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**MODELING *GBA1*-RELATED PARKINSON'S DISEASE
PATHOLOGY IN PATIENT-DERIVED MIDBRAIN ORGANOID**

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Abstract

Aggregation of α -synuclein protein in “Lewy bodies” and “Lewy neurites” and dopaminergic neuronal loss in the nigrostriatal system are the key neuropathological hallmarks of Parkinson’s disease. Mutations in *GBA1*, encoding the glucosylceramide-hydrolyzing enzyme glucocerebrosidase, cause Gaucher’s disease and are the most frequent genetic risk factor for Parkinson’s disease. However, a defined link between mutations in *GBA1* and Parkinson’s disease pathology is yet to be determined, largely because of the absence of experimental models able to recapitulate the defining neuropathological signatures of the disease.

Here, we present an innovative midbrain organoid culture system derived from subjects with *GBA1*-related Parkinson’s disease and Gaucher’s disease as the first patient-based model able to reproduce fundamental human neuropathological features of these disorders. We provide evidence of glucosylceramide accumulation due to glucocerebrosidase deficiency and, notably, of insoluble α -synuclein aggregation in inclusions reminiscent of Lewy bodies and Lewy neurites in *GBA1*-mutated organoids. We show that α -synuclein extracted from Parkinson’s samples displays seeding activity *in vitro* and, following inoculation in unaffected organoids, propagates the pathology. Finally, we demonstrate pharmacological rescue of pathological phenotypes, suggesting that midbrain organoids could represent valuable *in vitro* platforms for drug testing in Parkinson’s disease and potentially other Lewy body disorders still lacking a definitive therapy.

Chapter 1 Introduction

1.1 Parkinson's disease

In his seminal monograph from 1817, James Parkinson first described the symptoms of the “shaking palsy”, a condition that was subsequently defined nosologically and named after Parkinson himself by Jean-Martin Charcot. Today, Parkinson's disease (PD) is the most common, progressive, neurodegenerative movement disorder and the second-most frequent neurodegenerative disease affecting 2–3% of the population over the age of 65 (Poewe et al., 2017).

The clinical diagnosis of PD relies on the presence of motor features (*i.e.*, bradykinesia, rigidity, tremor, gait impairment, and postural instability). However, beyond the classical perception of PD as a pure disorder of movement, it has become clear that a complexity of non-motor features is part of the clinical spectrum, ranging from autonomic dysfunction (*e.g.*, cardiovascular, urogenital, and gastrointestinal manifestations), neuropsychiatric symptoms (*e.g.*, depression, apathy, anxiety, psychosis), cognitive dysfunction (*e.g.*, mild cognitive impairment, PD-dementia), sleep disorders (*e.g.*, REM sleep behavior disorder), sensory symptoms (*e.g.*, hyposmia, pain), which contributes considerably to overall burden (Schapira et al. 2017).

The cardinal neuropathological hallmarks of PD consist of dopaminergic neuronal loss in the mesencephalic substantia nigra (SN) and of proteinaceous inclusions containing aggregates of α -synuclein (α -syn) and other components within surviving neurons. Several other cell types and extra-nigral regions throughout the central and peripheral autonomic

nervous system are also affected, likely early in the disease (Jellinger, 2012).

The molecular pathogenesis underlying the clinical presentation involves multiple pathways and mechanisms (e.g., α -syn proteostasis, axonal transport, mitochondrial function, membrane trafficking, oxidative stress, calcium homeostasis, neuroinflammation), however the etiology of PD remains conjecture (Hirsch et al., 2013; Mullin and Schapira, 2015).

Symptomatic treatment in PD comprises dopamine replacement therapy and, at later disease stages, continuous dopaminergic stimulation to compensate for the intracerebral dopaminergic loss either with intrajejunal levodopa-carbidopa gel infusion, a subcutaneous apomorphine pump, or with deep brain stimulation. Thanks to advanced therapies, quality of life can be maintained into the latest stages of the disease. However, a curative therapy is yet to be discovered.

1.1.1 Epidemiology

PD is the second most common neurodegenerative disease after Alzheimer's disease and the most frequent movement disorder (GBD 2016 Neurology Collaborators, 2019). The prevalence of PD is 1-3% and 4% among people over the age of 65 and 85, respectively, with lower prevalence in Africa than in Europe and the Americas (de Lau et al., 2006; Winkler et al., 2010; Dotchin et al., 2008; Okubadejo et al., 2006). The median age-standardized incidence of PD in high-income countries averages between 11 and 13.9 per 100,000 people per year and increases continuously with age, reaching 160 per 100,000 people aged 65 years or older (Bower et al., 2000; Hirtz et al., 2007) (Figure 1.1a).

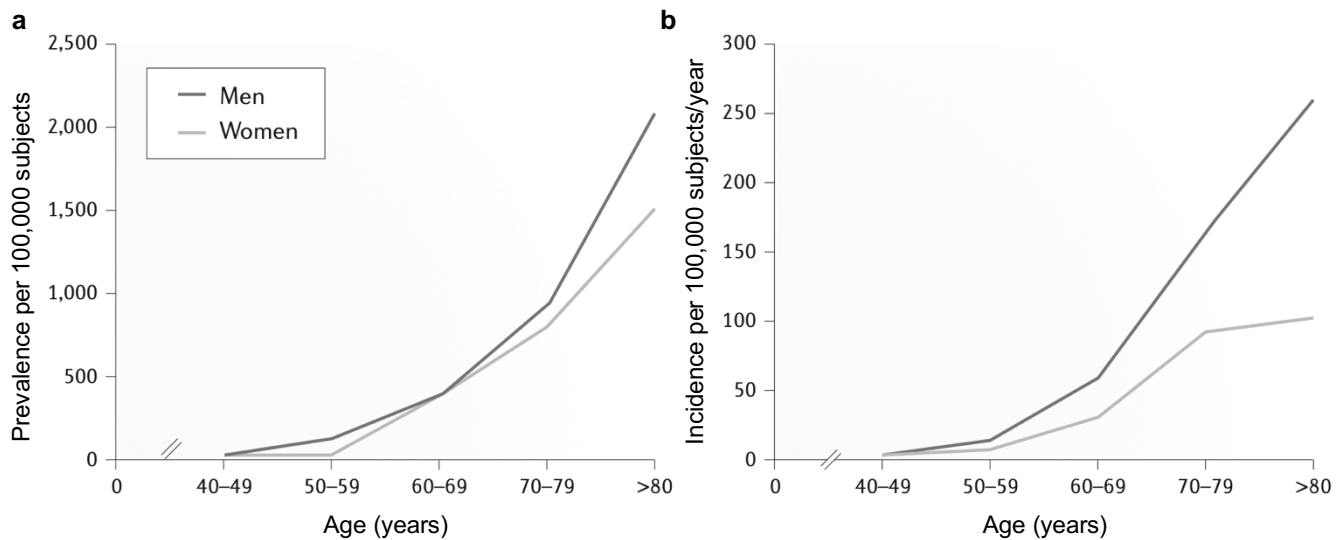
Prevalence and incidence rates of PD vary among studies by race and ethnicity, with sparse

and inconsistent data. In a study conducted on members of a large health maintenance organization in California, the age- and gender-adjusted rate per 100,000 people was highest in Hispanics (16.6), followed by non-Hispanic white people (13.6), Asians (11.3), and black people (10.2) (Van Den Eeden et al., 2003). A large cross-sectional study of US Medicare beneficiaries also reported higher prevalence and incidence rates in white people than in black people (Wright et al., 2010), whereas another work conducted on a culturally diverse community in New York City found higher incidence of PD in black people (Mayeux et al., 1995). The male to female incidence ratio ranges from 1.3 to 2.0 in most studies, with rates as low as 0.95 reported in Asia (Taylor et al., 2007) (Figure 1.1b).

