α-Synuclein pathology and mitochondrial dysfunction
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Chapter 1

General introduction

Preface

Although this year marks the celebration of two centuries of Parkinson’s disease research, the disease still remains poorly understood. Neurodegenerative disorders put a growing strain on health care with increasing incidence in our ageing population. While much research is conducted, there have so far been few successes in preventing or delaying the onset of these diseases. It is hoped that at the start of the 21st century, with advancing technological assistance and biological understanding, scientists will finally be able to solve some of biology’s major puzzles: to understand how the brain ages and how we can manage this process.

This thesis aims to elucidate a part of this puzzle with a focus on Parkinson’s disease and by using the latest technological developments and scientific insights in the field of molecular neuroscience. Dopaminergic neurons derived from induced pluripotent stem cells (iPSCs), engineered from skin biopsies, allow us to study these neurons in the process of degeneration for the first time in decades after their implication in Parkinson’s disease. Combining these techniques with advanced fluorescence microscopy allows us to follow the dynamics of mitochondrial trafficking in-vitro in neurons derived from Parkinson’s patient skin biopsies. The following introduction aims to provide a concise background to the work presented in this thesis.

Parkinson’s disease

Parkinson’s disease (PD) is the second most common neurodegenerative and the most common movement disorder in the world, affecting around 2% of the population over the age of 60. First described in 1817 by James Parkinson as paralysis agitans [1], the work
of French neurologist Jean-Martin Charcot provided a landmark in the understanding of the disease nearly 50 years later. He described early motor symptoms including tremors, rigidity, bradykinesia and postural instability [2]. He also ascertained that the disease was not of pyramidal origin, but it took the work of Arvid Carlsson in the late 1950’s to describe the underlying cellular processes using a model of monoamine depletion to immobilize animals [3, 4]. This pointed to the involvement of the dopaminergic system, specifically the substantia pars compacta (SNc), harboring the ventral midbrain A9 dopaminergic population. Progressive cell death of these neurons leads to diminished dopamine secretion in the putamen and caudate nucleus of the striatum, causing the underlying cause of the observed motor defects in PD. Onset of motor symptoms occurs when around 60% of the dopaminergic (DA) neuronal population and around 80% of dopamine is depleted [5]. This leads to a challenging conclusion, since it means that the disease is mostly studied at the end-stage when DA neurons are dying or dead and when most DA neurons are characterized by protein inclusions called Lewy bodies. Motor function deteriorates in most patients over a period of 10 years after diagnosis, during which non-motor effects like sleep disorders, depression and dementia can manifest [6]. This indicates that though DA neurons are mainly affected, other neuronal populations are also affected by the disease. In 2003, Braak et al. observed a specific pattern of disease spreading, now widely known as Braak staging of Parkinson’s disease [7]. The accompanying Braak hypothesis stipulates that Lewy bodies spread throughout the brain via the olfactory bulb and/or gastrointestinal tract, entering the medulla oblongata and pontine tegmentum at stage 1 and 2. Stage 3 and 4 show spreading to the midbrain, leading to lesions in the substantia nigra. Finally, stages 5 and 6 show development of lesions and inclusions in the neocortex (see Figure 1) [8]. This is a critical observation, since it indicates that substantia nigra DA neurons are not the first cell type affected by disease, but are likely more vulnerable to the disease pathology. Indeed, many patients report bowel irritability or changes in
olfaction up to a decade prior to disease [9], as predicted by the Braak hypothesis [10]. The observation that many patients develop dementia or depression, also adds evidence for the later stages described by Braak et al. [11]. It should be noted, however, that many patients do not strictly follow the pathway delineated by Braak et al. and PD can be highly heterogeneous in both development and progression of disease [12]. For this reason, the Braak hypothesis is frequently debated, and careful considerations should be made concerning the cause of PD. Nevertheless, the observation that spreading of disease occurs in PD and that the disease is progressive remains a widely accepted view. In this thesis, Braak staging will be approached from the latter view, a piece of evidence for spreading of pathology and the progressive nature of the disease.

