (Fischer et al., 2010; Y. Liu et al., 2011) are the most commonly used, but these require large plasmids, which can easily break during electroporation, resulting in fragmented constructs, or are costly and labour-intensive. A simple and straightforward method, perhaps not as optimal as the new techniques employing CRISPR/Cas9 genome editing, is the generation of reporter lines by nucleofection (Di Giorgio et al., 2008).

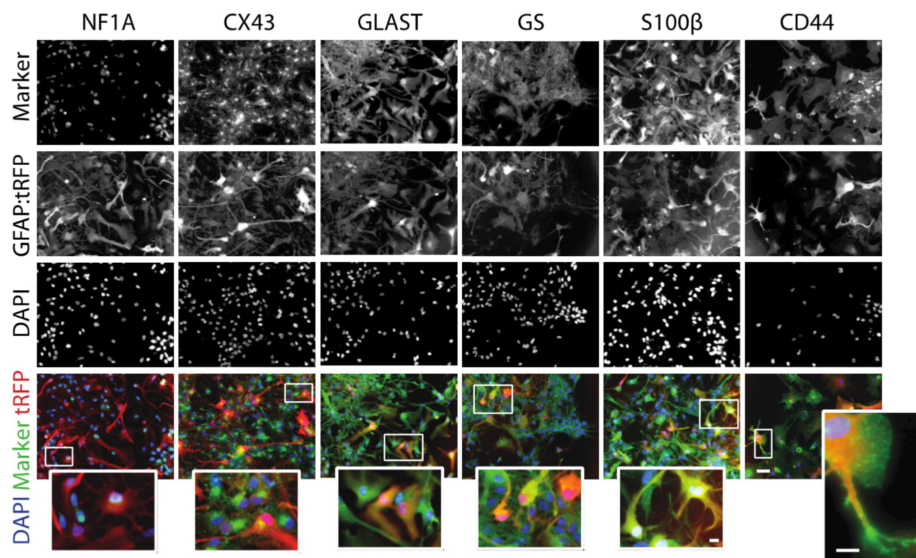

Figure 12.
a) Long-term characterisation of FACS-purified cultures of astrocytes generated from regionally defined neural progenitors. Panel of canonical markers expressed by iPSC derived astrocyte 40 days post-enrichment by FACS; at this time point, cultures are 130 DIV. Images show homogenous cultures of astrocytes co-expressing TagRFP and canonical markers NF1A, CX43, GLAST, GS, S100β and CD44. Nuclei are indicated with DAPI staining. Scale bar in main panel represents 20 µm and in insets 5 µm (bottom right panels).

In Paper IV, we generated a construct, driving TagRFP expression in our PSC lines, using the human GFA_{ABC1D} promoter fragment, previously shown to accurately direct expression in astrocytes of most brain regions, including the midbrain, and capable of

retaining specificity over (Y. Lee et al., 2008). The construct was electroporated into human ESC and iPSC lines and drug resistant clones were then individually picked and expanded (Figure 16a-b). We then differentiated these PSC reporter lines into midbrain NPCs, and subsequently differentiated those into astrocytes. At day 130 following enrichment by FACS, cultures contained cells co-expressing TagRFP with early (GFAP, nuclear factor 1-alpha, NF1A), intermediate (CD44, Connexin 43, CX43, S100 calcium-binding protein B, S100B), and mature (Glutamate aspartate transporter, GLAST, Glutamine synthetase GS) markers of astrocytes (Roybon et al., 2013) (Figure 12). At this stage, there was an almost complete overlap between the reporter and GFAP. Interestingly, during the pre-screening stage, at day 70, not all GFAP-expressing astrocytes co-expressed *GFA_{ABC1D}::TagRPF*. This lack of specificity might be a result of subpopulations of astrocytes being generated in the culture or due to the random insertions of the reporter construct. Moreover, the GFA_{ABC1D} promoter fragment is missing several regulatory elements compared to the full-length human promoter (Y. Lee et al., 2008), which may have resulted in loss of expression of the fluorescent reporter in some of these astrocyte subpopulations. It should also be noted that the specificity of the GFA_{ABC1D} promoter fragment was initially determined in rodents. Importantly, it is possible that the difference between reporter expression and GFAP also could be attributed to the dynamic expression of *GFAP* and to the usually short half-life of this intermediate filament protein, compared to the stable fluorescent proteins such as RFP (Chiu & Goldman, 1984).

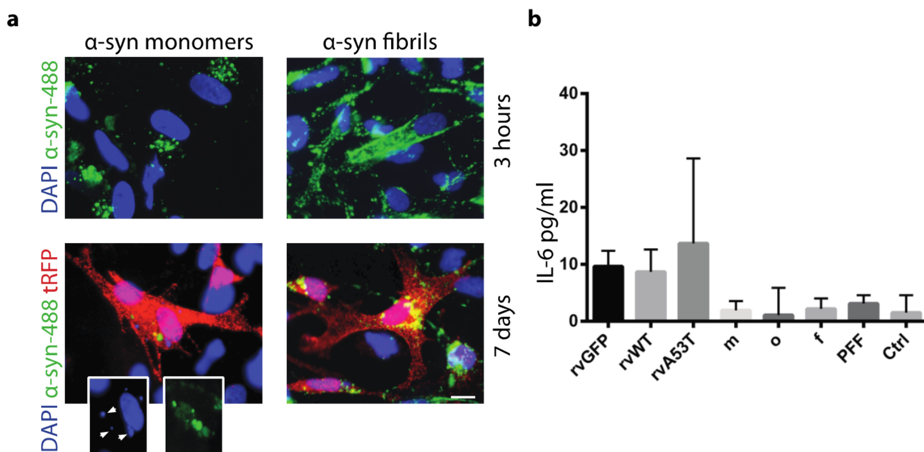

Figure 13.
a) Upper panel images show cellular localization of α-synuclein in 90 days old unpurified midbrain astrocyte cultures treated for 3 h with ATTO488-labeled α-synuclein species. Lower panel images show cellular localization of α-synuclein in cultures treated with ATTO488-labeled α-synuclein species, at 7 days. White arrowheads pinpoint DNA fragmentation. Scale bar represents 10 µm. b) Astrocytes overexpressing α-synuclein or treated with different species of α-synuclein did not release cytokine IL-6 (n=3).

α-Synuclein containing inclusions are observed in astrocytes of PD patient brains (Braak, Del Tredici, et al., 2003; Wakabayashi et al., 2000), but SNCA expression has not been observed in astrocytes. These observations raise the question of the origin of α-synuclein found in the inclusions in astrocytes. It has been suggested that α-synuclein released by neurons, is taken up by astrocytes (Braak et al., 2007). We found that exogenous Atto488-labelled α-synuclein (monomeric or fibril) is readily incorporated and degraded by human midbrain-derived astrocytes (Figure 13a). Interestingly, monomers accumulate as peri-nuclear inclusions (Bousset et al., 2013; H. J. Lee et al., 2010) (most likely in ER-Golgi), while fibrils also adhere outside the plasma membrane. This was also observed in an OLN-93 cell line model, suggesting the phenomena not to be cell type specific (Reyes et al., 2014).