The lifetime risk of developing PD for individuals aged 40 years in the USA was estimated to be 2% for men and 1.3% for women (Elbaz et al., 2002). PD is mainly a late-onset disorder, with an average age at onset around 60 years and a peaking incidence at around 80 years (Bower et al., 2000). Indeed, advancing age appears to be the single greatest risk factor for developing sporadic PD. The incidence of PD before the age of 50 years is low and is characteristic of rarer cases of “early-onset PD”, manifesting between 21 and 50 years, or “juvenile-onset PD”, manifesting before the age of 20 (Riboldi et al., 2022).

Increased risk of developing PD has been associated with various non-genetic factors, including exposure to pesticides (Langston et al., 1983; Baldi et al., 2003; Tanner et al., 2011; Ascherio et al., 2016), consumption of dairy products (Jian et al., 2014), use of methamphetamine (Callaghan et al., 2010; Curtin et al., 2015), history of melanoma (Liu et al., 2011), and history of traumatic brain injury (Marras et al., 2014), whereas a reduced risk has been reported in association with smoking or chewing tobacco (Morens et al., 1995; Hernán et al., 2001; O'Reilly et al., 2005; Thacker et al., 2007; Chen et al., 2010), consumption of caffeine (Ross et al., 2000; Ascherio et al., 2001; Liu et al., 2012) or tea (Ascherio et al., 2001; Hu et al.,

2007; Tan et al., 2008), higher serum urate concentrations (Davis et al., 1996; de Lau et al., 2005; Weisskopf et al., 2007; Chen et al., 2009; O'Reilly et al., 2010; Jain et al., 2011), physical activity (Chen et al., 2005; Logroscino et al., 2006; Thacker et al., 2008; Xu et al., 2010; Sääksjärvi et al., 2014; Yang et al., 2015), and use of non-steroidal anti-inflammatory drugs (Chen et al., 2003; Chen et al., 2005; Bower et al., 2006; Gao et al., 2011).



modified from Poewe et al., 2017

Figure 1.1 – Prevalence and incidence of Parkinson's disease.

- Prevalence of Parkinson's disease in men and women per 100,000 individuals.
- Incidence rate of Parkinson's disease per 100,000 persons/year. Data are derived from two meta-analyses, which used crude rates without adjustments for demographic differences or methodological differences between studies (Pringsheim et al., 2014; Hirsch et al., 2016).

1.1.2 Pathophysiology

The majority of the movement abnormalities seen in PD (*e.g.*, akinesia, bradykinesia, rigidity, and rest tremor) is thought to be caused by the loss of dopaminergic neurons projecting from the SN *pars compacta* to the striatum. Dopamine depletion in the basal ganglia results in the disruption of the connections to the thalamus and motor cortex, thus leading to clinical manifestations of parkinsonism.

In physiological conditions, the striatum and subthalamic nucleus are the primary entry points for cortical, brainstem and thalamic inputs into the basal ganglia. From these, information is conveyed to the principal basal ganglia output nuclei, globus pallidus internus and SN *pars reticulata*, which project to frontal areas of the cerebral cortex and to brainstem structures (*e.g.*, superior colliculus, pedunculopontine nucleus, parvocellular reticular formation).

Each basal ganglia nucleus is histologically distinctive. In the striatum, the most abundant cell type is the GABAergic medium spiny projection neuron, which accounts for 90–95% of all striatal neurons. Medium spiny neurons receive excitatory inputs from the cerebral cortex and the thalamus (centromedian and parafascicular nuclei), as well as intrinsic inhibitory inputs from several classes of striatal interneurons (*i.e.*, large cholinergic neurons, smaller cells containing somatostatin, neuropeptide Y, calbindin or nitric oxide synthetase) and extrinsic modulatory inputs from dopaminergic and serotonergic neurons projecting from the midbrain.

The neuronal cell groups in the globus pallidus externus and internus and in the SN *pars reticulata* are homogeneous, mainly consisting of large and GABAergic neurons which receive inputs from the striatum and the neighboring SN *pars compacta*. The subthalamic nucleus is a densely packed structure populated by excitatory glutamatergic neurons.

Of all basal ganglia structures, the striatum receives the most abundant cortical input, which

is topographically arranged. In primates, projections from motor areas (e.g., motor, premotor, and somatosensory cortices) terminate in the post-commissural putamen, prefrontal cortical areas terminate in the caudate nucleus and pre-commissural putamen, and limbic areas terminate preferentially in the ventral striatum (Wichmann and Delong, 2007).

The subthalamic nucleus is the second major recipient of excitatory cortical inputs. The primary motor cortex projects to the dorsolateral part of the subthalamic nucleus, premotor and supplementary motor areas project to the medial third, and prefrontal and limbic cortices project to the ventral and most medial portions of the subthalamic nucleus (Takada et al., 2001).

Striatal output determines the basal ganglia output nuclei (*i.e.*, globus pallidus internus and substantia nigra *pars reticulata*) via two separate pathways, the so-called “direct” and “indirect” pathways. The direct pathway is a monosynaptic circuit linking the striatum with the globus pallidus internus and the SN *pars reticulata*, whereas the indirect pathway is a polysynaptic pathway which involves the globus pallidus externus and the subthalamic nucleus *en route* to the globus pallidus internus and the SN *pars reticulata* (Albin et al., 1989; Alexander and Crutcher, 1990).

The direct pathway arises from monosynaptic projections from the striatum to the globus pallidus internus and the SN *pars reticulata*. These neurons express dopamine D1-receptors on their dendrites and on their axon terminals, which strongly modulate GABA release from the striatonigral and striatopallidal pathways (Trevitt et al., 2002).

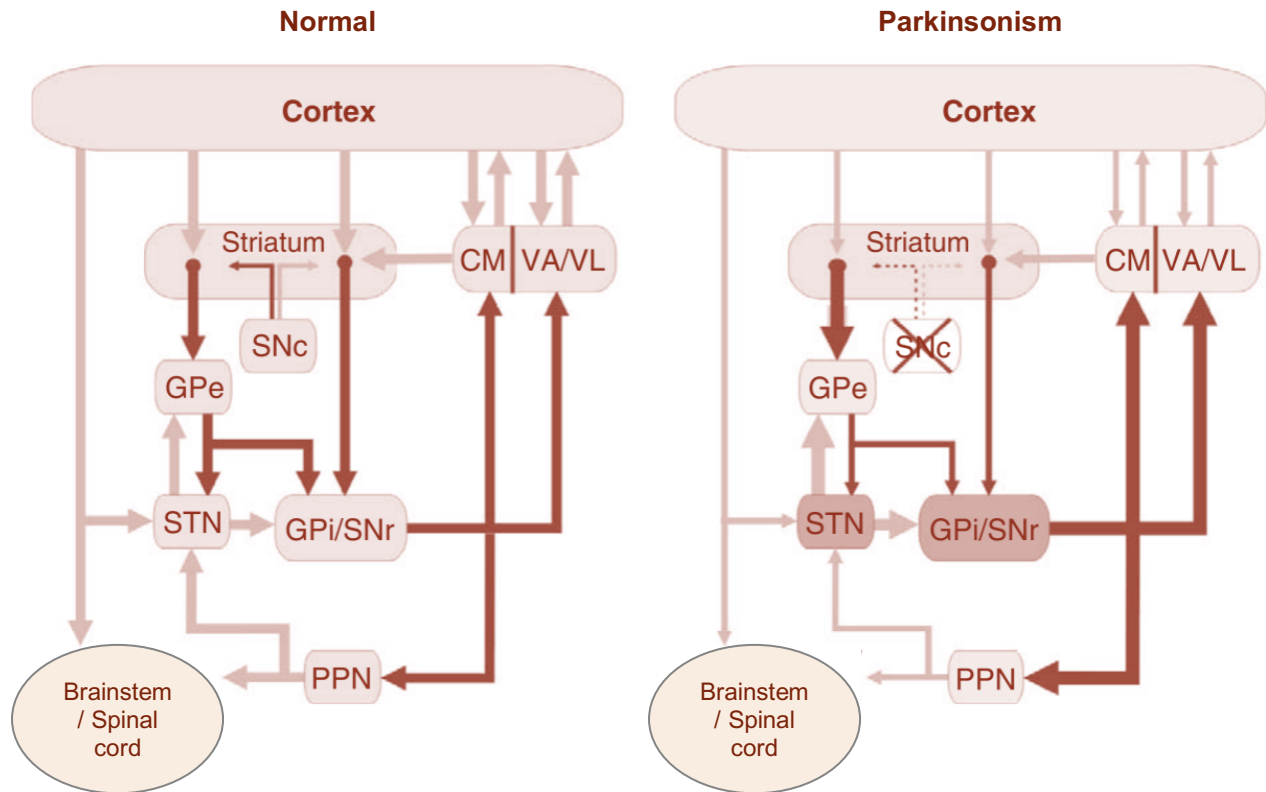
The indirect pathway arises from striatal projections to the globus pallidus externus. These neurons preferentially express dopamine D2-receptors. The indirect pathway is completed by additional projections from the globus pallidus externus to the subthalamic nucleus, and from the subthalamic nucleus to the globus pallidus internus and the SN *pars reticulata*. Rather than being a simple information relay, considerable processing occurs along the indirect pathway,