![Figure 1. Braak staging of Parkinson's disease. At stage I & II pathology spreading is observed in the pons and the olfactory bulb, after which disease pathology progresses towards the limbic systems (Stage III & IV). Finally, at stage V & VI disease pathology progresses to the neocortex. Modified after Braak et al. [8] ](image)

**Figure 1. Braak staging of Parkinson’s disease.** At stage I & II pathology spreading is observed in the pons and the olfactory bulb, after which disease pathology progresses towards the limbic systems (Stage III & IV). Finally, at stage V & VI disease pathology progresses to the neocortex. Modified after Braak et al. [8]

**Ageing and disease**

For many prevalent diseases, the largest risk factor is age. With an increase in life-expectancy, the incidence of age-related disorders increases, placing a growing burden on society. To tackle this epidemic,
efforts to understand the biology of ageing is vital. For many years cellular ageing was approached from the concept of genetic control of ageing, which was supported by the Hayflick studies in the 1960s [13]. However, ageing is now widely considered to be a complex process consisting of both internal and external factors leading to loss of physiological integrity. This loss of physiological integrity in ageing is currently characterized by the following hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication [14]. Many of the hallmarks mentioned in [14] are based on observations in cancer and stem cells, and may not be directly applicable to an ageing brain. In the brain, the cumulative acquisition of cellular damage under normal metabolic function is likely to result in cellular senescence. To assess hallmarks of PD, the next sections will discuss several contributors to this disease.

**Genetic contributors to Parkinson’s disease**

Though familial PD cases have provided more insight into the disease development and contributors, the disease development of both idiopathic and familial Parkinson’s remains poorly understood. Most PD cases (>90%) are thought to be sporadic, and some studies have linked the development of PD to environmental toxins [15] or genetic risk factors [16]. An overview of the most common genes involved in familial PD is depicted in Table 1.
Table 1. Most common familial PD genes and mutations, gene names and mutations with reported age of onset. Disease progression and special remarks are stated in the last columns.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutations</th>
<th>Average A/O</th>
<th>Progression</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SNCA</strong></td>
<td>Duplication [17]</td>
<td>~50</td>
<td>Slow</td>
<td>Dosage effect</td>
</tr>
<tr>
<td></td>
<td>Triplication [18]</td>
<td>~40</td>
<td>Rapid</td>
<td>Dementia, prominent motor symptoms</td>
</tr>
<tr>
<td></td>
<td>A53T [19]</td>
<td>40-50</td>
<td>Rapid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A30P [20]</td>
<td>~60</td>
<td>Slow</td>
<td>Mild dementia</td>
</tr>
<tr>
<td></td>
<td>E46K [21]</td>
<td>~45</td>
<td>Rapid</td>
<td>Dementia, visual hallucinations</td>
</tr>
<tr>
<td><strong>LRRK2</strong></td>
<td>G2019S [22]</td>
<td>~60</td>
<td>Slow</td>
<td>Occasional absence of Lewy bodies</td>
</tr>
<tr>
<td><strong>VPS 35</strong></td>
<td>D620N [22]</td>
<td>~60</td>
<td>Slow</td>
<td>Resembles idiopathic PD</td>
</tr>
<tr>
<td><strong>PARKIN</strong></td>
<td>Many [23]</td>
<td>20-40</td>
<td>Slow</td>
<td>Absence of Lewy bodies</td>
</tr>
<tr>
<td><strong>PINK1</strong></td>
<td>Many [24]</td>
<td>20-50</td>
<td>Slow</td>
<td>Resembles idiopathic disease</td>
</tr>
</tbody>
</table>

In 1997, Polymeropoulos et al. discovered the mutation G209A in the SNCA gene in familial PD, leading to an A53T mutation in the protein α-synuclein [19]. Shortly thereafter, α-synuclein was discovered as a major constituent in Lewy bodies [25, 26]. Currently, seven α-synuclein related mutations are known, including the G88C (A30P) [20] and G188A (E46K) [21, 27] mutations or duplications [17] and triplications [18] of the SNCA genomic region. While these mutations are present in the same gene, these mutations have varying disease characteristics. A53T mutations are associated with rapid progression and widespread motor pathology [28]. A30P mutations on the other hand display a milder phenotype, with later age of onset and the occurrence of mild dementia.
E46K mutations lead to rapidly progressing PD, similar to A53T mutations, but with added visual hallucinations [27]. Interestingly, triplication of the SNCA locus leads to earlier onset of disease compared to duplication, indicating there is a dosage effect in the expression of α-synuclein and disease onset [30]. A typical age of onset for SNCA triplication (four SNCA copies) is around 40 years, while SNCA duplication (three SNCA copies) onset is around 50 years of age. Disease progression in SNCA duplications is also slower and the disease pattern is highly heterogeneous [31], adding further evidence for a dosage effect.