Experimental models have shown that astrocytes can take up different forms of exogenous α-synuclein (monomeric, oligomeric)(Fellner et al., 2013; W. Zhang et al., 2005), resulting in the release of pro- and anti-inflammatory factors (Klegeris et al., 2006; H. J. Lee et al., 2010) and impaired mitochondrial function (Braidy et al., 2013). Interestingly, Song and colleagues found α-synuclein to accumulate only in protoplasmic astrocytes, and not in fibrous astrocytes, suggesting astrocytes involvement in PD might be astrocyte subtype specific (Song et al., 2009). To find out whether exogenous α-synuclein could trigger an inflammatory response in human ESC and iPSC-derived astrocytes, we over-expressed or treated astrocytes with different forms of human α-synuclein (wild type, mutant A53T, or recombinant monomers, oligomers, and fibrils), and evaluated release of IL-6 and nuclear translocation of nuclear factor k (data not shown). We did not observe any substantial release of IL-6 in response to α-synuclein uptake, in contrast to previous reports by the Lee lab (Figure 13b) (H. J. Lee et al., 2010). Lee and colleagues indeed observed that primary rodent astrocytes up-regulated chemokines and cytokines following treatment with conditioned media from α-synuclein over-expressing neuroblastoma cells. Although α-synuclein clearly was released by the over-expressing, and potentially dying neuronal cells, and transferred to the astrocytes, it is likely that the observed up-regulation of cytokines and chemokines was due to the uptake of other factors released by these cells, such as reactive oxygen species. Moreover, there are fundamental differences in both morphology and function between human and rodent astrocytes (Oberheim et al., 2009), as recently demonstrated by Needergaard lab (Han et al., 2013). Whether this inter-species heterogeneity also extends to the astrocyte specific response to injury and inflammation is currently unknown and needs to be further investigated. It is worth mentioning that previous reports have highlighted the fact that astrocytes release neurotrophic factor glial cell derived neurotrophic factor (GDNF) (Saavedra et al., 2006 {Sandhu, 2009 #287) and antioxidant glutathione (Sandhu et al., 2009), in response to dopaminergic neuronal damage, suggesting that supporting astrocytes also exhibit a neuroprotective function in the disease. Thus, future investigations should evaluate the release not only of

directly cytotoxic factors, but also that of neuroprotective ones as well as the interplay between human astrocytes and human microglia during neurodegeneration.

Taken together, the data presented in Paper IV show how astrocyte reporter lines can provide pure populations of astrocytes and how these can be used when modelling effects of α-synuclein on astrocytes.

Conclusions and Future Perspectives

Conclusions

In Paper I, we provides the first experimental evidence that different forms of α-synuclein can propagate from the gut to the brain. Consistent with previous investigations, we show that α-synuclein is propagated via a microtubule-associated transport-dependent manner. Paper I also provides support to the Braak hypothesis which advocates that Lewy pathology in PD starts in the enteric nervous system and spreads to the brain, where it leads to neurodegeneration primarily of dopamine neurons, resulting in locomotor impairment.

One of the aims of the work in this thesis has been to investigate whether glial cells can take up exogenous α-synuclein, adopt a detrimental phenotype, and contribute to neurodegeneration of dopamine neurons. Towards this aim, in Paper II, we generated a library of extensively characterized induced pluripotent stem cell lines and generated disease-relevant and defined cell types e.g. dopamine neurons and glia. These lines are summarised in Paper II, Figure 1. We envision that this type of resource can be applied to the research of biomarkers, patient stratification, as well as humanized high-throughput assays for drug discovery and environmental chemical safety assessment. Derived from a broad range of patients diagnosed with PD and MSA, the iPSC line libary provides a useful platform for identifying how genetics, and cell-type specific functions, contribute to neurodegeneration in PD and MSA.

This resource is used in Paper III, and allowed us to address SNCA expression in human oligodendrocytes. We found that α-synuclein transiently expressed in oligodendrocyte lineage cells during maturation. This study is the first to report on the generation of PD and MSA iPSC-derived oligodendrocytes, and follow-up investigations may also provide significant insights into the functional role of α-synuclein during development of neuronal lineages. Paper III supports earlier findings made in rodents by Richter-Landsberg and colleagues, of α-synuclein expression in oligodendrocytes (Richter-Landsberg et al., 2000). This discovery challenges the conjecture that α-synuclein found in GCIs, in MSA, has a neuronal origin, and suggests it may instead originate within the oligodendrocytes themselves. It remains to be determined how significant these findings are in order to better understand the mechanisms behind MSA.

Paper IV shows that exogenous α-synuclein is taken up and sequestered intracellularly by iPSC-derived human astrocytes, and does not contribute to

inflammation. In order to show this we generated astrocyte reporter lines, from which highly pure populations of astrocytes were obtained. We also differentiated regionally defined progenitor cells into mature human midbrain astrocytes that were capable of releasing cytokines and chemokines in response to stressors.

Future perspectives

The work presented in this thesis raises a number of questions that would be interesting to pursue. A string of recent scientific advances, during a relatively short time, has been developed, this include the creation of the first human embryonic stem cells by James Thomson (Thomson et al., 1998), the induction of human iPSC lines (Takahashi & Yamanaka, 2006) enabling patient specific model systems (Takahashi & Yamanaka, 2006), and the recently developed gene editing techniques (Doudna & Charpentier, 2014), with the potential for precise correction and manipulation of the genome (Doudna & Charpentier, 2014). These discoveries, potentiated by synergistic effects, have presented unprecedented opportunities for disease modelling of PD.

Considering that non-neuronal cells, including but not limited to oligodendrocytes in MSA, contribute to the PD pathology, our iPSC library will allow for disease mechanism analyses and drug screens practically with all cell types involved in the development of the disease. Moreover, three-dimensional cell culture models also called organoids (S. H. Choi et al., 2014; Y. H. Kim et al., 2015; Pasca et al., 2015), and transplantation of iPSC-derived neural cells into the rodent brain are still other promising pre-clinical experimental strategies to be pursued using PD-iPSC lines, to reveal the contribution of genetic factors to the disease (Avaliani et al., 2014; Pomeshchik et al., 2015; Thompson & Bjorklund, 2015; Wang et al., 2013). In addition, isogenic lines could be created by CRISPR-Cas9 which would serve as excellent controls in order to dissect the contribution of specific pre-defined mutations compared to those associated with the genomic landscape.

The reporter lines we generated in Paper IV could efficiently be used in order to devise new protocols for robust generation of human PSC-derived astrocytes from other brain regions (e.g. dorsal and ventral forebrain), provided that reporter expression is confirmed in cortical and striatal astrocytes. Furthermore, we have already developed and tested a strategy in which the *GFA_{ABC1D}::TagRFP* is delivered into cell lines by lentiviral delivery. This approach enables large-scale isolation of pure patient-specific astrocyte cultures for studies concerning patient stratification or identification of divergent functional pathways through application of transcriptome analysis.

In experimental cell replacement therapies, graft survival and sufficient circuit integration is of pivotal importance (Daviaud et al., 2013; J. Liu & Huang, 2007). To address such issues, co-transplantation strategies, where neurons are transplanted

together with glia, has been proposed (Noble et al., 2011). To this aim, fluorescent-based reporter lines such as that presented in this thesis, is of interest. Of note, transplantation of regionally specific astrocytes, might further improve graft survival and successful integration.

In the enteric nervous system, enteric glial cells are intriguingly positioned in the submucosal plexus surrounding neurons and contacting intestinal epithelial cells (Clairembault et al., 2014; Jessen & Mirsky, 1980). GFAP expression is increased in the intestinal tracts of PD patients (Devos et al., 2013), correlating with increased levels of pro-inflammatory cytokines (Devos et al., 2013; Ruhl et al., 2001). Multiple lines of evidence support Braak hypothesis, thus, future investigations should consider investigating enteric glia and enteric gliosis as potential therapeutical targets.

Material and methods

General considerations

Ethical considerations

All procedures were conducted in accordance with national and European Union directives. Animal housing and procedures as well as the derivation of mouse ESC lines and generation of human iPSC lines, the latter using viral-mediated gene delivery, were conducted in accordance with ethical permit approved by Malmö-Lund Committee for animal research, as well as the Swedish Work Environment Authority (Arbetsmiljö verket).

Human Tissues

Brain lysates injected into rodents in the propagation study, were prepared from the brain of a neuropathologically confirmed PD case. Dr. Elisabet Englund at the Brain Bank of Lund University Hospital provided brain tissue. The patient died of acute aspiration and subsequent cardiac arrest due to advanced PD. Fresh substantia nigra was dissected out (20 h postmortem) and immediately frozen and stored at –80 °C.

