Chapter 6

Summary and discussion

In two hundred years after the first description, the understanding of Parkinson’s disease (PD) has evolved from a classical motor disease to a disease with complex heterogeneous symptoms [1]. Pathological hallmarks such as Lewy bodies (LB) and neuronal cell death have been found outside the substantia nigra pars compacta (SNc), and it is now accepted that PD pathology involves large regions of the nervous system [2]. This complexity is also reflected in our current understanding of the cause of disease: a complex interplay between genetic and environmental factors [1]. Despite a large increase in understanding of the molecular and cellular basis of the disease there are no treatments that slow the neurodegenerative process, and management of disease symptoms by administration of L-Dopa has been used for over 50 years [3]. In part, this is attributable to difficulties with proper clinical diagnosis of a disease that develops slowly over many years, and consequently results in the study of only the latter stages of disease [4]. Recent advancements in genetics and biotechnology, such as the discovery of genetic contributors to PD [5, 6] and the introduction of induced pluripotent stem cells (iPSCs) [7] and embryonic stem cells (ESCs) [8], have presented new opportunities in understanding PD [9]. In this dissertation, patient-derived iPSCs carrying the PD donors’ genetic background were used to study the early onset of familial PD.

Chapter 1 provides a concise introduction into the history and current literature of PD research. This is followed by a study in a neuroblastoma cell PD model in Chapter 2, which focusses on mitochondrial trafficking in these cells. To validate results of this study in iPSCs, a method for purification of iPSC derived dopaminergic (DA) neurons is introduced in Chapter 3. In Chapter 4 mitochondrial trafficking
in these neurons is assessed, to validate the findings of Chapter 2. Finally, Chapter 5 introduces a method based on the Hutchinson–Gilford progeria syndrome, to accelerate the in-vitro ageing of hiPSC- and hESC-derived cells. Box 1 contains an overview of the findings of this dissertation, which will be discussed in the following sections.

**Box 1. Main conclusions and findings of this dissertation.**

**Mitochondrial trafficking in Parkinson’s disease**

As highlighted in the general introduction, the selective vulnerability of SNc DA neurons is likely attributable to a combination of constant pace-making activity and a complex axonal arbor [10]. Many proteins critical to synaptic function are produced in the soma and require functional anterograde transport along the axon [11]. Conversely, retrograde transport is necessary for the removal of dysfunctional organelles and the transport of neurotrophic factors [12]. We hypothesized that SNc DA neurons are more vulnerable to mitochondrial trafficking deficiencies as mitochondrial activity in these neurons is high and trafficking processes are more complex compared to other neuronal cells. Mitochondrial trafficking deficiencies have been associated with many different neurodegenerative disorders [11], but the underlying mechanisms and effects of these deficiencies differ and have not been completely explored. A heterozygous mutation in the *DNM1L* gene, for
instance, results in severe infantile neurodegenerative disease characterized by microcephaly and abnormal brain development [13]. DNM1L encodes the protein Drp1, which is involved in normal mitochondrial fission [14]. Mutations in the middle domain of Drp1 lead to elongated mitochondria, resulting in an incorrect distribution of mitochondria in neurons [15]. Interestingly, DNM1L mutations primarily affect neuronal cells, indicating that correct mitochondrial fission is essential for their function, development and maintenance through proper axonal trafficking [16]. Conversely, compound heterozygous and homozygous mutations in the protein mitofusin 2 (Mfn2) lead to the axonal peripheral sensorimotor neuropathy Charcot-Marie-Tooth neuropathy type 2A [17]. Improper mitochondrial fusion by Mfn2 leads to small fragmented mitochondria, which display significant impairment in neuronal mitochondrial trafficking [18]. Strikingly, conditional knockout mice for Mfn2 in DA neurons display nigrostriatal degeneration in a dying-back phenotype [19]. In these mice, increased mitochondrial fragmentation led to a decrease of transport within the axon. Similar to this, the early onset familial PD mutations in PINK1 and Parkin also result in abnormal mitochondrial morphology and trafficking deficiencies [20, 21]. These mutations illustrate the importance of a fine balance between fusion and fission of mitochondria required for proper mitochondrial dynamics.