In addition to α-synuclein, other familial PD genes have been identified, such as mutations in leucine-rich repeat kinase 2 (LRRK2), leading to autosomal dominant PD [22], or acting as risk factor depending on the mutations [32]. The fact that LRRK2 can also be a risk factor for PD, hints that both genetic and environmental cues can contribute to disease.

Vacuolar protein sorting-associated protein 35 (VPS35) mutations have only recently been discovered and interestingly lead to disease progression resembling idiopathic PD [33, 34]. As a major component of a membrane protein-recycling retromer complex, VPS35 is thought to play a role in mitochondrial turnover, causing mitochondrial fragmentation and cell death.

Juvenile PD with an onset below 30 years of age is mainly caused by homozygous PARK2 mutations [23]. Lewy bodies are absent in this form of PD and progression of disease is slow. The second largest cause for juvenile PD are mutations in the protein PINK1, with similar disease characteristics as idiopathic PD [24]. Both PARK2 and PINK1 are involved in mitochondrial turnover and lead to mitochondrial dysfunction at early age.

**Synucleins**

The synuclein family consists of the three genes SNCA, SNCB and SNCG, encoding the proteins α-, β- and γ-synuclein. Synucleins are
predominantly found in neural tissues, and are enriched at presynaptic nerve terminals [35]. In neurons they are abundantly expressed and by some estimates are thought to make up 1% of the cytosolic protein content [36]. Human α-synuclein has 140 amino acids, and is believed to be natively unfolded [35], although recent research has suggested it might be present in a tetrameric structure [37]. The structural properties of α-synuclein are depicted in figure 2.

![Figure 2. Structural properties of α-synuclein. Known mutations are located in the N-terminal phospholipid domain, which is characterized by KTKEGV repeats. This is followed by the hydrophobic NAC domain and flanked by the acidic C-terminal domain.](image)

The central portion of the protein, called the non-amyloid-β component (NAC), is hydrophobic and prone to aggregation [38]. The C-terminus is highly acidic, and the N-terminus consists of 11-residue slightly dissimilar repeats (XKTKEGVXXX), which influence α-synuclein aggregation properties. Interactions with acidic phospholipid surfaces are mediated via the N-terminal domain. Anionic solvents like sodium dodecyl sulfate (SDS) can also interact with this domain, making it more challenging to study these proteins in Western blot or immune labeling. Upon interaction with phospholipid surfaces, conformational changes cause synuclein to fold into an amphipathic α-helix. This property likely sensitizes α-synuclein to dimerization. Under normal physiological conditions α-synuclein can change from a membrane bound state to an unfolded state. The initial phase of dimerization that occurs is reversible, but dimers exhibit high stability and can act as a trigger in a self-assembly process [39]. This leads to a thermodynamically favorable state in which synuclein can form oligomers, and subsequently fibrils. Finally, the
formation of these fibrils is hypothesized to lead to the formation of Lewy bodies (see Figure 3.).

**Figure 3. Model for α-synuclein aggregation.** Membrane bound state and natively unfolded monomers can dimerize, which leads to formation of oligomers. This is followed by the formation of fibrils, ultimately leading to the formation of Lewy bodies. Arrows indicate the favorable conformational state.

The exact functions of the synuclein protein family remain to be elucidated, but involvement in vesicle snaring and exocytosis has been established [40]. Mouse α-synuclein knockout models display mild cognitive impairment [41], but even triple synuclein knockout mice show no morphological aberrations or decrease in synapse number [42]. This suggests that synucleins have a modulatory role and are not essential for neuronal function or survival. Considering its abundant expression pattern, it is likely that the synuclein family is involved in the modulation of many cellular processes.

**α-Synuclein and protein inclusions**

The propensity of α-synuclein to oligomerize, fibrillize and form aggregates has been studied in detail since its implication in PD. While Braak staging revealed a step-wise disease progression, the underlying mechanism for spreading pathology remained unknown. In 2008, Li et al. showed the presence of Lewy bodies in post mortem brains of PD patients that received embryonic nigral transplants 10 to 15 years before [43]; these findings indeed point to a spreading of pathology into the newly transplanted dopamine neurons from surrounding tissue. The transplanted fetal cells contained α-synuclein positive inclusions and additional experiments showed that this was the case only in long-term
grafts (>10 years after grafting) [44]. This spread of pathology is thought to be the result of prion-like properties of α-synuclein, which has been demonstrated in various model systems. The ability of α-synuclein to transfer from cell to cell has been demonstrated in cell models [45] and recently in rat models. By injecting rats with post mortem PD patient brain lysate or α-synuclein fibrils near the enteric plexus, α-synuclein was able to spread to the brain via the vagus nerve [46]. This find is supported by epidemiological evidence that shows chronic duodenal ulcer patients undergoing a truncal vagotomy have a reduced risk of developing PD [47].