For the generation of iPSC lines, human fibroblasts were collected by skin punch biopsy from healthy individuals and individuals diagnosed with PD and MSA. Diagnosis of primary degenerative Parkinsonism fulfilled current criteria for probable PD or MSA. Fibroblast cell lines used to generate iPSC lines CSC-21B, CSC-21C and CSC-22A were commercially obtained from the Coriell institute. The mutations (*PARKIN* p.*C253Y*, *PARKIN p.R275W*, *LRRK2 p.G2019S*, *LRRK2 p.R1441C*, *GBA p.L444P*, *GBA p.N370S* and *PINK p.Q456X*) were confirmed by direct DNA sequencing (Eurofins Genomics).

In the study of *SNCA* expression in oligodendrocyte lineage cells, tissue from pons was used. Human tissues from three healthy and three individuals diagnosed with MSA, with confirmed GCIs in oligodendrocytes, were obtained from the New York brain bank at Columbia University (New York, USA).

Microscopy, imaging and artwork

Images of stained tissues were acquired using a BX53 Olympus microscope using settings for bright field or epifluorescence imaging. Most cultured cells were imaged

with an inverted Olympus IX73 epifluorescence microscope, equipped with Hamamatsu C11440 Orcaflash 2.8 camera. Some cultures were imaged using laser scanning confocal microscope (Leica) or automated plate runner (Trophos). For each acquisition at the same magnification and combination of antibody probes, identical settings were used for exposure time, digital gain, etc.

Laser Scanning Confocal microscopy

Confocal microscopic images of double-labelled medulla oblongata were captured using a Leica SP8 Scanning confocal microscope using a HyD detector and sequential scanning to avoid bleed-through. Solid-state lasers at wavelengths 488- and 552- and 650 nm were used to excite the respective fluorophores. Pinhole was retained at Airy 1 for all acquisitions. For each acquisition at the same magnification, identical settings were loaded for laser power and gain. Post-acquisition, deconvolution was performed using the "Deconvolution" plugin for ImageJ [developed by the Biomedical Imaging Group (BIG), EPFL, Switzerland http://bigwww.epfl.ch/] utilizing the Richardson–Lucy algorithm and applying point-spread functions (PSFs) calculated for the specific imaging equipment using the Gibson and Lanni model in the PSF Generator (BIG, EPFL, Switzerland http://bigwww.epfl.ch/algorithms/psfgenerator/). The same PSF models and deconvolution parameters were applied to all image stacks at the same magnification. Orthogonal projections were generated using ImageJ (v1.48p) without further modifications of the images.

Automated imaging and image analysis using Metamorph

Whole well images were acquired using the Plate Runner HD from Trophos, at a resolution of 2046 x 2046 pixels and 4092 x 4092 pixels). Overview images were presented without post processing.

Automated quantitative image analysis of fluorescent and stained reporter line astrocyte cultures was performed using MetaMorph Software V7.8.6.0 (Molecular Devices). Quantitative analysis of stained hPSC-derived astrocyte cultures was performed using the Multi-Wavelength Cell Scoring application. For a specific marker, positive cells were selectively identified as having clear signal intensity above local background. Intensity thresholds were set blinded to sample identity.

Figures and artwork

Vector graphics and schematics were created using Adobe illustrator CS5 and figures were arranged and compiled using Adobe InDesign CS5 (Adobe Systems, San Jose, USA). Some figures were arranged using Canvas Draw 3 (ACD Systems, Seattle, USA). When applicable artwork and schematics where processed using Adobe Photoshop CS5. 3D model of α-synuclein was generated using UCSF Chimera software version 1.8 (RBVI, UCSF)

Statistics

All quantitative data were analyzed using Prism 6.0 (developed by GraphPad Software, San Diego, USA http:// www.graphpad.com). In general, sample groups were subjected to one-way analysis of variance (ANOVA) and unpaired *t-test*.

When investigating *SNCA* expression in oligodendrocyte lineage cells, each single experiment was started from pluripotent stem cell stage for mouse and human cells, and freshly harvested brain cells for mouse primary cultures. Per experiments, 5-7 random fields of view were counted, at low magnification (10X objectives). Sometimes, images from up to 3 separate wells were analyzed. Sample groups were subjected to un-paired *t-test*.

In FRAP experiments groups were analyzed using one-way ANOVA for difference among the groups, then groups were compared in-between pairwise by student's *t-test*.

General methods and related tables

RT-qPCR

RNA extraction was carried out using Trizol, followed by RNA purification through columns according to manufacturers instructions (EZNA total RNA Kit I, VWR) and for the very low content material, RNeasy microkit (Qiagen) columns were used. For classic reverse transcription and generation of cDNA, Super Script III reverse transcriptase kit was employed (Invitrogen). Resulting cDNA was subsequently processed using the Bio-rad CFX-96 apparatus, using a protocol customized according to the instructions of the SsoFast™ EvaGreen® Supermix (Bio-Rad). Relative quantification was applied using *GAPDH* as reference gene. The list of primers used in this thesis is listed in table 2.

Table 2.
Human and viral RT-qPCR primers utilised throughout this thesis. Relevant paper

Gene name	Abbreviation	Direction	Sequence	Paper
Engrailed 1	EN1	Forward (F)	GTGTCTGCCCACCTCTTCTC	II
		Reverse (R)	GCAGTCTGTGGGGTCGTATT	
Forkhead box protein A2	FOXA2	F	CCGTTCTCCATCAACAACCT	II
		R	GGGGTAGTGCATCACCTGTT	
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	F	GAAATCCCATCACCATCTTCCAGG	II
		R	GAGCCCCAGCCTTCTCCATG	
Homeobox protein Hox-B4	HOXB4	F	ACACCCGCTAACAAATGAGG	II
		R	GCACGAAAGATGAGGGAGAG	
LIM homeobox transcription factor 1, alpha	LMX1A	F	AGAGCTCGCCTACCAGGTC	II
		R	GAAGGAGGCCGAGGTGTC	
Homeobox protein OTX2	OTX2	F	GAAGCTCCATATCCCTGGGTGGAAAG	II
		R	CCATGACCTATACTCAGGCTTCAGG	
Paired box protein Pax-6	PAX6	F	GGCAACCTACGCAAGATGGC	II
		R	TGAGGGCTGTGTCTGTTCGG	
Myc avian myelocytomatosis viral oncogene homolog	cMYC	F	AGCAGAGGAGCAAAAGCTCATT	III & IV
		R	CCAAAGTCCAATTTGAGGCAGT	
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	F	GAAATCCCATCACCATCTTCCAGG	III
		R	GAGCCCCAGCCTTCTCCATG	
Kruppel-factor 4	KLF4	F	GACCACCTCGCCTTACACAT	III & IV
		R	GTTGGGAACTTGACCATGATTG	
Myelin basic protein	MBP	F	TTAGCTGAATTCGCGTGTGG	III
		R	GAGGAAGTGAATGAGCCGGTTA	
NKX Homeobox 2	NKX2.2	F	TGCCTCTCCTTCTGAACCTTGG	III
		R	GCGAAATCTGCCACCAGTTG	
POU Class 5 homeobox 1	Oct-04	F	AGTGAGAGGCAACCTGGAGA	III & IV
		R	GTGAAGTGAGGGCTCCCATA	
Oligodendrocyte transcription factor 2	OLIG2	F	GGAGCGAGCTCCTCAAATC	III
		R	CACTGCCTCCTAGCTTGTCC	
Platelet-derived Growth factor receptor, alpha	PDGFRA	F	TCTGCTGGACTGAGAAGTTTCATC	III
		R	CTATCCACACTGTCAAACAGGTTG	
Alpha-synuclein	SNCA	F	CAACAGTGGCTGAGAAGACCA	III
		R	GCTCCTTCTTCATTCTTGCCCA	
SRY (sex determining region Y)-box 2	SOX2	F	ACACTGCCCCTCTCACACAT	III & IV
		R	GGGTTTTCTCCATGCTGTTTCT	
Virus specific	rv-pMX	R	TATCGTCGACCACTGTGCTG	IV
Virus specific	rv-KLF4	F	CCGCTCCATTACCAAGAGCT	IV
Virus specific	rv-SOX2	F	GCCCTGCAGTACAACTCCAT	IV
Virus specific	rv-cMYC	F	CCACTGGTCCTCAAGAGGTG	IV
Virus specific	rv-pMX #2	R	CCCTTTTTCTGGAGACTAAATAAA	IV
Virus specific	rv-OCT4	F	CCCCAGGGCCCCATTTTGGTACC	IV

Immunochemistry

Immunocytochemistry of cell cultures was performed after a 30-minute fixation period with 4% PFA. Immunohistochemistry was performed after an o.n. fixation of tissue perfused with 4% PFA and stored in 30% sucrose solution before sectioning. Brain tissue was sectioned using a microtome, while small pieces of intestine and vagus nerve were sucrose-embedded before being sectioned using a cryostat. Samples were blocked for 1 hour in blocking buffer containing 10% donkey serum in PBS supplemented with 0.1% for cell cultures or 0.2% for tissues, of Triton-X100. Antigens were then probed by incubation with primary antibodies diluted in the blocking solution, o.n. at 4°C. The next day, samples were washed three times for 10 min. with PBS, then incubated for 1 hour with secondary antibodies diluted in PBS, at RT, then washed and incubated with DAPI to stain nuclei. The antibodies used in this thesis are listed in Table 3.