particularly in the subthalamic nucleus, where cortical inputs are integrated with inputs from the globus pallidus externus (Takada et al., 2001).

The current model of the basal ganglia circuitry predicts that activation of the direct pathway reduces inhibitory basal ganglia output from targeted neurons with subsequent disinhibition of related thalamocortical neurons, therefore resulting in increased activity in appropriate cortical neurons. By contrast, activation of the indirect pathway leads to a greater inhibitory basal ganglia output on thalamocortical neurons (Figure 1.2).

The SN *pars compacta* does not directly participate in the transfer of information along the basal ganglia–thalamocortical pathways, but rather modulates the circuitry by providing dopaminergic inputs to the striatum. Neurons in the SN *pars compacta* show a tonic pacemaker-like firing pattern providing a tonic background of dopamine which serves to modulate the balance between direct and indirect pathways. In this function, dopamine modulates the activity of the globus pallidus externus and the SN *pars reticulata* via facilitation of transmission over the direct pathway and inhibition of transmission over the indirect pathway (Gerfen, 1995).

It has been postulated that in conditions of dopamine depletion in the nigrostriatal pathway, such as in the case of PD, the indirect inhibitory pathway mediated via dopamine-D2 striatal receptors is overactive, functionally disinhibiting the subthalamic nucleus, whereas the direct inhibitory pathway mediated via dopamine-D1 striatal receptors is underactive (Figure 1.2). As a result, the main pallidal-thalamic outflow pathway provides excessive inhibitory input to the thalamus, which causes suppression of thalamo-cortical-spinal pathway, manifested clinically by parkinsonism. The general pathophysiologic model in which overactivity along the indirect pathway is a major contributor to parkinsonism is supported by the demonstration that lesions of the subthalamic nucleus, globus pallidus internus, or SN *pars reticulata* reverse some or all signs of parkinsonism (Bergman et al., 1990).



modified from Wichmann and DeLong, 2007

Figure 1.2 – Motor circuit pathophysiology.

Schematic overview of the anatomical connections within the basal ganglia circuitry in physiological conditions (left) and activity changes associated with parkinsonism (right).

Abbreviations: GPe, globus pallidus externus; STN, subthalamic nucleus; GPi, globus pallidus internus; SNr, substantia nigra *pars reticulata*; SNc, substantia nigra *pars compacta*; PPN, pedunculo pontine nucleus; CM, centromedian nucleus of the thalamus; VA, ventral anterior nucleus of the thalamus; VL, ventrolateral nucleus of the thalamus. Gray arrows indicate excitatory connections, black arrows indicate inhibitory connections.

1.1.3 Neuropathology

Fundamental neuropathological features of PD include neurodegeneration in specific brain areas, primarily in the SN, and widespread intracellular protein accumulation, particularly enriched in aggregated α -syn. Although neither the loss of neuromelanin-containing dopaminergic neurons in the SN nor the deposition of α -syn in neurons is specific for PD, these two major findings are specific for a definitive diagnosis of PD when present in combination (Dickson et al., 2009).

1.1.3.1 Neurodegeneration

Neuronal loss occurs preferentially in certain neuronal population within specific brain regions. In early-stage disease, dopaminergic degeneration is restricted to the ventrolateral SN (*i.e.*, A9 or nigrostriatal pathway) with relative sparing of dorsal and medial cell groups within the ventral tegmental area (VTA) (*i.e.*, A10 or mesolimbic pathway), but becomes more widespread by end-stage disease (Fearnley and Lees, 1991; Damier et al., 1999; Dickson et al., 2009) (Figure 1.3a-d). The dramatic loss of these dopaminergic neurons even early in the disease suggests that the degeneration in the ventrolateral SN starts before the onset of motor symptoms (Dijkstra et al., 2014; Iacono et al., 2015). It is accepted that motor manifestations of PD begin to emerge when approximately 80% of striatal dopamine content (or 60% of nigral dopaminergic neurons) are lost, and that the course of the disease is associated with the progressive reduction of striatal dopamine content.

In addition to a depletion of striatal dopamine, PD brains also exhibit a reduction in norepinephrine and serotonin due to the degeneration of neurons located in the locus coeruleus

and in the raphe of the pons and medulla. As such, PD comprises a progressive loss of all three types of monoaminergic cells, although biochemical changes extend beyond the loss of monoamines only, as indicated by a reduction in other neurotransmitters as well as enzyme activities for their synthesis. In fact, the cholinergic nucleus basalis of Meynert undergoes neuronal and volume loss, and hypocretin-positive hypothalamic neurons that regulate sleep and appetite degenerate at later disease stages. Neurodegeneration has also been described in glutamatergic projections from the thalamus to the basal ganglia and from the presupplementary motor cortex to the premotor cortices.

1.1.3.2 *Lewy pathology*

The abnormal deposition of α -syn in intracellular inclusions within neuronal somas and processes, respectively termed “Lewy bodies” (LBs) and “Lewy neurites” (LNs) (Goedert et al., 2013), is a signature of PD neuropathology (Braak et al., 2003) (Figure 1.3e-g). The presence of Lewy pathology in the SN *pars compacta*, along with clinical features of parkinsonism, is usually considered essential for the neuropathological diagnosis of PD. However, patients with juvenile PD may not present LBs (*i.e.*, patients with mutations in *PARKIN* or *LRRK2* gene) (Poulopoulos et al., 2012; Kalia et al., 2015). Thus, the presence of LBs is a critical pathologic marker to confirm the diagnosis, but is not necessary and is not pathognomonic for PD. Moreover, LBs are found in 8–17% of neurologically normal people over 60 years, with their incidence rate increasing with age as in PD (Frigerio et al., 2011).