Taken together, there is ample evidence that supports the Braak hypothesis, cell-to-cell transfer of α-synuclein, and gastro-intestinal involvement in the development of PD.

**Protein homeostasis**

Aberrant protein homeostasis leads to conformational changes in tertiary protein structure, incorrect folding of proteins or non-functional degradation of proteins. Each of these processes has been studied for PD, but no clear underlying mechanism has arisen to describe the development of disease. Chaperone folding proteins have been implicated in many neurodegenerative diseases and in PD it has been observed that heat shock proteins (HSP) are present in Lewy bodies [48, 49]. Apart from α-synuclein, Lewy bodies contain neurofilaments, various ubiquitinated proteins, and HSP70 and HSP90. Furthermore, proteasomal degradation of α-synuclein has been linked to HSP27, HSP40, HSP70, and HSP90 [49]. It is hypothesized that proper function of HSPs can prevent occurrence of inclusions and avert development of PD [50], but as of yet none of the HSP genes have been associated to PD risk in large GWAS studies. Further investigation is required to elucidate the causal relationship between HSPs and PD, and check their validity as a therapeutic target.

One prominent gene involved in protein degradation has been linked to PD. Mutations in glucocerebrosidase (GBA1) cause lysosomal
dysfunction and lead to increased risk for α-synuclein aggregation [51]. Currently, GBA1 mutations are considered the most widely distributed genetic risk factors for PD, with reported GBA1 mutations in over 5% of PD patients. Estimates conclude that GBA1 mutations can lead to a 20- or 30-fold increased risk for PD development [52]. GBA1 mutations are also implicated in Gaucher disease [53] in which recessive mutations can cause lipid storage disease. Heterozygous carriers of Gaucher disease associated GBA1 mutations have an increased risk for PD. Mutations in Gaucher disease are usually associated with loss of function or out of frame mutations that disrupt protein production. GBA1 mutations associated with PD, on the other hand, are hypothesized to induce a gain of function [51]. The underlying mechanism of GBA1-related PD is still debated, and it remains a protein of interest since disease closely mimics the pathology of idiopathic PD with a late onset and relatively slow disease progression.

Another protein implicated in PD involved in protein homeostasis is LRRK2. While being a major risk factor for PD, around 20% of heterozygous mutation carriers do not develop disease. Furthermore, carriers that do get PD can present highly heterogeneous disease patterns and display differences in neuropathology or even have an absence of Lewy bodies [30]. Apart from PD, LRRK2 mutations are also implicated in other neurodegenerative disorders, including Lewy body pathology, tauopathies, and Alzheimer’s disease [54, 55].

**Mitochondrial dysfunction in Parkinson’s disease**

The link between PD and mitochondrial dysfunction was elucidated by a chemistry student who attempted to produce synthetic opiates and injected himself with the substance 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [56]. Several days after, he suffered from what appeared to be early onset PD, and the connection between drug use and the disease was made. MPTP is converted by glia cells into MPP+ by monoamine oxidase-B, and subsequently taken up by dopamine
transporters of DA neurons. The mechanism by which MPP+ exerts its effects is attributed to interference with complex I of the electron transport chain in mitochondria [56]. This leads to the production of reactive oxygen species (ROS), a decrease of ATP production and a subsequent toxic increase in cellular calcium levels. Several toxins causing mitochondrial respiratory chain inhibition have since been found to induce PD-like symptoms, such as the pesticide rotenone [57] and the herbicide paraquat [58]. Many pesticides are now epidemiologically linked to the development of PD and most are linked to dysfunction of complex I [15, 59]. The involvement of complex I is further supported by the finding that complex I activity is reduced in muscles and brain of PD patients [60-62]. Mild inhibition of complex I can lead to increased production of ROS, which can in turn lead to DNA damage, RNA damage, and protein damage.