Tissue sections were, in general, stained free-floating on a shaking table. Vagal nerve sections were stained after mounting on glass slides. Some sections were not fixed and immediately mounted on slides to evaluate the natural signal of fluorescently labelled proteins.

SDS and Native PAGE Western blotting

Protein concentrations were measured using Bradford Bio-Rad protein assay kit according to the manufacturer's recommendations (Bio-Rad, USA). The absorbance measurement was performed following manufacturer's protocol on a 96-well plate reader (Biochrome Asys Expert 96 micro plate reader, Cambridge, UK). Proteins were loaded and separated over 4-20% Mini-Protean TGX Precast Gels (Bio-Rad, USA), then transferred to nitrocellulose membranes using Trans-Blot Turbo System (Bio-Rad). After blocking in 5% skim milk, membranes were incubated o.n. with primary antibodies at 4°C followed by incubation with peroxidase secondary antibodies (Vector Labs). Blots were developed using Clarity Western ECL Substrate (Bio-Rad) and the protein levels were normalized to α-tubulin. Membranes were then blocked with 5% skim milk diluted in PBS-Tween 20.

For native PAGE, samples were homogenized in the presence of protease inhibitors using a Fast- Prep-24 homogenizer (MP Biomedicals, Santa Ana, CA, USA) in lysis buffer (20 mM Tris/HCl pH 7.4, 100 mM NaCl, 0.4 % (w/v) sodium dodecyl sulfate and 0.2 % (v/v) Triton X-100). Samples were then mixed with Native PAGE loading buffer and separated over 8–16 % polyacrylamide gels before being transferred to nitrocellulose membranes.

Table 3
Alphabetical list of antibodies used throughout this thesis. Relevant paper is indicated in the far right column.

Antibody	Isotype	Company	Catalouge number	Dilution	Paper
α-synuclein	mouse	Santa Cruz	SC-12767	1:200-1:1000	I & III
α-synuclein	mouse	Invitrogen	18-0215	1:700	I
α-synuclein-1 (WB)	mouse	BD Bioscience	610786	1:1000	I
choline acetyltransferase	Goat	Millipore	AB144P	1:500	I
CD44	Mouse	BD Bioscience	555478	1:30	II
CX43	Rabbit	Sigma-Aldrich	C6219	1:500	II
FOXA2	Goat	Santa Cruz	SC-6554	1:100-1:250	II & IV
GFAP	Rabbit	Dako	Z033401-2	1:5000	II & IV
GFAP	Mouse	Sigma-Aldrich	G3893	1:100	II
GLAST/EAAT1	Sheep	R&D	AF6048	1:200	II
Glutamine synthetase	Mouse	Millipore	MAB302	1:1000	II
HOXB4	Rat	DSHB	I12 anti-hoxb4	1:80	II
LMX1A	Rabbit	DSHB	50.5 5A5	1:500	II
NF1A	Rabbit	Active motif	39036	1:100	II
OTX2	Goat	R&D	AF1979	1:500	II
S100β	Mouse	Sigma-Aldrich	S2532	1:500	II
tagRFP	Rabbit	Evrogen	AB234	1:5000	II
AFP	Mouse	Sigma-Aldrich	A8452	1:200-1:500	III & IV
α-synuclein	Rabbit	Santa Cruz	SC-7011-R	1:200	III
α-synuclein	Mouse	BD Bioscience	610787	1:200	III
α-synuclein (WB)	Rabbit	Cell Signaling	2642	1:500-1:1000	III
β-III-Tubulin / TUJ1	Rabbit	Covance	PRB-435P	1:200	III & IV
CNPase	Mouse	Sigma-Aldrich	C5922	1:200	III
GAL-C	Mouse	Millipore	MAB342	1:200	III
GAL-3	Rat	Gift from H. Leffler to T. Deierborg		1:300	III
Ki67	Rat	eBIOSCIENCE	14-5698	1:50	III
MBP	Chicken	Millipore	AB9348	1:100	III
NG2	Mouse	BD Bioscience	554275	1:200	III
NKX2.2	Mouse	DSHB	74.5A5	1:50	III
4	Mouse	Gift from J. Goldman		1:50	III
Oct-04	Mouse	Millipore	MAB4401	1:200	III
OLIG2	Rabbit	Millipore	AB9610	1:200	III
PDGFRA	Rabbit	Santa Cruz	SC-338	1:200	III
PDGFRA	Rat	eBIOSCIENCE	12604-013	1:100	III
SSEA-4	Mouse	Life technologies	A14766	1:200	III
SOX2	Goat	Santa Cruz	SC-17320	1:200	III
SOX10	Goat	R&D	AF2864	1:200	III
IBA-1	Rabbit	Wako	019-19741	1:500	III
RIP	Rabbit	Millipore	MAB1580	1:200	III
LMX1A	Rabbit	Abcam	AB139726	1:200	IV
SMA	Mouse	Sigma-Aldrich	A2547	1:500	IV
TH	Mouse	Millipore	MAB318	1:2000	IV

Cell culture

Cell culture was carried out in a P2 level room. Cultures were grown in specific media, in an incubator set at 37°C with a CO_2 level of 5%. The media composition differed depending on whether the cells were of mouse or human origins, and their cellular identity. The composition of the media used to grow and differentiate the cells is listed below, in table 4.

Table 4. Composition of cell culture media used throughout this thesis

MEF - Mouse embryonic feeder medium	Final concentration	500ml	Company
DMEM		475 ml	Thermofisher
FBS (heat in-activated)	10%	5 ml	Thermofisher
WiCell - Wisconsin cellular medium	Final concentration	500ml	Company
DMEM / F12		475 ml	Thermofisher
L-Glutamine	1%	5 ml	Thermofisher
NEAA	1%	5 ml	Thermofisher
β-mercapto ethanol	50 µM	1:1000	Thermofisher
NIM - Neural induction medium	Final concentration	500ml	Company
Adv. DMEM / F12	50%	237 ml	Thermofisher
Neurobasal	50%	237 ml	Thermofisher
L-Glutamine	1%	5 ml	Thermofisher
B27 w.o Vitamin A	2%	10 ml	Thermofisher
N2	1%	5 ml	Thermofisher
P/S	1%	5 ml	Thermofisher
NDM - Neural differentiation medium	Final concentration	500ml	Company
Neurobasal		475 ml	Thermofisher
L-Glutamine	1%	5 ml	Thermofisher
B27 w.o Vitamin A	2%	10 ml	Thermofisher
N2	1%	5 ml	Thermofisher
P/S	1%	5 ml	Thermofisher
Heparin 2 mg/ml	2 µg/ml	500 µl	Sigma-Aldrich
GIM - Glial induction medium	Final concentration	500ml	Company
Adv. DMEM / F12		475 ml	Thermofisher
L-Glutamine	1%	5 ml	Thermofisher
NEAA	1%	5 ml	Thermofisher
P/S	1%	5 ml	Thermofisher
B27 w.o Vitamin A	2%	10 ml	Thermofisher
Heparin 2 mg/ml	2 µg/ml	500 µl	Sigma-Aldrich
N2 medium	Final concentration	500ml	Company
Adv. DMEM / F12		475 ml	Thermofisher
N2	1%	5 ml	Thermofisher
L-Glutamine (200mM)	1%	5 ml	Thermofisher
Sodium pyruvate	1%	5 ml	Thermofisher
P/S	1%	5 ml	Thermofisher

Key Methods used in Paper I

Preparation of α-synuclein species for injection into the guts of rat

Brain lysate from a PD patient

A lysate containing aggregated as well as mono- and oligo-meric forms of α-synuclein was prepared FROM the freshly frozen substantia nigra tissue of a patient diagnosed with PD. The tissue was mechanically homogenized in sterile PBS at 4 °C, vortexed, sonicated and centrifuged. The supernatant was aliquoted and samples kept frozen at −80 °C. Before injection, each sample of supernatant lysate was further diluted in PBS.