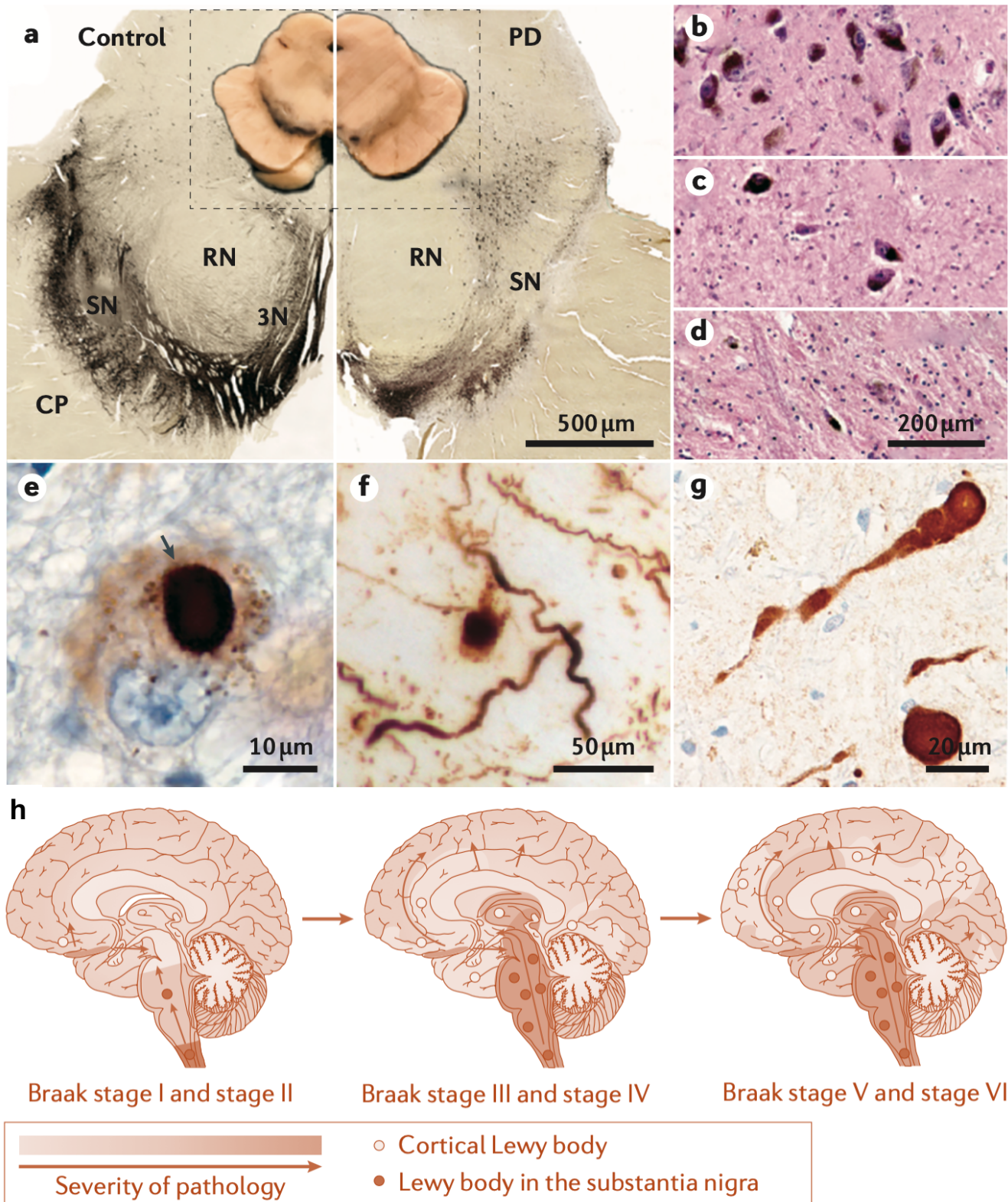
The neuroanatomical regions that accumulate Lewy pathology in most patients with clinical PD follow a preferential distribution pattern as the disease progresses, and it has been

suggested that LBs survive for 6-16 months in degenerating neurons. According to Braak and colleagues, α -syn pathology in PD patients often starts in dorsal motor vagal nucleus and olfactory bulb (stage 1 and 2), before reaching the SN and the brainstem sensory relay centers (e.g., serotonergic raphe nuclei, adrenergic cerulean nuclei, and cholinergic brainstem complex) in a topographically predictable sequence (stage 3 and 4). This spreading process terminates in the neocortex (stage 5 and 6) (Braak et al., 2003; Halliday and McCann, 2010) (Figure 1.3h).

There is a strong correlation between the topography of Lewy pathology and the clinical presentation. Indeed, before the first clinical manifestations of stage 3 and 4-related degeneration of dopaminergic neurons in the midbrain, the so-called “premotor phase”, primary PD-related sensory, sleep, and autonomic symptoms may arise, preceding the motor phenotype of a few decades.

Even though more than a century has passed since LBs and LNs were first described as major hallmarks of PD, much remains to be understood about their precise composition, the processes that lead to their formation, propagation and clearance, and their role in the pathogenesis of PD.

A growing body of evidence suggests that the aggregation of α -syn, one of the most abundant proteinaceous constituents of LBs and LNs, is a trigger of LB formation and one of the key mechanisms that drive neurodegeneration and pathology progression in PD. In the seminal work by Spillantini et al. (1997), abnormal filamentous structures of α -syn were discovered to accumulate within LBs and LNs of patients with PD and dementia with LBs. Since then, elucidating the sequence of events that trigger the fibrillization of α -syn to form LBs, their contribution to neuronal dysfunction and degeneration, and their role in disease progression has been a subject of intense debate and active investigation.



modified from Poewe et al., 2017

Figure 1.3 – Neuropathology of Parkinson's disease.

- a. Depigmentation of the substantia nigra in PD compared with control (insets) and selective loss of neurons expressing tyrosine hydroxylase in the ventrolateral parts of the substantia with sparing of the more medial and dorsal regions.
- b-d. Hematoxylin and eosin staining of the ventrolateral region of the substantia nigra showing a normal distribution of pigmented neurons in a healthy control (b) and diagnostically significant moderate (c) and severe (d) pigmented cell loss in PD.
- e-g. Immunohistochemical staining of α -syn showing intracytoplasmic Lewy bodies (e), more diffuse, granular deposits of α -syn (e, f), Lewy neurites in cell processes (f), extracellular dot-like α -syn structures (f) and α -syn spheroids in axons (g).
- h. Progression of α -synuclein aggregation in PD as theorized by Braak et al., 2013.

Abbreviations: 3N, 3rd nerve fibers; α -syn, α -synuclein; CP, cerebral peduncle; RN, red nucleus.

A complex series of events that extend beyond an increase in α -syn protein levels have been implicated in the process that underlies α -syn misfolding, the transition from its physiological soluble state to fibrillized pathological forms, and the formation and maturation of Lewy inclusions (Fares et al., 2021). Multiple post-translational modifications, including α -syn phosphorylation, ubiquitination, nitration, and truncation, have been called into question as key players (Fares et al., 2021). Additionally, recent evidence has shown that LBs and LNs are also enriched in non-proteinaceous components, including lipids, cytoskeletal proteins, organelles, and membranous fragments, and that their interaction with α -syn likely modulates the formation and polymorphic organization of inclusions and downstream effects of neurodegeneration (Ikenaka et al., 2019; Shahmoradian et al., 2019; Mahul-Mellier et al., 2020). Indeed, α -syn pathology in *post-mortem* brains of patients with PD acquires heterogeneous conformations, ranging from oligomers, protofibrils, fibrils, and mature LBs (Spillantini et al., 1998; Paleologou et al., 2009; Roberts et al., 2015). Moreover, LBs present a wide heterogeneity of morphology, structure, and molecular composition across brain regions (Forno and Norville, 1976; Langston and Forno, 1978; Gibb et al., 1991), and whether this diversity reflects distinct pathologies or rather represents a continuum of maturation stages of LBs is still to be determined.

1.1.3.2.1 Structure and composition of Lewy bodies

Early identification and classification of LBs was based on conventional histological techniques, such as hematoxylin and eosin staining, which distinguished different aspects of LBs according to whether they were found in the brainstem or in cortical regions. Typically, brainstem LBs exhibit a dense, spherical structure with a hyaline, eosinophilic core and a pale halo (Baba et al., 1998; Beyer, 2006). Conversely, cortical LBs and LNs lack a central halo and

are therefore more difficult to detect by hematoxylin and eosin. Imaging of LBs by means of transmission electron microscopy showed that, even though both brainstem and cortical LBs display a similar ultrastructural composition of filamentous proteins and lipids, fibrils in LBs irradiate at the periphery around a dense core, whereas cortical LBs have a more diffuse and disorganized distribution of filamentous fibrils (Kosaka, 1978; Baba et al., 1998; Wakabayashi et al., 1998).