Further evidence for mitochondrial dysfunction in PD is provided by mutations in the PINK1 and PARK2 genes, coding for the protein PINK1 and Parkin. In mitochondria with intact membrane potential, PINK1 is internalized into the inner mitochondrial membrane, where it is cleaved and inactivated by PARL [63, 64]. Mitochondria that lack sufficient membrane potential are unable to internalize PINK1 and, after recruitment of Parkin, can go into mitophagy or mitofission [65]. Mutations in either PINK1 or PARK2 lead to early onset (<40 years) PD with risk of additional features including depression and dementia [23, 24]. While non-motor symptoms are observed, the specific degeneration of DA neurons in the substantia nigra pars compacta emphasizes the vulnerability of these cells. A link between α-synuclein and ROS production has yet to be elucidated, but theories about underlying mechanisms are diverse, ranging from direct effects of α-synuclein oligomers on the mitochondrial membrane [66, 67], effects on mitophagy [68, 69], to indirect effects on mitochondrial trafficking in neurons [70, 71].
Environmental contributors to PD
The implication of mitochondrial toxins has led to the theory that environmental factors are contributors to the incidence of sporadic PD. While herbicides and pesticides show a clear connection to PD [58], other chemicals and heavy metals have also been implied in idiopathic PD [72, 73]. While not entirely conclusive, an etiologic study by Tanner et al. on monozygotic and dizygotic twin pairs established that a genetic component is not evident for idiopathic PD [74]. These results were confirmed in another twin cohort, suggesting environmental factors play an important role in the etiology of sporadic PD [75]. Indeed, the occurrence of PD has been linked to higher exposure to pesticides [76] and rural living [72]. Other chemicals implied in PD are organic solvents, such as the solvent trichloroethylene, which is associated with an increased risk of PD upon repeated exposure [77]. It should be noted that while evidence for an environmental disease component is strong, development of sporadic PD is likely attributable to a complex interplay between genetic susceptibility and environmental factors.

Axonal transport and Parkinson’s disease
Neuronal axons can cover large distances and axonal trafficking to distal parts is essential to the function of neurons. The unique architecture of the neuronal cell requires information from the soma to be transported for lengths of over a meter in some neurons. The axonal trafficking process is complex and makes neuronal cells vulnerable to trafficking deficiencies. Many neurodegenerative diseases, including PD [78, 79], have been linked to altered axonal transport [80-82]. The basic mechanism for transport requires a cytoskeletal network and the presence of motor proteins. In neurons, long-range axonal transport is mainly dependent on microtubules, the presence of kinesins for anterograde and the presence of dyneins for retrograde trafficking. Cargo (e.g. vesicles, proteins, mitochondria) is bound to kinesins or dyneins and subsequently transported over microtubules. Direction is caused by polarity of the
microtubule, whereby kinesins move to the plus-end while dyneins move to the minus-end. Transport rates can vary greatly from fast trafficking of organelles (~1 µm/s), to slow trafficking of structural proteins (<0.1 µm/s) (See also Figure 4) [83].

Figure 4. Schematic representation of axonal trafficking. Anterograde trafficking takes place via kinesins, while retrograde trafficking is facilitated by dyneins. Trafficking takes place along microtubules, which are organized with a distal plus-end and minus-ends near the soma.

Synucleins are predominantly transported via slow trafficking and axonal transport of synuclein slows significantly with ageing [84, 85]. This trafficking retardation was proposed to lead to α-synuclein aggregation formation over time, hampering normal axonal trafficking dynamics [86].