Expression and purification of recombinant α-synuclein

The expression and purification of human wild type α-synuclein was performed as reported by Ghee and colleagues (Ghee et al., 2005). Briefly, bacteria (Escherichia coli strain BL21 ;DE3) were transformed with an expression vector pET3a encoding wild type α-synuclein, and grown in LB medium to an optical density of 0.8 arbitrary units. The bacteria were then lysed by sonication and lysates clarified by centrifugation. α-Synuclein was precipitated by addition of ammonium sulfate and collected by centrifugation. The resulting pellet was resuspended and loaded onto a diethylaminoethyl (DEAE) column, before being eluted by a gradient of 0–500 mM NaCl. After clarification through a Superdex 75 HiLoad 26/60 column (GE healthcare), pure α-synuclein was filtered through sterile 0.22-µm filters and stored at −80 °C. α-Synuclein concentration was determined using spectrophotometry, using an extinction coefficient of 5,960 M^{-1} cm^{-1} at 280 nm.

Assembly of monomeric α-synuclein into oligomers and fibrils

For oligomer formation, α-synuclein (200 µM) was incubated in buffer A (50 mM Tris–HCl, pH 7.5, 150 mM KCl) at 4 °C, without shaking, for 7 days. Oligomeric α-synuclein was separated from the monomeric form of the protein by size-exclusion chromatography (Superose 6 HR10/300, GE Healthcare). For fibril formation, α-synuclein was incubated in buffer A at 37 °C under continuous shaking in an Eppendorf Thermomixer set at 600 r.p.m. Assembly was monitored continuously in a Cary Eclipse spectrofluorimeter (Varian Inc., Palo Alto, CA, USA) in the presence of Thioflavin T with an excitation wavelength set at 440 nm and an emission wavelength set at 480 nm with an averaging time of 1 second.

Fluorescent labelling of monomeric, oligomeric and fibrillar -synuclein assemblies and BSA

Monomeric and oligomeric α-synuclein assemblies in buffer A were buffer exchanged using NAP10 desalting columns (GE Healthcare) to phosphate-buffered saline (PBS) buffer. α-Synuclein labelling was performed with Atto-550 NHS ester fluorophore following the manufacturer's instructions (Atto-Tec Gmbh) using a protein:label molar ratio of 1:2 as such two Atto molecules per α-synuclein monomer, whether monomeric, oligomeric or fibrillar form. Unreacted fluorophore was removed using NAP10 desalting columns. For fibrillar α-synuclein labelling, fibrils were centrifuged twice, resuspended in PBS and labelled as described above. Unreacted fluorophore was removed by a final cycle of two centrifugations and resuspension of pelleted fibrils in PBS. Lyophilized bovine serum albumin (BSA) was dissolved in PBS and labelled with ATTO-550 dye using a protein:dye ratio of 1:2 following the same procedure as described for monomeric α-synuclein.

Animals, injection of human α-synuclein and surgery

In the study of α-synuclein propagation, we used adult wild type Sprague Dawley rats (~250 g; purchased from Charles River Laboratories). The animals were kept with food and water *ad libitum* under a 12-h light/12-h dark cycle before surgery. Directly before surgery, animals were anesthetized using isoflurane (2–4 %) and their body temperature was kept constant using a heat pad. Injections of α-synuclein samples into the gut were made by inserting Hamilton syringes into the intestine wall at the location of the myenteric plexus of the stomach and duodenum. Animals (n=3 per group; 4 groups) were injected with 3 µl at 5 sites, for each different forms of α-synuclein: PD brain lysate (2 µg/µl of total protein content), monomer (1 µg/µl), oligomer (1 µg/µl) or fibril (1 µg/µl). Control animals (n=3) were injected with BSA diluted in PBS to a final concentration of 1 µg/µl. Great care was taken to avoid injections into the intestinal lumen. Following injections, animals were sutured and returned to normal housing conditions. At 12-h, 24-h, 3-days or 6 days animals were killed. The animals were anesthetized with an overdose of sodium pentobarbital and transcardially perfused with a physiological saline solution followed by an ice-cold 4% PFA solution. Brains, intestines and thoracic vagal nerves were dissected out and stored o.n. in 4% PFA before transferring to 30% sucrose solution. Transverse serial sections of the medulla oblongata, 30 µm thick, were cut using a microtome (Leica, SM 2010R). Vagal nerves were divided into proximal (0–15 mm), medial (25–40 mm) and distal (50–65 mm) segments (in relation to the skull). Transverse and longitudinal sections of 14 µm were generated using a cryostat (Leica, CM 3050S). Some sections were immediately mounted and cover-slipped for the detection of the injected proteins fluorescent tags.

Fluorescent recovery after photobleaching, FRAP

FRAP was performed using a Zeiss LSM 510 confocal microscope system running 2009 Zen software with 63Å~/1.4 Oil DIC Plan-apochromat objective and solid state 488-nm argon laser for excitation and bleaching of fluorophores. Constant temperature at 35–37 °C, humidity and 5 % CO_2 atmosphere was maintained using a heated stage and chamber system (CTI Controller 3700 digital, temp controller 37-2). FRAP was performed based on conventional FRAP procedure with minor modifications (Figure 14)(Axelrod et al., 1976; Snapp et al., 2003). Before each imaging session, media were replaced with imaging buffer composed of HBSS supplemented with 5 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM HEPES, pH 7.4. Data was collected from 256 x 256 pixels. Pre- and post-bleaching images were collected using 0.8% laser power at a rate of 5 Hz with pinhole set to maximum, to avoid additional photobleaching and phototoxicity. Each experiment started with the collection of 4 baseline images. Bleaching was then performed with 100 % laser power for 5 iterations on part of neurites located 100–200 µm away from the cell body. Following photobleach, fluorescence intensities were recorded for 44 s in bleached, cell body reference and background regions. For each group; expressing α-synuclein-BiFC-Venus (n = 4), α-synuclein-Venus (n = 5), Actin-GFP (n = 6) and Venus alone (n = 5), three cells were imaged. Values averaged from the three cells in each experiment constitute one n. To investigate whether mobility was dependent on microtubule dynamics, separate experiments were performed in which cells were treated with 10 nM of vinblastine, a microtubule destabiliser for one hour before imaging, α-synuclein-BiFC-Venus (n = 2), α-synuclein- Venus (n = 3), Actin-GFP (n = 5) and Venus alone (n = 5).