Amyloid-like properties of filaments within both cortical and brainstem LBs, including the cross- β structure found in amyloid plaques and neurofibrillary tangles in brains of individuals with Alzheimer's disease, were initially hypothesized based on the high content of fibrillar constituents rich in insoluble β -sheets and were later confirmed by strong reactivity of amyloid-specific dyes, such as Thioflavin-S (Irwin et al., 2013). Moreover, both brainstem and cortical LBs were found to exhibit a strong immunoreactivity for ubiquitin and neurofilament (Goldman et al., 1983; Baba et al., 1998; Spillantini et al., 1998).

Until 1997, the nature of the protein building blocks of the filamentous structures found in LBs remained unknown. Following a genome-wide linkage analysis which identified *SNCA* as the first gene linked to familial PD (Polymeropoulos et al., 1997), α -syn was discovered to be the main constituent of Lewy inclusions (Spillantini et al., 1997). Subsequent characterization studies have demonstrated that α -syn within LBs is largely detergent-insoluble and presents several post-translational modifications, with phosphorylation at serine 129, ubiquitination, nitration, and N-terminal and C-terminal truncations being the most typical (Fujiwara et al., 2002; Hasegawa et al., 2002; Anderson et al., 2006).

To date, more than 100 proteins have been identified in LBs along with α -syn by immunohistochemical staining and, more recently, a total of 296 proteins have been detected

by proteomic analyses of isolated cortical LBs (Leverenz et al., 2007; Wakabayashi et al., 2013; Fares et al., 2021). Proteins consistently found in LBs are involved in several cellular processes, such as the ubiquitin-proteasome system, synaptic transmission, vesicular transport, mitochondria homeostasis, autophagy, intracellular trafficking, oxidative stress, signal transduction, and apoptosis. In particular, the ubiquitin-binding protein p62 has emerged as a predominant constituent of LBs (Kuusisto et al., 2001).

In addition to proteins, LBs were found to be enriched in lipid constituents in the form of membrane fragments, dysmorphic organelles (e.g., mitochondria, lysosomes, autophagosomes), and vesicular structures, with which α -syn fibrils likely interact as they convert into LBs (den Jager, 1969; Forno et al., 1976; Watanabe et al., 1977; Gai et al., 2000; Shahmoradian et al., 2019).

1.1.3.2.2 Formation of Lewy bodies

Since ageing is the main risk factor for developing PD, it has been hypothesized that the process of LB formation occurs over several months or even years. Indeed, the average lifespan of a LB from its formation to neuronal death has been suggested to be 6.2 months (Greffard et al., 2010). Much of the current knowledge on the complex spatiotemporal mechanisms that regulate LB formation derives either from histological studies on *post-mortem* brain sections of subjects with PD or from reductionist models of α -syn aggregation. However, the difficulty to reproduce and comprehensively dissect this phenomenon has hindered our understanding of the origin and development of these inclusions.

A proposed theory for the *de novo* formation of LBs has been extrapolated by combining evidence collected from pathology studies and experimental approaches (Mahul-Mellier et al.,

2020; Fares et al., 2021) (Figure 1.4). In physiological conditions, α -syn predominantly localizes to the presynaptic compartment in unfolded and α -helical forms bound to membranes. Ageing, genetic predisposition, or a toxic environmental insult – or a combination of the three – that increases α -syn levels, induces its misfolding or translocation from the presynaptic terminal, impairs pathways of protein degradation or mitochondrial homeostasis, could trigger *de novo* LB pathology. Specifically, the increase in α -syn levels and the induction of post-translational modifications that facilitate α -syn aggregation (*e.g.*, truncation at the C-terminal) could initiate nucleation-dependent polymerization events, which can be summarized into five stages:

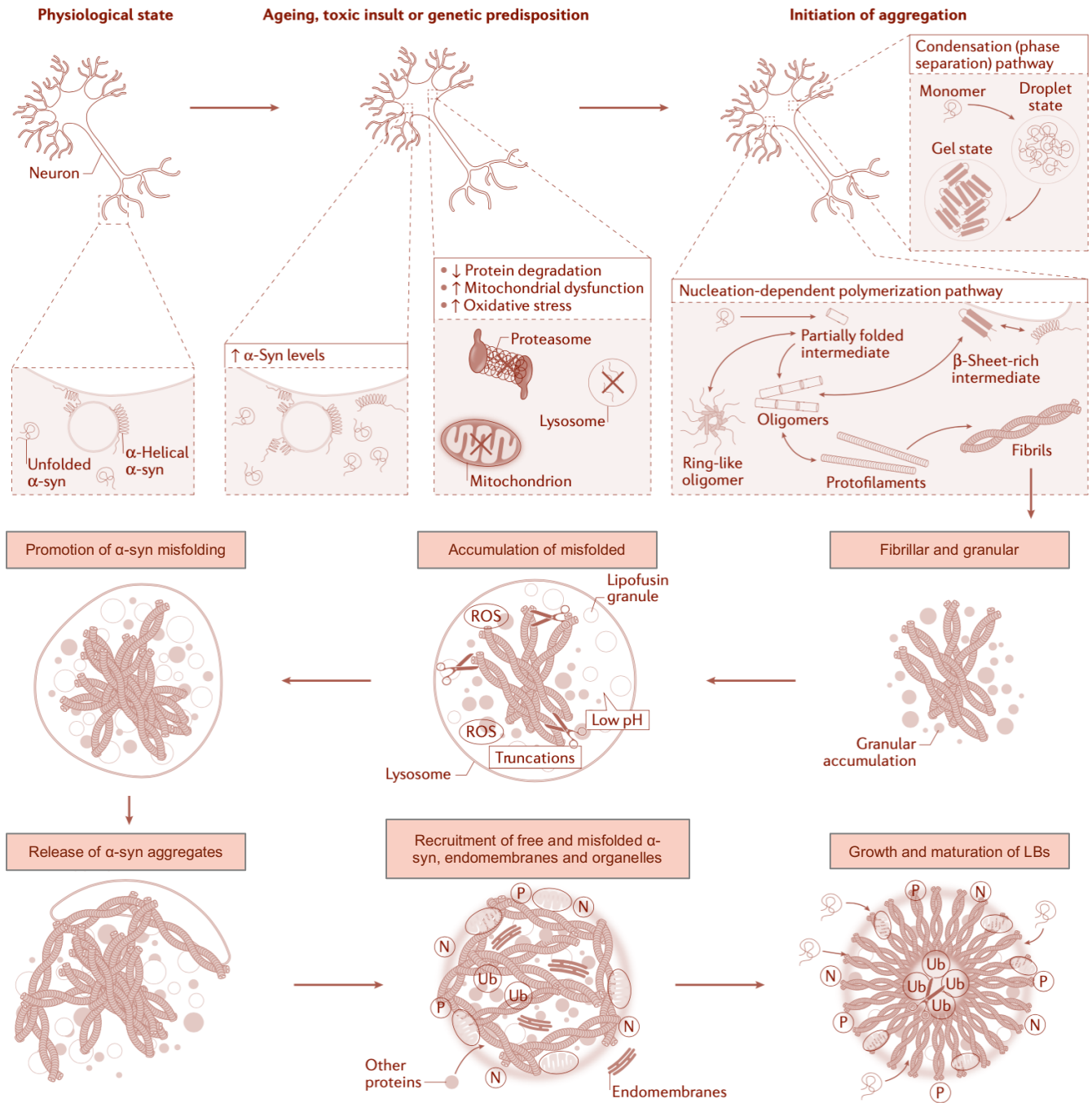
- 1) conversion of cytosolic and membrane-bound α -syn monomers into partially folded or β -sheet-rich intermediates;
- 2) assembly of nucleation-competent oligomers;
- 3) formation of protofilaments;
- 4) formation of fibrils;
- 5) elongation of fibrils by incorporation of α -syn monomers.