Selective vulnerability of SNc DA neurons likely arises from the size of their largely unmyelinated axonal arbor, estimated to be ten times larger compared to other neurons [87]. Apart from this complex axonal architecture, SNc DA neurons express L-type Ca^{2+} channels that regulate the robust pacemaker properties of these neurons resulting in high neuronal activity [88, 89]. Taken together, the cellular architecture and intrinsic pacemaking properties place SNc DA neurons under constant
Mitochondrial integrity in distal regions of neuronal cells is entirely dependent on proper retro- and anterograde trafficking of these organelles. Therefore, the complexity of the axonal arbor in SNc DA neurons makes these cells vulnerable to trafficking deficits. Another link is provided by the observation that PINK1 is involved in mitochondrial trafficking, fission and fusion through interaction with the MIRO/Milton complex [90]. The MIRO/Milton complex is an essential adaptor complex involved in mitochondrial trafficking, coupling mitochondria to kinesin 1 [91]. A link between mitochondrial trafficking, PINK1 mutations, and early onset PD provides evidence for mitochondrial trafficking involvement in this disease [92]. Equally important to anterograde trafficking is retrograde trafficking. Indeed, the implication of axonal trafficking in PD is supported by a heterozygous mutation in DCTN1, a subunit of the dynactin complex, which anchors vesicles to retrograde dynein motor proteins. Mutations in DCTN1 lead to late onset PD with mild cognitive impairment [93]. While the process of mitophagy, i.e. the elimination of damaged mitochondria, can take place at distal regions of the axon [94], proper retrograde trafficking remains essential to remove dysfunctional or damaged mitochondria. Presence of damaged mitochondria at distal parts of the cell leads to oxidative damage at these sites, resulting in damaged mitochondrial DNA and oxidation of proteins at these sites. Presynaptic terminals, which are characterized by a high energy demand and Ca\(^{2+}\) accumulation, are crucially dependent on functional trafficking of mitochondria. The link between intracellular trafficking and α-synuclein pathology has been investigated, and trafficking has been shown to be impaired upon expression of α-synuclein [95]. The inhibiting effect of oligomeric α-synuclein on tubulin polymerization is a mechanism that has been proposed [96], and a recent study has shown a direct effect of α-synuclein on retrograde trafficking [97]. Lysosomal dysfunction in an iPS-based PD model is also demonstrated to occur through trafficking disruptions by α-synuclein [98],
further adding to the hypothesis that trafficking is impaired in PD pathology. The bridge between mitochondrial dysfunction and trafficking impairment has been illustrated by assessing the mitochondrial dynamics in mouse cortical neurons overexpressing human α-synuclein. In this model, overall transport velocity was reduced in axons of cells expressing A53T α-synuclein [99]. However, the underlying trafficking dynamics, i.e. retrograde and anterograde trafficking, and the presence of this deficiency has not yet been characterized in a human PD model. The focus of this thesis will be primarily on α-synuclein and mitochondrial trafficking in relation to PD.

**Therapies for Parkinson’s disease**

Groundbreaking research in the early 1950s by Arvid Carlsson [3] showed a reduction of Parkinsonian symptoms by administering the dopamine precursor L-DOPA in a Parkinsonian animal model. The discovery led to the most common and effective line of therapy of PD [100]. While other drugs like monoamine oxidase B inhibitors [101] or dopamine agonists [102] are also used, the side effects associated with these treatments make them less suitable compared to L-DOPA [103-105]. Another line of therapy used is the surgical insertion of brain electrodes [106]. Deep brain stimulation has proven effective in patients with unstable L-DOPA responses, but possible complications during surgery and the fact that it merely treats symptoms of disease make it less commonly used. Addressing the underlying cause of PD symptoms, the loss of ventral midbrain DA neurons, was explored in the 80’s by injecting fetal brain grafts into the brains of PD patients aiming to restore striatal DA levels [107]. While initial results were positive, later trials reported no significant benefit within a short period following engraftment [108]. Furthermore, the high demand for fetal tissue, six to eight embryos for one brain [109, 110], made the technique impractical for routine therapy. Due to side effects and ethical concerns, fetal transplant therapies in PD were put on halt for over a decade, but have recently gained renewed interest
Advancements in the stem cell field have provided methods that can provide a potential source for DA neurons. The introduction of DA neurons derived from embryonic stem cells (ESCs) in 2003 [112, 113], and the later discovery of induced pluripotent stem cells (iPSCs) [114] have created an inexhaustible source of new cells. The ability of these cells to differentiate into DA neurons makes them a suitable candidate for transplantation studies. Apart from stem cell based methods, transdifferentiation has also been proven feasible using skin fibroblasts [115, 116], providing an even more accessible source of DA neurons.