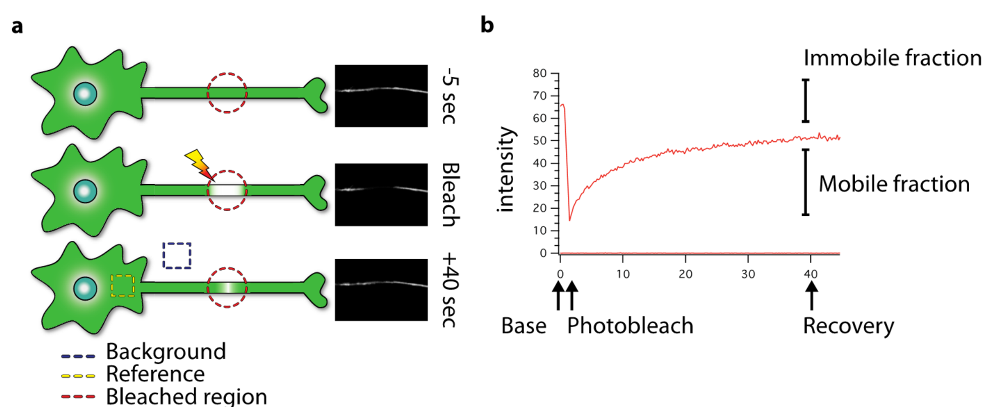

Figure 14.
a) Schematic depicting how the FRAP experiment was performed. Panels to the right show representative images of neurites before, during and after fluorescent recovery. b) Graphical representation of parameters considered for analysis.

Model fitting/FRAP data analysis

Raw intensity signals were, for each time point, normalized by subtracting the average background intensity and correcting for acquisition bleaching by adjusting to loss of fluorescence within the non-bleached reference region (Phair et al., 2004). Normalized data from each cell were then individually fitted to appropriate models. The type of model used for each protein was chosen to describe the bleach corrected and normalised intensity data. For this, data from cells in α-synuclein-BiFCVenus, α-synuclein-Venus and Actin-GFP groups were fitted to an exponential chemical-interaction model as described by Eq. (1) (Phair et al., 2004) as α-synuclein and actin are known to interact with other proteins. In contrast, vinblastine-treated cells, and those expressing Venus alone, were fitted to the empirical diffusion model described by Ellenberg's Eq. (2) (Ellenberg et al., 1997). From this, the mobile fraction was deduced with correction for gap ratio, Eq. (3). Fittings of FRAP curves that deviated significantly from normalized raw data were excluded from further analysis. All curve fittings and normalisations were performed in Igor Pro v6.32A using the plugin K_FRAPcalc v9 [developed by the European Advanced Light Microscopy Network (EAMNET)—EMBL—Germany http://www. embl.de/eamnet/]. (1) Single exponential model according to Phair single exponential, double normalization (2) For calculation of mobile fraction with correction for gap ratio (3) Ellenberg's I final will be considered as the mobile fraction.

$$I(t) = y_0 + Ae^{-\tau_1 t} \quad (1)$$

(1) Single exponential model according to Phair single exponential, double normalization

$$I(t) = I_{final}\left(1 - \left(\frac{w^2}{w^2 + 4\pi Dt}\right)^{½}\right) \quad (3)$$

(2) Ellenberg – I final will be considered the mobile fraction.

$$Mob = \frac{\left(\frac{-A}{1 - (y_0 + A)}\right)}{Gap\, ratio} \quad (2)$$

(3) For calculation of mobile fraction with correction for gap ratio

Key Methods used in Paper II

Generation and characterisation of iPSC lines

Culture of primary human fibroblasts

Cells were cultured and expanded in conventional fibroblast growth medium composed of DMEM and 10% fetal bovine serum before cryo-banking. All lines tested negative for mycoplasma

Derivation of induced pluripotent stem cell lines from human fibroblasts

Proliferating fibroblasts were expanded in conventional media and expanded prior to cryo-banking. Only low passage fibroblasts (<8 passages) were used in the generation of iPSC lines. Fibroblasts were seeded in plates one day before transduction with the reprogramming viruses. The iPSC lines were generated using two different strategies for delivery of the Yamanaka reprogramming factors. CSC lines 1 to 9 were generated by retroviral delivery, as described by (Boulting et al., 2011), Fibroblasts were transduced twice (day 0 and day 1) with retroviruses (multiplicity of infection 10) prepared from the plasmids pMXs-hKLF4, pMXs-hOCT3/4 and pMXs-hSOX2 (Addgene). iPSC lines CSC-1A, CSC-2B, 2C, CSC-6A, CSC-8A, CSC-8B, CSC-8C, CSC-8F, CSC-8S, CSC-9A and CSC-9B were generated using an additional fourth retrovirus prepared from the pMXs-hc-MYC plasmid (Addgene). In the second approach, CSC lines 10 to 22 were generated by delivering the four Yamanaka factors using Sendai viruses. Here CytoTune™-iPS 1.0 (for lines CSC-21B, CSC-21C and CSC- 22A) and 2.0 kits (Thermofisher scientific) were used according to manufacturer's instructions, with minor modifications such as scaling of the culture format. Following transduction media were changed daily to fresh fibroblast growth media until day 6, when cells were re-seeded on irradiated mouse embryonic fibroblasts (CF-1 MEF, GlobalStem) in WiCell media composed of advanced DMEM/F12, 10% Knock-Out Serum Replacement (v/v), 2 mM L-glutamine, 1% non-essential amino acids (NEAA, v/v), 50 µM β-mercaptoethanol and 20 ng/ml fibroblasts fgrowth factor (FGF2). After 2-4 week, once colonies appeared, they were picked individually, each colony clone was individually and expanded and bio-banked before testing of pluripotency.

Expression of Stem cell pluripotency genes

Each clonal iPSC line was evaluated for expression of stem cell pluripotency-associated antigens by probing cultures using antibodies, anti-OCT3/4, anti-SSEA4, anti-NANOG and anti-TRA1-81. The staining procedure is described in detail under general methods.

Alkaline Phosphatase activity

Alkaline Phosphatase activity test was performed using the 86R-1KT (Sigma Aldrich), according to the manufacturer recommendations.

Cytogenic analysis - Karyotyping

The G-banding analysis for all lines was performed in a clinical diagnostic setting. 15-25 metaphases were analysed according to the ISCN 2013 12 by at the department of genetics, at the hospital of Lund, Sweden (Medicinsk service / Labmedicin, Klinisk genetik och biobank).

Down-regulation of viral transgenes and loss of viral agents

Levels of reprogramming factor mRNA in human iPSC lines generated with retroviruses were quantified by RT-qPCR at approximately passage 13. Levels were compared with those of non-transduced fibroblasts and a human embryonic stem cell line (H13). Relative quantification was applied using GAPDH as reference. The iPSC lines generated by Sendai viral delivery of reprogramming factors were evaluated for remaining virus using the anti-Sendai antibody.

Finger print analysis

Fingerprinting analysis was outsourced to IdentiCell STR profiling service, department of molecular medicine, Aarhus university hospital Skejby, Denmark.

Spontaneous differentiation of iPSC into the three germ layers

Human iPSC were grown for two weeks as embryonic bodies (EB) in low-attachment multi-well plates in WiCell supplemented with 20 ng/ml FGF2. EBs were then re-seeded in 96-well plates (Greiner Bio-One) coated with 0.1% gelatin in DMEM medium containing 10% fetal bovine serum (FBS) and 1% P/S for subsequent spontaneous differentiation over two weeks. Media were changed every 2-3 days.

Telomerase activity TRAP assay

To further validate the “stemness” of generated iPSC lines a telomeric repeat amplification protocol (TRAP) was employed. For this purpose a telomerase activity assay TRAPeze (S7700, Millipore) was used. Briefly, total protein was extracted from each of the generated iPSC clones as well as from corresponding parental fibroblast lines. Active telomerase in the samples were allowed to add a number of telomeric repeats (GGTTAG) onto the substrate oligonucleotide by incubation for 30 minutes at 30°C before amplification of the extended products by PCR. The resulting ladder of products was visualized by separation through PAGE run over a 12% gel at 72V for 3 hours. The ladder of products with six base increments started at 50 nucleotides and each sample contain a 36 base pairs (bp) internal standard.

Generation of relevant cell types

Generation of human neural precursor cells

Generation of regionally specified human midbrain NPCs was performed as described in Paper IV

Generation of TH expressing human neurons

To generate TH-expressing neurons, floor-plate NPCs where cultures were dissociated on D12 and plated at high density on plates coated with 20 µg/ml Poly-ornithinin (PO) and 50 µg/ml Laminin (lam). Neuronal differentiation medium (NDM) was composed of Neurobasal, 2% B27 minus vitamin A, 2mM L-glutamine and 1% P/S and was added every 2-3 days. NDM was supplemented with 20 ng/ml brain derived neurotrophic factor (BDNF), 10 ng/ml GDNF, 5 ng/ml cerebral derived neurotrophic factor (CDNF), 200 µM ascorbic acid (AA), 500 µM db-cAMP to induce differentiation into TH-positive cells. The cells were matured for two weeks, and fixed on day 30 for immunocytochemistry.