An alternative explanation of LB formation entails the conversion of α -syn monomers to amyloid fibrils via a condensation pathway (Ray et al., 2020; Hardenberg et al., 2021) (Figure 1.4). According to this recently proposed model, α -syn undergoes liquid-liquid phase separation to form a droplet state, by which α -syn aggregates are formed due to a highly saturated environment and gradually mature into amyloid-rich hydrogels.

In either case, the accumulation of misfolded α -syn may induce the cell's attempt to degrade the fibrillar structures via different pathways, including the lysosomal pathway. However, the

environment within lysosomes could further exacerbate the aggregation of α -syn. Indeed, a low pH and the presence of reactive oxygen species, lipofuscin granules, and proteases could promote the truncation and misfolding of α -syn, fragmentation of already formed fibrils, or the production of α -syn species highly prone to aggregation.

The lysosomes engulfed with insoluble fibrillar material – so called “secondary lysosomes” – could then break down and release their content into the cytosol. The expelled fibrils would readily incorporate free α -syn and recruit misfolded α -syn monomers, thereby acting as nucleation centers. The growing aggregates could interact with and/or grow on membranes of nearby organelles (*e.g.*, mitochondria) and recruit them into nascent inclusions. This phase of this model would correspond to the gradual transformation of inclusions with disorganized structure - so called “pale bodies” – to mature LBs with a refined ultrastructure consisting of a dense core and radiating α -syn fibrils (Kuusisto et al., 2003). LBs would then continue to grow in size by actively trapping soluble proteins and organelles and would undergo post-translational modifications in the cell’s attempt to degrade pathological accumulations (*e.g.*, ubiquitination, phosphorylation at α -syn S129, nitration, truncation).



modified from Fares et al., 2021

Fig. 1.4 – *De novo* formation of Lewy bodies.

In the physiological state, α -syn at the presynaptic terminal exhibits unfolded and α -helical membrane-bound forms. A precipitating factor causing an increase in α -syn levels, or impairing mitochondrial homeostasis or protein degradation (e.g., a toxic environmental insult, ageing, genetic predisposition) may trigger LB pathology, ultimately resulting in the initiation of nucleation-dependent polymerization events. Consequently, α -syn undergoes conformational changes to form β -sheet-rich intermediates, which further assemble into oligomers and, ultimately, mature fibrils. α -syn fibrillization may also arise from condensation of α -syn into amyloid structures via phase separation. In either case, the disorganized fibrillar or granular accumulations of α -syn are then engulfed within lysosomes for attempted degradation. Truncation of α -syn may further promote α -syn aggregation within “secondary lysosomes”, which eventually break down and release the α -syn aggregates in the cytoplasm. The released fibrils incorporate soluble α -syn and recruit organelles, endomembranes and other proteins, gradually leading to the formation of mature LBs with a dense core and radiating α -syn fibrils and undergoing multiple post-translational modifications.

Abbreviations: N, nitration; P, phosphorylation; PTM, post-translational modification; ROS, reactive oxygen species; Ub, ubiquitin.

1.1.3.2.3 Propagation of α -synuclein pathology

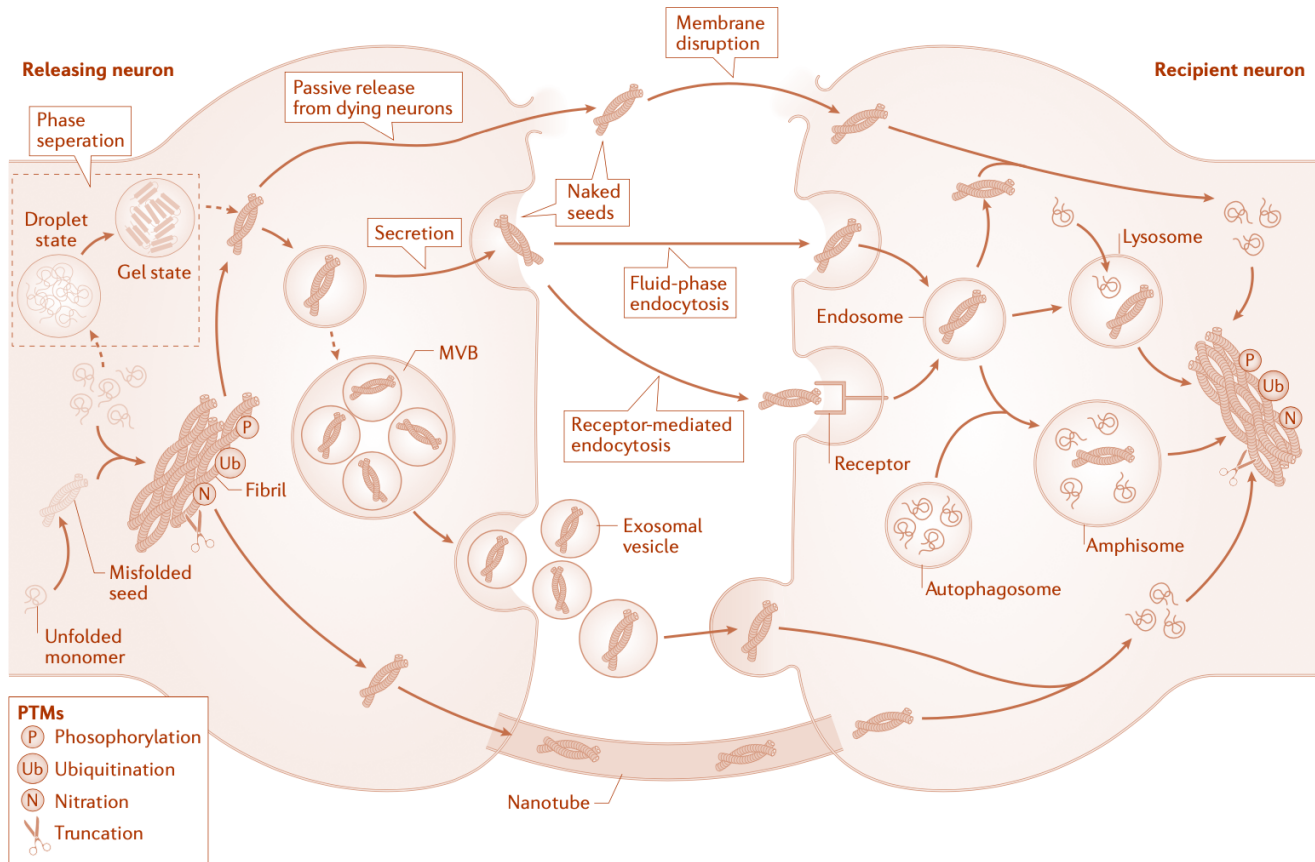
The evidence that, in individuals with PD, Lewy pathology is also observed in extra-nigral brain regions, peripheral and autonomic nervous structures, and even extra-nervous peripheral tissues, and that a sequential pattern of spreading of fibrillar α -syn pathology in the brain of patients correlates with disease progression, have suggested that α -syn may have the ability to propagate in a prion-like manner (Braak et al., 2003; Jellinger, 2003; Dickson et al., 2010; Goedert 2015). A proposed model for the spreading of LB pathology that emerged from evidence from cellular and animal models of α -syn propagation (Desplats et al., 2009; Luk et al., 2009; Volpicelli-Daley et al., 2011; Luk et al., 2012; Masuda-Suzukake et al., 2013; Mahul-Mellier et al., 2020) suggests that *de novo* formed α -syn fibrils are released from neurons in which nucleation-dependent fibrillization or phase separation events occurred, and then enter surrounding neurons where they induce conformational changes of the native α -syn monomers and abnormal aggregation (Fares et al., 2021) (Figure 1.5).

The α -syn seeds could be released from neurons via passive diffusion through plasma membrane pores, active secretion by non-classical endoplasmic reticulum-Golgi apparatus-independent exocytosis (Jang et al., 2010) or via recycling endosomes (Liu et al., 2009), incorporation into vesicles of multivesicular bodies and release as exosomes (Danzon et al., 2012), or transcellular communication through nanotubes (Agnati et al., 2010).