Cell models for Parkinson’s disease research

PD disease models have contributed a large part of our understanding of the disease. These models range from animal models [117] and cell based models [118] to phospholipid membrane models [119]. Animal models have been extremely valuable in explaining PD pathogenesis [120], but there are limitations to these model organisms [121]. MPTP induced toxicity, for instance, presents a different pathology depending on the mouse strain used [122], and is different from human MPTP toxicity [121]. MPTP primate models offer a striking resemblance to human toxicity [123], but high costs and ethical concerns surrounding the use of these models limit their use. Rodent transgenic human α-synuclein models have produced mixed results, mainly display pathology outside the SNc, and do not cover all clinical aspects of PD pathology [124]. PD cell models overcome some of the challenges faced in PD animal experiments, and have been instrumental for discovering the complex pathways involved in the disease process [118]. A human genetic background, cost-effectiveness and the lack of ethical concerns create an advantage for PD cell-models compared to animal models. The most widely used cell line in PD research is the SH-SY5Y neuroblastoma cell line [125]. It is a subline of the SK-N-SH cell line, which was originally derived from a metastatic tumor biopsy [126]. While use of a mitotic tumor cell line might seem undesirable, SH-SY5Y cells can be differentiated to
a more neuronal state by using retinoic acid [120]. The resulting populations display a higher expression of dopaminergic markers, and subsequent treatment with phorbol esters increases this expression even further [127]. These properties, combined with the cost-effectiveness, ease of culturing and reproducibility make SH-SY5Y cells an excellent tool for the elucidation of biochemical pathways involved in PD [118]. However, SH-SY5Y cells do have shortcomings when used as a PD cell model. Endogenous expression of DA neuronal markers and expression of α-synuclein are low compared to SNc DA neurons [128], and the proliferative nature of these cells is in sharp contrast to a post-mitotic neuronal state. The discovery of ESCs and iPSCs have provided a solution for this problem, by being able to differentiate these cells into ventral midbrain DA neurons resembling their human counterparts [129]. Though this method is more laborious and more costly compared to SH-SY5Y cells, the ability to study endogenous expression of disease causing proteins in a PD patient genetic background holds great promise. The amount of publications using iPSCs and ESCs in the field of PD research is still relatively low, but with the arrival of new differentiation methods, the technique is bound to provide new insights into the disease process [130]. Additionally, transdifferentiation holds great promise as a source for DA neurons to be used in disease modeling. While the availability and the neuronal yield of direct reprogramming protocols is low, the presence of epigenetic marks associated with aging make these neurons more useful in the study of late onset disorders [131].

**Generation of dopamine neurons**

Animal studies have elucidated many of the pathways involved in embryogenesis and have been crucial in creating protocols to generate DA neurons from ESCs and iPSCs [132, 133]. The intrinsic and extrinsic signaling pathways involved can be reproduced by supplementing cell culture media with a variety of defined factors, thereby mimicking early embryogenesis. After formation of neuroepithelium in the neural tube,
regional specific generation of mesencephalic DA neurons takes place under the influence of two extrinsic signaling centers: fibroblast growth factor 8 (FGF-8) signaling from the isthmus induces the development of DA neurons at the anterior-posterior axis of the neural tube [134], while sonic hedgehog (SHH) signaling from the floor plate positions these neurons along the dorsal-ventral axis [135]. Additionally, early WNT signaling promotes early DA neurogenesis, proliferation and maturation (see Figure 5A).

**Figure 5.** (A) Generation of ventral midbrain DA neurons during development. While FGF-8 positions DA neurons along the anterior-posterior axis, SHH positions these neurons on the ventral plane at the floor plate. WNT signaling promotes early DA neurogenesis and proliferation. (B) Schematics of dual-SMAD based dopaminergic differentiation of human pluripotent stem cells, coupled with a schematic plot illustrating changes in protein expression levels during this differentiation.
Trophic factors like glial- [136] and brain- [137] derived neurotrophic factor (GDNF/BDNF), appear to be essential for the survival of these DA neurons as demonstrated in various mouse knockout studies [138, 139].

To mimic the in-vivo process of regional specification during in-vitro dopaminergic differentiation of ESCS a two-step protocol has been developed based on the signaling pathways employed by the above-described factors (Figure 5B) [129, 140]. The first step covers the neural induction of ESCs by dual inhibition of SMAD signaling with small molecules [140]. SB431542 blocks phosphorylation of the receptors ALK4, ALK5 and ALK7, inhibiting activin and TGFβ pathways. LDN-193189, a small molecule inhibitor of ALK2 and ALK3, blocks BMP signaling, promoting neuralization of primitive ectoderm. In the second step, the resulting neural stem cell-like populations are subjected to Wnt pathway inhibition using CHIR99021, a potent GSK3 inhibitor, and are supplemented with FGF-8 and SHH to increase the efficiency of DA cell differentiation [129]. During dual SMAD inhibition, expression of pluripotency markers such as NANOG and OCT3/4 and SOX2 decreases, while expression of midbrain marker LMX1A and FOXA2 increases. At around 7 days, expression of the neuronal marker BIII-tubulin increases, and the cells start to display neuronal structures. This is followed by tyrosine hydroxylase (TH) expression around 15-20 days of differentiation, and increases until around day 30 of differentiation. Upon maturation, neurons become electrically active and start to display markers such as GIRK2. After maturation, the resulting DA neuron populations are supplemented with trophic factors BDNF, GDNF and the γ-secretase inhibitor DAPT [141] to sustain long-term in-vitro culture. Although this DA differentiation protocol has been shown to be the most effective in generating ventral midbrain DA neuronal populations, variability between different ESC and iPSC lines appears to be considerable [142], and are likely caused by differences in neural differentiation potential between iPSC lines [143].
Outline of the thesis