Generation of human oligodendrocytes

Generation of human oligodendrocytes was performed as described for Paper III.

Generation of human astrocytes

Generation of human astrocytes from regionally specified NPCs was performed as described for Paper IV.

Key Methods used in Paper III

Cell culture

Primary mouse cultures

Primary forebrain cultures used for investigating oligodendrocytes protein expression were prepared from 1-3 day old wild-type mice pups. Primary cells were cultured in conventional medium for three weeks, before FACS and ICC analysis.

Generation of rodent and human oligodendrocytes.

Mouse ESC-derived oligodendrocytes were generated accordingly as described by Czepiel and colleagues (Czepiel et al., 2011). To generate embryonic bodies, ESC aggregates were cultured free-floating in EB medium for 8 days. This medium was composed of Knock-out DMEM, 15% FBS, 1% NEAA, 2-mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µM 2-mercaptoethanol.

Induction of mNPCs, the 8-day-old EBs were cultured in N2 medium for 7-8 days. The N2 medium was supplemented with 20 ng/ml FGF2, and 20 ng/ml epidermal growth factor (EGF). To induce oligodendrocyte differentiation, mNPC EBs were plated onto PDL/Lam coated wells in N2 medium supplemented with 10 ng/ml PDGF for another 4 days, the first two days this medium also contained 20 ng/ml FGF2 and 20 ng/ml EGF. To enhance the maturation of oligodendrocytes, 30 ng/ml T3 and 10 ng/ml NT-3 were added during the final phase of differentiation (6 days).

To induce oligodendrocyte differentiation using human iPSC, cultures were grown as EBs in WiCell medium with 20 ng/ml FGF2 and 1 µM Y27632. Two to four days later, the medium was switched to neural induction medium containing 200 nM LDN-193189 and 10 µM SB-431542 for 4 days. Neuralized EBs were then also exposed to ventralizing agents by adding 100ng/ml SHH-C II and SAG (1 µM, Millipore) in addition to the LDN and SB, for 4 days. EBs were then treated with SAG only. A final glialisation step was then applied using a DMEM/F12-based medium, as previously published (Hu et al., 2009). This medium was supplemented with 10ng/ml each of PDGF-AA, IGF1, NT3 and 60 ng/ml T3, and 5ng/ml HGF to enhance the oligodendrocyte maturation process (Douvaras et al., 2014). EBs were subsequently seeded on PLO/ lam-coated 96 well plates for the remainder of the differentiation.

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Extraction of cell nuclei from adult human brain

Tissue from human postmortem brains of healthy or diseased individuals were homogenized and centrifuged as described by Spalding and colleagues (Spalding et al., 2005) to separate out the nuclei fraction. Tissue samples were homogenised and centrifuged to yield a nuclear fraction. SOX10+ oligodendrocyte lineage nuclei were then isolated by FACS using a BD InfluxTM cell sorter (BD Bioscience). The purity of the isolated fraction was assessed by reanalysis. Isolated nuclei fractions were collected and further processed for RT-qPCR analysis.

Key Methods used in Paper IV

Generation of astrocyte reporter lines

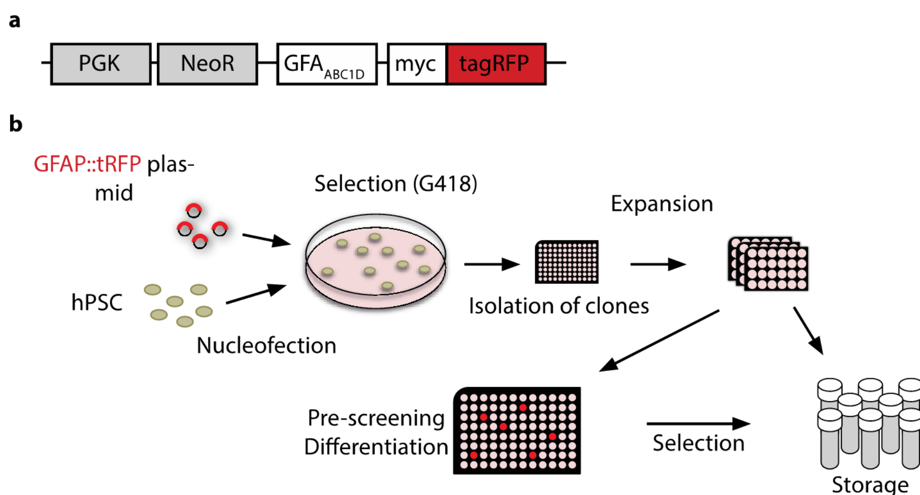

Figure 17.
a) a) Schematic representation of the construct generated and used to generate astrocyte pluripotent stem cell reporter lines. b) Strategy employed for generating astrocyte reporter lines from human embryonic stem cells. Nucleofection of reporter construct into PSCs followed by selection for resistant clones using geneticin. After expansion of the clones pre-screening was preformed for selection of clones co-expressing GFAP and GFA_{ABC1D}::TagRFP.

Design and cloning of the GFA_{ABC1D}::tagRFP construct

The pBluescript-GFA_{ABC1D} was a gift from Prof. Michael Brenner (University of Alabama-Birmingham, USA). This construct encodes a 681 bp fragment of the human GFAP promoter resulting in selective expression in murine astrocytes throughout the CNS (Y. Lee et al., 2008). The *PGK::Neo-Hb9::GFP* plasmid was a

gift from Prof. Hynek Wichterle (Columbia University of New York, USA), and was previously used to generate a hESC line reporting on motor neurons (Di Giorgio et al., 2008). The GFA_{ABC1D} promoter fragment was inserted in place of the Hb9 promoter using Sal1 and Asc1 restriction sites. Then, the tagRFP was inserted in place of the GFP using Asc1 and NheI restriction sites. The *PGK::Neo-*GFA_{ABC1D}*::tagRFP* construct was checked by restriction and sequencing.

Creation of transgenic human ES and iPS cell reporter lines

Nucleofection, selection and pre-screening were performed as described by Placantonakis et al,. (Figure 17) (Placantonakis et al., 2009); briefly, colonies of each single cell line (H13 and NAS2) were dissociated into single cell cultures, concentrated and electroporated (AMAXA, B-016 program) with the linearized vector (linearized using Not1 single restriction site). Nucleofected cells were seeded and grown on a monolayer of irradiated CF6NeoR MEFs in WiCell medium supplemented with 20 µM Y-27632 and 20 ng/ml FGF2. Once colonies were formed, selection was applied by adding increasing concentrations of antibiotic G418 to the medium. Every 3 days, G418 concentrations were increased two-fold, to a final concentration of 400 µg/ml. Resistant colonies were picked and expanded separately for storage and pre-screening. Here, cultures were considered positive once RFP expression was observed. Pre-screening was carried out using the midbrain differentiation protocol described below.

Cell culture

Maintenance of Stem cell cultures (mouse ESC, human ESC, human iPSC)

Human embryonic stem cell line H13 was obtained from the Harvard Stem Cell Institute; the human iPSC line NAS2 had been previously generated and characterization by Devine and colleagues (Devine et al., 2011). Human ESCs and iPSCs were routinely expanded at 37 °C under 5% CO_2 on irradiated CF1 MEFs (GlobalStem), in WiCell media supplemented with 20 ng/ml FGF2. Culturing media was generally changed every other day, and colonies were regularly passaged using dispase. Upon thaw from cryo-bank, 20 µM of the Rho-Kinase inhibitor (Y-27632) was added to the media, in order to increase cell recovery.