The α -syn fibrils released in the extracellular space could then be internalized by neighbouring neurons via direct disruption of the plasma membrane (Lashuel et al., 2002; Mahul-Mellier et al., 2015), receptor-mediated or adsorptive endocytosis (Desplats et al., 2009; Volpicelli-Daley et al., 2011; Holmes et al., 2013), or fusion of the exosomal and plasma membranes.

Once inside the recipient neuron, non-membrane-bound α -syn seeds could directly interact with free α -syn monomers and induce their fibrillization. Alternatively, membrane-bound α -syn fibrils could either disrupt endosomal membranes and access the cytoplasm or they could interact with endogenous α -syn in autophagosomes, thus forming amphisomes, or with α -syn monomers delivered by chaperone-mediated autophagy (Lee et al., 2014). The interaction between α -syn seeds and endogenous α -syn would result in further fibrillization events that eventually damage the endosomal membranes and grant cytoplasmic access to the seeds.

Free α -syn seeds in the cytoplasm of the recipient neuron could undergo C-terminal truncation, a key event that would recruit the bulk of endogenous α -syn monomers (Desplats et al., 2009; Volpicelli-Daley et al., 2011). Growing fibrillar intracellular structures could undergo post-translational modifications (e.g., phosphorylation at S129, ubiquitination, p62 tagging) and fragmentation which would be responsible for structural changes that drive the transition from fibrils to mature LBs (Mahul-Mellier et al., 2020). α -syn fibrillization could be transmitted to other neurons via release of novel seeds generated by fragmentation events, thereby establishing a vicious cycle of pathology spreading among interconnected neurons (Fares et al., 2021).



modified from Fares et al., 2021

Figure 1.5 – Spreading of fibrillar α-synuclein pathology.

De novo-formed α-syn fibrils undergo multiple post-translational modifications (e.g., phosphorylation, ubiquitination, nitration, truncation) and fragment into misfolded seeds. α-syn seeds are then released in the intercellular space by various mechanisms (e.g., passive diffusion, active secretion, vesicular incorporation and release as exosomal vesicles, direct translocation via nanotubes) and ultimately internalized by neighbouring neurons (e.g., disruption of the plasma membrane, fluid-phase or receptor-mediated endocytosis, fusion of plasma-exosomal membranes). Once inside the recipient neuron, α-syn seeds interact with α-syn monomers and promote further fibrillization events, ultimately leading to the formation of LBs.

1.1.3.3 Neuromelanin

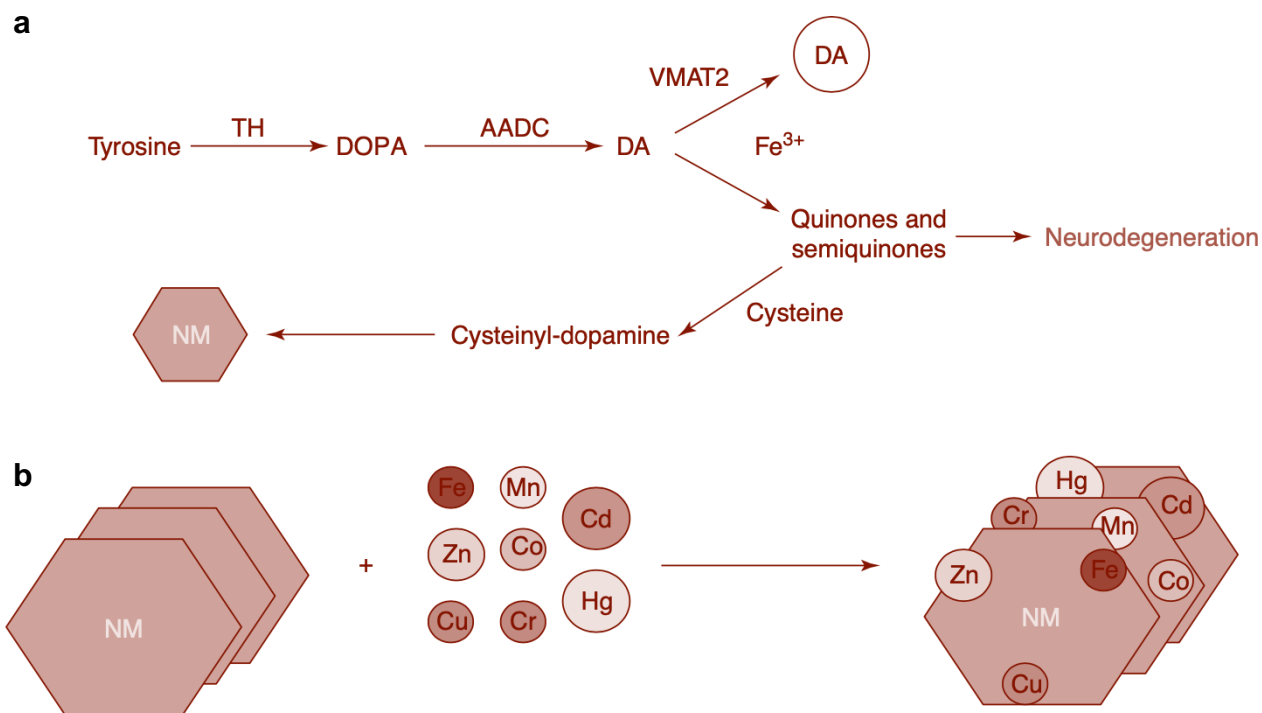
In the human midbrain, neuromelanin (NM) contained in dopaminergic neurons confers the SN its characteristic pigmentation. Pigmentation of this area becomes apparent after the third year of life and increases linearly with ageing (Foley and Baxter, 1958; Fenichel and Bazelon, 1968; Mann and Yates, 1974; Cowen, 1986; Zecca et al., 2001; Zecca et al., 2004; Zecca et al., 2008). In PD, NM-containing neurons show an intrinsic vulnerability to the pathogenic processes and are selectively lost (Gibb et al., 1990; Pakkenberg et al., 1991). Indeed, NM concentrations in PD brains drop to <50% of those in age-matched controls (Zecca et al., 2002). Therefore, much effort has been put into the characterization of NM and its reproduction in PD-relevant models (Kastner et al., 1992).

In the biosynthetic pathway of eumelanin found in melanocytes, the conversion of tyrosine to L-dopa and then to dopa-quinone is catalyzed by tyrosinase (TYR) (Sánchez-Ferrer et al., 1995). Since TYR mRNA and promoter activity have been detected in the SN (Xu et al., 1997, Tief et al., 1998), it has been proposed that NM biosynthesis may as well be mediated by TYR. However, immunohistochemical staining of TYR in the SN did not reveal protein expression (Ikemoto et al., 1997). Moreover, albinos lacking expression of TYR due mutations in the *TYR* gene have normally pigmented SN (Foley et al., 1958), therefore suggesting TYR-independent alternative pathways of NM production.

It has long been debated whether the synthesis of NM is due to enzymatic oxidation or auto-oxidation of dopamine derivatives (Zecca et al., 2001). In physiological conditions, dopamine is synthesized from tyrosine by tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC), and is taken up into vesicles by the vesicular monoamine transporter 2 (VMAT2). Alternative enzymatic actions have been suggested in the biosynthesis of NM, including

oxidation of dopamine mediated by TH or peroxidase, and further oxidation to the respective quinones that are possible precursors of NM (Haavik et al., 1997, Okun, 1997). Alternatively, NM could derive from non-enzymatic auto-oxidation of catechols to quinones with the addition of a thiol, as it has been demonstrated to occur in the brain (Fornstedt et al., 1986). Excess dopamine can interact with iron to form quinones and semiquinones, that react with proteins and lipids to cause peroxidation and neuronal damage (Zecca et al., 2003). When quinones react with cysteine to form cysteinyl-dopamine, which can be further converted into NM, neurodegeneration can be avoided. Consequently, NM removes excess cytosolic catecholamines that are not accumulated by synaptic vesicles, thus preventing the subsequent damage. Therefore, it has been suggested that the production of NM is a protective mechanism in which damaged organelles, proteins and lipids, perhaps including dopamine-glutathione, are targeted for lysosomal degradation (Zecca et al., 2003) (Figure 1.6a).