To investigate if mitochondrial trafficking is also involved in cellular pathology of familial PD with mutations in the SNCA gene, we established both a neuroblastoma cell model and an iPSC in-vitro PD model. In Chapter 2, SH-SY5Y cells were used to generate an in-vitro model using transgenic overexpression of α-synuclein mutants. This model uses neuroblastoma cells with a high mitotic index that can be lowered by exposing the cells to retinoic acid [22], resulting in neurite outgrowth and axon formation. Chapter 2 demonstrates that retrograde mitochondrial trafficking is significantly impaired in the A53T mutant expressing cells in comparison to control at 6 days in vitro (DIV); both retro- and anterograde
trafficking are impaired at 8 DIV. Trafficking in the A30P mutant and WT α-synuclein was not significantly impaired compared to control, but the cells did display increased ROS production associated with mitochondrial dysfunction. Impairment of normal mitochondrial dynamics has been previously reported in PD animal models and cell models [23-25], but a directional bias and the time-dependent changes in mitochondrial dynamics had not yet been elucidated. Furthermore, α-synuclein induced trafficking impairment and ROS production can be rescued using the small interfering peptide davunetide (NAP) [26].

Although expression of mature DA markers has been reported in SH-SY5Y, expression levels are low and represent less than half of the proteins associated with mature DA neurons [27]. Therefore, to further assess trafficking impairment, proper ventral midbrain dopaminergic neurons derived from iPSCs were used. These differentiated DA neurons contain more in-vivo like axonal structures, which are way more complex than the simple axonal structures in the neuroblastoma cell model, and have a physiologically normal neuronal expression pattern. Additionally, these neurons have a very low mitotic index, can be cultured for prolonged duration and display spontaneous electrical activity. Chapter 4 describes a decrease in mitochondrial trafficking in DA neurons from PD patients compared to an age-matched control, similar to the defects described in Chapter 2. However, while wild-type α-synuclein displays no trafficking deficiencies in the SH-SY5Y overexpression model, iPSC DA neurons harbouring an SNCA triplication do reveal trafficking deficiencies. This is likely the result of prolonged culture duration, higher levels of oxidative metabolism, and intrinsic electrical activity in these neurons.

However, the mechanisms by which α-synuclein causes these trafficking differences remain elusive and numerous candidates for trafficking deficiencies have been described. Figure 1 illustrates several mechanisms related to mitochondrial trafficking impairments.
**Figure 1. Proposed mechanisms leading to mitochondrial trafficking impairments.** Increased ROS production is a result of distal accumulation of impaired mitochondria. Increased Ca\(^{2+}\) influx leads increased cellular stress and to microtubule anchoring of mitochondria by syntaphilin. \(\alpha\)-Synuclein-mediated disorganisation of microtubules and actin is thought to contribute to trafficking deficiencies. Using NAP stabilizes microtubules and rescues the observed trafficking deficiencies in our SH-SY5Y PD cell model.

Different species of \(\alpha\)-synuclein oligomers increase calcium influx in SH-SY5Y cells through a pore-forming mechanism, which can indirectly lead to cell damage [28]. Direct disruption of isolated mitochondrial membranes by \(\alpha\)-synuclein has also been reported, but no evidence for this model has been presented in cell models [29]. Combined with the intrinsic pace-making activity of DA neurons through L-type calcium channels, \(\alpha\)-synuclein pore-formation can result in high calcium levels in \(\alpha\)-synuclein-overexpressing cells and make these neurons more vulnerable to cellular stress [30]. Additionally, increased calcium influx can directly disrupt neuronal trafficking of mitochondria, arresting mitochondria in distal regions of the axon by anchoring syntaphilin [31, 32]. Other mechanisms for disruption of trafficking might result from the interaction of \(\alpha\)-synuclein with microtubule associated protein tau (MAPT) and actin [33, 34]. Overexpression of \(\alpha\)-synuclein resulted in slowed actin polymerization, thereby decreasing reassembly of actin filaments [33]. MAPT on the other hand aggregates under the influence of \(\alpha\)-synuclein fibrils, thereby inhibiting its function to stabilize microtubules [35]. Single
nucleotide polymorphisms in the \textit{MAPT} locus have been frequently associated with PD, underscoring its implication in disease [6, 36]. Incorrect trafficking of mitochondria can lead to distal accumulation of impaired mitochondria, ultimately leading to an increase in ROS production in axonal regions. To enhance microtubule stability, a small protein named davunetide (NAP) can be used, which is based on the activity dependent neuroprotective protein (ADNP). Introduced as a possible therapy for progressive supranuclear palsy [37], NAP is currently considered as a therapeutic in Alzheimer’s disease [38] and has been shown to alleviate PD pathology in an \(\alpha\)-synuclein mouse model [26]. The eight amino-acid peptide (NAPVSIPQ) has been shown to provide microtubule stability and reduce tau phosphorylation [37, 39]. Protecting the cytoskeleton and increasing the microtubule-tau interaction [40], NAP is likely able to counter the negative effects of \(\alpha\)-synuclein on mitochondrial trafficking.