Generation of regionally specified neural progenies

One day before the start of differentiation, human ESC and iPSC colonies were harvested and seeded in low adherence flasks in Wicell medium supplemented with FGF2 and Y-27632. One to two days later when embryoid bodies had formed, patterning toward regionalized neural progenitors was initiated (day 0, D0). For patterning into midbrain (floor-plate) identity, neural induction media (NIM) was

applied. NIM was composed of advanced DMEM/ F12:Neurobasal (1:1) supplemented with 2% (v/v) B27 minus vitamin A, 2 mM L-glutamine, 1% nonessential amino acids (NEAA) and penicillin/streptomycin (P/S) (all from Life Technologies). Midbrain identity was directed using a protocol modified from Kriks et al (Kriks et al., 2011) and Kirkeby et al (Kirkeby et al., 2012a), by supplementing NIM with 0.1 µM LDN-193189 (D0-8; Stemgent), 10 µM SB-431542 (D0-6; Sigma- Aldrich), 200 ng/ml SHH-C25II N-terminus (D0-12; Life Technologies), 0.8 µM CHIR-99021 (D0-12; Stemgent) and 1 µM of smoothened agonist (SAG; D2-12; Millipore). Media was replaced every other day. Patterning toward spinal cord lineage fate was directed using protocols modified from Amoroso et al and Lamas et al., (Amoroso et al., 2013; Lamas et al., 2014) and was started in WiCell media (D0 to D4) with a gradual shift (D4-6) to glial induction media (GIM) composed of advanced DMEM/F12 with 2% (v/v) B27 plus vitamin A, 2 mM L-glutamine, 1% (v/v) NEAA and P/S. Media were supplemented with 0.1 µM LDN-193189 (D0-D6) and 10 µM SB-431542 (D0-6), 1 µM of retinoic acid (RA; D2-D12, Sigma-Aldrich), 200 ng/ml SHH-C25II N-terminus (D6-D12), 10 ng/ml BDNF (D6-D12, Life Technologies), 0.4 µg/ml ascorbic acid (D6-D12, Sigma-Aldrich). For both midbrain and spinal patterning, medium was changed every other day. Some NPC cultures were fixed for immunocytochemistry on day 12 of differentiation

Generation of midbrain and spinal cord astrocytes.

To generate astrocytes, EBs containing regionalised NPCs were expanded for 1 month as free-floating cultures in GIM. This expansion media was supplemented with 20 ng/ml FGF2 and 100ng/ml EGF (Nelson et al., 2008), and changed biweekly. After one month, glial progenitor cells (GPCs) were seeded at high density in flasks coated with PO and Lam. Plated cells were passaged weekly and grown in GIM supplemented with 1% FBS allowing spontaneous maturation of GPCs into astrocytes. On day 70, astrocyte cultures were fixed and immunocytochemistry was performed for GFAP. For longterm characterisation, reporter line astrocytes were FACS sorted on day 90 and subsequently cultured until D130

Enrichment of astrocyte populations

Flow cytometry analysis and enrichment of astrocyte populations by FACS

Flow cytometry analysis was performed on days 70, 90, 110 and 130 to assess the number of reporter positive cells. For longterm characterisation of astrocytes, populations were FACS sorted on day 70, the purified astrocyte cultures were then kept until day 130. For enrichment and flow cytometry and FACS, cells were dissociated and resuspended in FACS buffer supplemented with FBS. Samples were analysed and sorted using a BD FACSAria III (BD Biosciences) with FACSDiva v8.0

software (BD Biosciences). The cytometer was set up using a 100 µm nozzle at standard pressure of 20 psi and a frequency of 30.0 kHz and was calibrated daily using BD FACSDiva Cytometer Setup and Tracking (CS&T) software and CS&T Research Beads (BD Biosciences). For sorting, drop delay was optimised with Accudrop beads (BD Biosciences) in "fine tune" sort mode. 7-Aminoactinomycin D (7AAD) was excited by the blue laser (488 nm/20 mW) and emission was detected through a 695/40 bandpass (BP) filter. RFP was excited by the yellow/ green laser (561 nm/50 mW); emission at 610/20 BP. PMT were set using unstained and fully stained cells and emission was detected as the area of fluorescence intensity. In short, gating strategy was separating out 1) single cells based on FSC-W/FSC-A and SSC-W/SSC-A 2) live and dead cells based on uptake of 7AAD and 3) RFP positive cells compared to non-reporter expressing astrocytes. Compensation was set up using single-positive cells. Each analysis was based on 10,000 to 20,000 events. Samples were sorted at 4°C directly into GIM medium supplemented with 1% (v/v) FBS. Re-analysis of test-samples was performed to ensure sort purity (>97%) and viability (>95%) of non-enriched populations. Sorted cells were then seeded onto PO / lam coated plates.

Functional assays

Detection of cytokine- and chemokine-release by dot-blot protein array and ELISA

FACS-purified astrocytes were seeded in GIM medium containing 1% FBS (v/v) and kept in a incubator at 37 degrees. Two days later cultures were treated with either 1% FBS or 10 ng/ml IL-1β (R&D). After 7 days, the media was harvested for subsequent analysis by protein array or enzyme-linked immunosorbent assay (ELISA). For IL-6 ELISA, samples were diluted 1:10 before performing the assay according to manufacturer's guidelines (KHC0061, Life Technologies). Lastly, absorbance was measured by spectrophotometry at 450 nm. The human cytokine array panel A (ARY005, R&D Systems) was performed strictly according to manufacturer's guidelines. Here, intensities were measured using the ChemiDoc software (Bio-Rad) and then normalized to the mean intensities of reference spots

Preparation of human α-synuclein species and assay for Astrocytic protein uptake.

Recombinant human α-synuclein (S10012, rPeptide) was labelled with Atto-488 NHS ester fluorophore following manufacturer's instructions (#38371, Sigma). For fibril formation, unlabelled human aSYN and atto-488 labelled aSYN (50uM, 5:1 ratio) were incubated in buffer (165 µl 50 mM Tris, 250 mM NaCl, pH 7.5) for 7 days at 37 °C (in the dark). The solution was continuously shaken (300 rpm) for 3 days, left overnight without shaking and then shaken for 4 additional days. Preparations were evaluated by electron microscopy to confirm fibrillation. Before

treatment of astrocytes, fibrils were sonicated twice 30 s at 40% amplitude (QSonica, 125A-220). Astrocyte cultures were then incubated with 0.7 μM of either monomers or fibrils for either 3 or 7 days.

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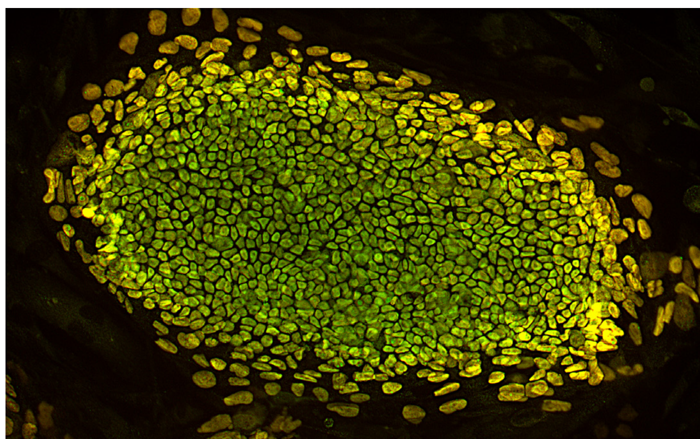

Co-expression of OCT4 (red) and NANOG (green) in induced pluripotent stem cells derived from a patient skin biopsy.

B E Staffan Holmqvist

Started his university studies with a course in geology. In 2008 he earned his bachelor in molecular biology when defending his thesis "A study on the response of Schwann cells after axonal damage". During his master studies Staffan had the opportunity to be part of a study in the Li laboratory, which resulted in him defending his thesis "Assessment of long-distance propagation of Parkinson's disease pathology", earning his master degree in 2011. This work lead to the start of his doctoral studies under the supervision of Laurent Roybon and Jia-Yi Li.

On September the 6th 2016, Staffan will defend his doctoral thesis.

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