Parkinson’s disease is associated with α-synuclein pathology and mitochondrial dysfunction, but the mechanisms linking these pathological aspects of PD are currently still unclear. The aim of this thesis is to elucidate part of these mechanisms using novel technologies and to determine how α-synuclein pathology contributes to mitochondrial dysfunction. For this purpose, we generated α-synuclein-overexpressing neuronal cell models to study mitochondrial function. Using iPSC technology, we further show that long-term in-vitro generated neurons show similar pathologies as those observed in our overexpressing cell models. Finally, to mimic age-associated pathology, we introduce an experimental model to effectively age cells derived from iPSCs, since these cells must be considered rejuvenated as a consequence of the iPSC induction process.

In Chapter 2, we have used SH-SY5Y cells overexpressing wild-type, A30P and A53T α-synuclein to examine the effect of α-synuclein on mitochondrial trafficking. We demonstrate that cells overexpressing A30P and A53T α-synuclein show an increase in ROS production and that trafficking in A53T α-synuclein expressing cells is significantly reduced, first in retrograde trafficking, and at a later stage also in anterograde trafficking. Treatment with davunetide, a microtubule-stabilizing peptide, rescues trafficking deficiency and decreases ROS production.

To validate the findings of our SH-SY5Y experiments, we use PD-patient derived iPSCs to study similar processes in the DA neurons produced from these iPSCs. To this aim, long-term culture protocols were used to prevent the usage of mitochondrial toxins and to study cells with endogenous α-synuclein expression. In order to be able to perform experiments on purified cell suspensions of DA neurons, we developed a new method to purify neuronal cells based on metabolic capacity, as described in Chapter 3. Using glucose deprivation and lactate supplementation effectively enriches neuronal populations, leading to
post-mitotic neuronal populations without the use of toxic mitotic inhibitors or need for strenuous cell sorting. The resulting neuronal cultures are functional and have a significantly higher percentage of neuronal cells. Crucially, the resulting populations can be cultured for prolonged periods of time without interference of proliferating cells.

Using our purified long-term cultured neurons, we show in Chapter 4 that PD patient-derived neurons, obtained via iPSC technology present a similar mitochondrial deficiency as observed in the SH-SY5Y cells described in Chapter 2. The familial PD-derived iPSCs, from respectively a PD4 (SNCA triplication) and a PD1 (A53T mutation) patient, were successfully differentiated into DA neurons and were analyzed at 90 days in-vitro to assess mitochondrial trafficking. Mitochondrial trafficking appears to be significantly reduced in both PD1- and PD4-derived DA neurons compared to control, but not between PD1 and PD4 DA neurons.

The need to culture cells for prolonged periods of time when studying aging-related pathologies is challenging in in-vitro research using iPSCs, since they are known to generate rejuvenated differentiated cells. Also the need to use mitochondrial toxins, and proteasome inhibitors makes it a complicated model to work with. Therefore, we have used transgenic overexpression of progerin, the disease-causing protein in the accelerated aging Hutchinson-Gilford progeria syndrome, to “age” our rejuvenated, iPSC-derived DA neurons (Chapter 5). We show that progerin-overexpressing PD patient-derived DA neurons exhibit an increase in α-synuclein load. Unfortunately, these DA neurons appeared to be unsuitable to study the progerin-induced effects on mitochondrial activity and cellular aging features in detail as they are more difficult to culture. Therefore, we have included studies on cardiomyocytes, since these cells are relatively easy to differentiate and have high mitochondrial activity. The resulting cultures demonstrate that progerin-expressing cells have folded nuclei, a higher incidence of double stranded DNA breaks and a higher production of ROS.
Finally, in **Chapter 6** the results of the studies described in this thesis have been discussed. Furthermore, suggestions for future work are proposed and the relevance of this work in the stem cell field is discussed.

**References**


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