It remains to be elucidated whether one or several of the above mentioned mechanisms are responsible for the observed trafficking deficiencies in Chapter 2 and 4. It is clear, however, that mitochondrial trafficking deficiencies precede neuronal cell death in these cell models for PD.

\textbf{Energy metabolism in human neurons derived from iPSC}

Energy metabolism of human pluripotent stem cells is extensively studied and is well recognized as being an important roadblock on the way to pluripotency [41]. While many somatic cells use oxidative phosphorylation (OXPHOS) as an important source of energy [42], a switch to a glycolytic metabolism is characteristic of stem cells [43]. This process is preceded by a burst in OXPHOS activity, which provides a metabolic gate to pluripotency [44]. Self-renewal in cultured ESCs and iPSCs is characterized by glycolysis, lactate production and the presence of round mitochondria near the nucleus [45]. Glycolysis-mediated histone acetylation in pluripotent stem cells has been revealed to play a role in
maintaining pluripotency [46], and is currently recognized as an active regulatory mechanism of the pluripotent state [47]. Conversely, differentiating pluripotent stem cells to fibroblasts causes elongation of mitochondria accompanied by a shift to OXPHOS [45].

In Chapter 3, these characteristics of stem cells and their somatic progeny were utilized to purify neuronal cells based on their metabolic capacity to use lactate as a substrate. The ability of cells to metabolize lactate varies between different cells types and is dependent on the presence of specific transporters and enzymes to metabolize lactate. By using glucose deprivation and lactate supplementation (GDLS), neuronal cells can be purified from their undifferentiated counterparts as illustrated in Chapter 3. Purified cultures displayed over 90% purity, while normal cultures contained fewer than 40% of neuronal cells at 60 DIV. While various articles have reported higher initial yields, estimates are usually done below 40 DIV [48, 49]. Contaminating populations often arise at later time points, and reproducing results reported by other labs can be challenging due to differences in culture methods or because of intrinsic differences between iPSC lines [50]. Intrinsic differentiation potential varies between pluripotent cells, which can be illustrated by spontaneous differentiations [51]. H9 ESCs have a propensity towards an ectodermal lineage, which makes these cells suitable for neuronal differentiations. HUES9 ESCs on the other hand spontaneously differentiate to the mesoderm and endoderm lineage, making these cells suitable for cardiomyocyte differentiations [51]. To homogenize differentiated populations cells are often cell-sorted based on surface markers [52], but these techniques are laborious and cell-straining. The introduction of GDLS creates a simple purification method to serve as an alternative tool to homogenize different neuronal cultures. Figure 2 highlights the principles on which GDLS neuronal purification is based.

Neuronal metabolism of lactate was emphasized in the astrocyte-neuron lactate shuttle (ANLS) hypothesis, proposed in 1998 by Pellerin [53]. While the theory initially sparked critical reviews [54], refinements
have been made over the years [55], and recent in-vivo evidence of a lactate gradient has created renewed interest for the theory [56, 57]. Current understanding of the ANLS theory leans heavily on the cellular distribution of monocarboxylate transporters (MCT). In the brain, the high affinity MCT2 is found predominantly in neuronal cells, while the lower affinity MCT1 and the low affinity MCT 4 are present on astrocytes and oligodendrocytes [58, 59]. The shuttle is linked to neuronal activity by modulating glucose metabolism in astrocytes via the excitatory amino acid transporter (EAAT) 1 and 2. Glutamate released by neurons is taken up by EAAT1 and 2 and stimulates glucose uptake by astrocytes, leading to an increase in astrocytic glycolysis. MCT1 and 4 transport lactate from the soma to the extracellular space, to be taken up by neurons by MCT2. Next, lactate is converted to pyruvate by lactate dehydrogenase and enters the tricarboxylic acid (TCA) cycle providing the cell with ATP [60]. While lactate has long been associated with stress responses of the brain which are characterized by increased cerebral energy demand, OXPHOS increases have been recently reported in neurodevelopment and in normal brain function [56, 61]. In in-vitro studies, exogenous lactate contribution to neuronal OXPHOS has been estimated to be around 75%, while only 25% was contributed to glucose metabolism [62].
Fig 2. Metabolic selection by glucose deprivation and lactate supplementation (GDLS).

(A) Principles of a neuronal lactate shuttle. MCT1 and 4 transport lactate to the extracellular space, to be taken up by the high affinity transporter MCT2. (B) While differentiated neurons are able to metabolise lactate, progenitor cells and undifferentiated cells cannot metabolise lactate. Consequently, mitochondrial OXPHOS and subsequent ATP production, are lower compared to neuronal cells. (C) Combining the principles from (A) and (B) using GDLS results in metabolic selection of neuronal cells.
In GDLS, a combination of the above described processes allows the purification of neuronal populations from iPSC differentiations. Undifferentiated cells are heavily dependent on glucose, while differentiated neurons can depend on MCT2-mediated transport of lactate. Contaminating astrocytes are less efficient at transporting and metabolising lactate, leading to purified cultures of neuronal cells. GDLS thereby provides the stem cell community with a new, economical and time-efficient tool to purify neuronal cells. Furthermore, GDLS adds an important piece of evidence to the ANLS hypothesis.

Progerin-induced ageing in iPS cell culture applications

The road to pluripotency leads to many cellular alterations, including the above described metabolic alterations [43]. These alterations result in iPSCs stemness, but also rejuvenate these cells [63]. While this makes iPSCs suitable for long-term culture and transplantation purposes, it hampers the ability to use these cells in disease modelling. Removal of epigenetic marks associated with ageing, telomere extension, increased nuclear stability and increases in autophagy are all associated with the rejuvenation process and render differentiated cells less suitable to study late-onset diseases [63, 64]. To overcome this challenge, Miller et al. introduced a method to age cells using the protein progerin [65]. Progerin was discovered as the disease causing protein in the Hutchinson-Gilford Progeria Syndrome (HGPS) and its expression recaptures many hallmarks associated with cellular ageing. While the approach by Miller et al. provides an easily applicable method, there are some drawbacks to their use of modified RNA. The transfection-induced toxicity by this method causes high level of cell death even in control cultures and, consequently, can only be applied for a limited number of days [65]. To provide another toolset for cell ageing studies based on progerin expression, Chapter 5 introduces a lentiviral approach to achieve expression. Using this approach, expression of progerin can be maintained for over 30 DIV without major toxicity associated with the
expression method. This allows the cells to be studied for prolonged culture duration and shows an indirect effect on mitochondrial function. The use of cardiomyocytes (CMs) was considered for practical reasons, since these cells are relatively easy to differentiate, are post-mitotic, and have a high mitochondrial metabolism. Moreover, due their large flat soma, progerin expression and its effects can be easily observed and evaluated with immunocytochemistry. At the end of chapter 5, a conditional promoter system specific for CM is introduced, which is currently being used to validate earlier findings and assess bioenergetics function using the Seahorse XF analyser. Figure 3 captures the principles of iPSC rejuvenation, and the rationale behind progerin-induced ageing.

Figure 3. Progerin-induced ageing in stem cell culture applications. Aged somatic cells are characterized by nuclear deformation, genomic instability and decreased mitochondrial integrity. The process of reprogramming effectively rejuvenates these somatic cells and their differentiated counterparts. This
improves nuclear folding and protein homeostasis, increases telomere length and removes senescent epigenetic marks, and increases mitochondrial integrity. Using progerin expression, several of the hallmarks associated with senescent cells are recaptured.

Cellular ageing was thought to be a process governed by cell division after the Hayflick experiments in 1961 which indicated that cells do not divide indefinitely [66]. At around fifty cell divisions, fibroblast cells slow their doubling speed and eventually enter cellular senescence. The concept of replicative senescence remained and the underlying mechanisms have been well studied over the years. Amongst the complex processes underlying cellular ageing are: a decrease in mitochondrial integrity [67], nuclear instability [68], telomere attrition [69] and altered protein homeostasis [70]. Because interplay between these processes usually results in cellular senescence, the combined effects of these processes are difficult to study. Decreased nuclear stability as seen in HGPS, can indirectly lead to mitochondrial stress, which in turn can lead to increase in ROS production and DNA damage. For further reading on the processes underlying ageing, a review by Lopez-Otin et al. provides an excellent overview [71].

Chapter 5 highlights hallmarks of ageing that are more easily quantifiable, such as genomic instability and mitochondrial integrity. Skin fibroblast cells from aged individuals often present impaired protein homeostasis, nuclear deformation, genomic instability, epigenetic alterations, shortened telomeres and decreased mitochondrial integrity [72]. These hallmarks are countered by iPSC reprogramming, which increases protein homeostasis, increases mitochondrial integrity, improves nuclear folding and extends telomeres [63]. Expressing progerin reverses part of the rejuvenation process, leading to an increase in nuclear deformation and genomic instability and a decrease in mitochondrial integrity. While the model does not recapitulate all hallmarks of cellular senescence, it is more suited compared to conventional methods which often use toxins [73] or impair functions of
the DNA repair machinery [74]. In post-mitotic cell cultures, cells are less affected by DNA damage and the ability to model ageing using progerin provides a good alternative. Future experiments using progerin will focus on mitochondrial respiration to determine which complexes are most affected. In these experiments performed in collaboration with the Dept. of Experimental Cardiology UMCG, the focus will be on exposing the link between nuclear deformation and mitochondrial dysfunction.

**Future research**

Further investigation of mitochondrial trafficking in iPSC-derived DA neurons could make use of mitochondrial labelling in individual DA neurons. This can be achieved using a tissue-specific promoter, in this case the human synapsin promoter [75] or the human tyrosine hydroxylase promoter [76], and generating stable cell lines with this construct. Mixing labelled iPSCs with non-labelled iPSCs will allow the study of retro- and anterograde trafficking in PD DA neurons in-vitro, as it will allow mitochondria to be followed even in dense neuronal cultures. Comparing the trafficking dynamics of PD DA neurons to control DA neurons, can provide insights into the early events that lead to PD. Next, rescue of the trafficking deficiencies by davenutide might clarify involvement of the microtubule system in the observed trafficking deficiencies. Further studies of DA neurons in these settings could involve the use of brain organoids. First described in 2012, these organoids are the product of self-organization of neural stem cells, and effectively model a mini-brain in a spinning bioreactor [77]. Since then, brain organoids from different brain regions have been produced, and midbrain organoids contain functional dopaminergic and melanin-producing neurons [78, 79].

Another revolution in biomedical research recently took place involving the use of the prokaryotic clustered regularly interspaced short palindromic repeats/Cas (CRISPR/Cas) adaptive immune system of bacteria [80] to facilitate genome editing. While genome editing methods were already available [81, 82], the ease of use and efficacy of CRISPR-based genome editing have promoted its widespread use. Combining
iPSC- and CRISPR-technology will allow researchers to study disease-causing mutations in a similar genetic background. Genome-wide association studies have identified several single-nucleotide polymorphisms (SNP) in the \textit{SNCA} and \textit{MAPT} loci [6], but the mechanisms underlying the potential increased risks associated with these SNPs have not yet been elucidated. Using CRISPR on hESCs or hiPSCs to induce these SNPs and compare these cells to their isogenic controls, will provide new insight into the disease mechanisms. In 2016, Soldner et al. have proven the feasibility of this approach by showing that a PD-associated SNP causes increased α-synuclein expression via the modulation of a distal enhancer element [83]. Combining CRISPR technology to generate PD lines with isogenic controls and researching these cells in ventral midbrain organoids will surely lead to a better understanding of PD.

Other advancements in cell culture have come in the form of more specific cell culture media. It is now widely recognized that functional maturation of specific cell types in-vitro is dependent of the mineral and nutrient mixture provided in the medium and recently this awareness has generated a new neuronal medium that supports synaptic function [84]. By mimicking the composition of cerebrospinal fluid, the brain’s normal physiological levels of salts, glucose, osmolarity and pH can be reached. This facilitates the functional maturation of differentiated neuronal cultures. However, while this media composition uses a lower glucose level compared to the widely used Neurobasal™ media, it still has a low amount of carboxylates in the form of sodium pyruvate. To more carefully mimic a brain physiological condition, lactate supplementation should be considered as an addition to this medium. In mixed culture conditions lactate can be produced and exported by astrocytic cells, but in more pure differentiations these cells are underrepresented. Furthermore, a study on the rat MCT2 illustrates that this transporter has a higher affinity for lactate [85], making it a suitable energy substrate alongside sodium pyruvate.
Finally, the second half of this decade marks the revival of nigrostriatal grafting. Similar trials as undertaken by the end of the 90's century were halted after mixed results [86-88]. Nevertheless, renewed interest partially fuelled by iPSC technology has resulted in the approval for engraftment of fetal mesencephalic dopamine neuronal precursor cells. While the number of patients enrolled is low, anticipation of these studies is high, as these studies pave the way for the use of human ESC-derived Neural Precursor Cells. Set to start in 2017, a study using these cells will run until 2020, and time will tell if the clinical benefits outweigh the potential risks. But with constant improvements in neuronal cell culture and the ever-increasing knowledge of PD, we might finally be able to prevent and/or cure the disease in at the beginning of the third century of PD research.

References


55. Laughton JD, Bittar P, Charnay Y et al. Metabolic compartmentalization in the human cortex and hippocampus: evidence for a cell- and region-
specific localization of lactate dehydrogenase 5 and pyruvate dehydrogenase. **BMC neuroscience.** 2007;8:35.


64. Rando TA, Chang HY. Aging, rejuvenation, and epigenetic reprogramming: resetting the aging clock. **Cell.** 2012;148:46-57.