The chemical structure of NM presents groups with a strong chelating ability, which allows NM to bind to a variety of toxic agents, including MPP^+ , a metabolite of MPTP (D'Amato et al., 1986), the herbicide paraquat (Lindquist et al., 1988), dopaminergic-blocking agents like chlorpromazine, haloperidol and imipramine (Salazar et al., 1978), and a variety of metals, including zinc, copper, manganese, chromium, cobalt, mercury, lead, cadmium, and in particular iron (Zecca et al., 1994; Zecca et al., 1996). By chelating toxic metals in the cytosol and forming stable complexes, NM blocks toxic effect of metals, including Fenton's reaction and enzyme inactivation (Figure 1.6b). The ability of NM to act as a black hole capable of chelating redox-active metals and a wide variety of toxic agents suggests that it could be a high-capacity storage trapping system, and as such might prevent neuronal damage (Zecca et al., 2003).



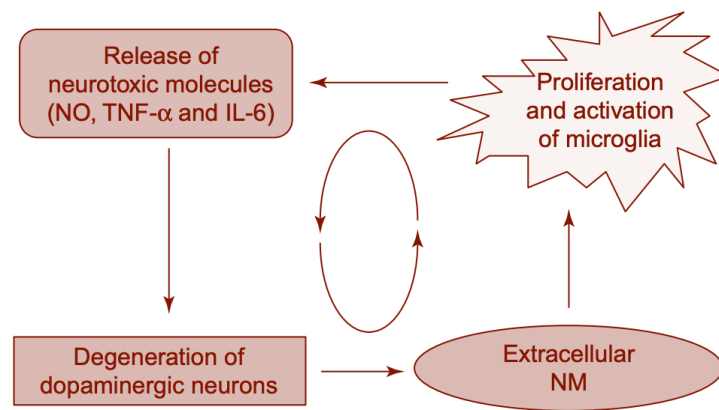
modified from Zecca et al., 2003

Figure 1.6 – The protective role of neuromelanin.

- Dopamine produced from tyrosine by catalysis of TH and AADC is taken up into vesicles by the VMAT2. Excess dopamine can interact with Fe^{3+} to form quinones and semiquinones, which cause neurodegeneration. If dopamine byproducts react with cysteine to form cysteinyl-dopamine and are ultimately incorporated into NM, neurodegeneration can be avoided.
- Neuromelanin chelates exogenous metals released in the cytosol in reactive and potentially toxic forms, forming stable complexes.

Abbreviations: AADC, aromatic amino acid decarboxylase; Cd, cadmium; Co, cobalt; Cr, chromium; Cu, copper; DA, dopamine; DOPA, dihydroxyphenylalanine; Fe^{3+} , ferric iron; Hg, mercury; NM, neuromelanin; Mn, manganese; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2; Zn, zinc.

However, NM may also have a potential neurotoxic role (Figure 1.7). Indeed, following neuronal damage, due to genetic or environmental factors, NM is released in insoluble extra-neuronal granules that induce microglia activation and proliferation (McGeer et al., 1988; Langston et al., 1999), with production of nitric oxide, tumor necrosis factor- α and interleukin 6, which damage other neurons (Wilms et al., 2003). Therefore, although various mechanisms such as environmental toxins and genetic factors can initiate neuronal damage in PD, chronic release of NM from dying neurons may trigger a neuroinflammatory response, potentially leading to a subsequent aggravation of neurodegeneration (Zecca et al., 2003).



modified from Zecca et al., 2003

Figure 1.7 – The toxic role of neuromelanin.

Following neuronal damage, NM is released in the extracellular environment and induces microglial activation, with subsequent production of neurotoxic molecules (e.g., TNF- α , IL-6, NO), which damage other neurons. Newly degenerating neurons will release NM, therefore establishing a chronic condition of neuroinflammation and neurodegeneration.

Abbreviations: IL-6, interleukin 6; NM, neuromelanin; NO, nitric oxide; TNF- α , tumor necrosis factor- α .

1.1.4 Genetics

While apparently mostly sporadic, PD has a well-defined genetic component in at least 5-10% of cases (Di Fonzo et al., 2018).

A genetic contribution to the etiology of PD is suggested by the observation that 20-25% of patients with PD have at least one first-degree relative with PD and that first-degree relatives of patients with PD have a 2.3 times increased risk of developing PD.

Familial forms of PD have been associated with causative mutations in a number of genes (Table 1.1).

The known monogenic forms of PD are rare and all together account for approximately 5-10% of PD cases in most populations (Riboldi et al., 2022). Many of the recently discovered genetic risk alleles, on the other hand, are much more common, but have a relatively weak effect, increasing the likelihood to develop sporadic PD only by a factor of 1.2– 2.0. Thus, in contrast to high-penetrance mutations, these small-effect risk factors are not useful to predict an individual's risk to develop PD.

Locus	Gene	Chromosome	Inheritance	Clinical presentation
PARK1-4	<i>α-Synuclein</i>	4q21	AD	PD, Lewy body dementia
PARK2	<i>Parkin</i>	6q25.2-27	AR	Early onset PD
PARK3	<i>Unknown</i>	2p13	AD	Typical PD
PARK5	<i>UCH-L1 (?)</i>	4p14	AD	Typical PD
PARK6	<i>PINK1</i>	1p36	AR	Early-onset PD
PARK7	<i>DJ-1</i>	1p36	AR	Early-onset PD
PARK8	<i>LRRK2</i>	12q12	AD	Typical PD
PARK9	<i>ATP13A2</i>	1p36	AR	Early-onset parkinsonian-pyramidal syndrome with dementia
PARK10	<i>Unknown</i>	1p32	Unknown	Typical PD
PARK11	<i>GIGYF2 (?)</i>	2q36-37	AD	Typical PD
PARK12	<i>TAF1</i>	Xq21-q25	X-linked	Typical PD
PARK13	<i>Omi/HTRA2 (?)</i>	2p12	Unknown	Typical PD
PARK14	<i>PLA2G6</i>	22q	AR	Adult-onset dystonia parkinsonism
PARK15	<i>FBXO7</i>	22q	AR	Early-onset parkinsonian-pyramidal syndrome
PARK16	<i>(?)</i>	1q32	Unknown	Typical PD
PARK17	<i>VPS35</i>	16q11.1	AD	Typical PD
PARK18	<i>EIF4G1</i>	3q27.1	AD	Typical PD
PARK19	<i>DNAJC6</i>	1p31.3	AR	Early-onset PD atypical
PARK20	<i>SYNJ1</i>	21q22	AR	Early-onset PD atypical
PARK21	<i>CHCHD2</i>	7p11.2	AD	Typical PD

modified from Di Fonzo et al., 2018

Table 1.1 – PD-associated genes, chromosomal location, pattern of inheritance, and clinical phenotype.

1.1.4.1 *GBA1* gene

Mutations in the *GBA1* gene (1q22) are the main genetic risk factor for PD identified to date (Sidransky et al., 2009), exhibiting penetrance of 10-30% (Rana et al., 2013; Anheim et al., 2012). Mutations in *GBA1*, either in homozygous or heterozygous form, are found in 5-15% of Caucasian PD cases and 25% of Ashkenazi Jewish patients with PD, and confer a ~ 20-fold increase in the risk of developing PD with respect to the general population (Schapira, 2015; Zhang et al., 2018). PD patients carrying a *GBA1* mutation are generally indistinguishable clinically from patients with idiopathic PD, however they tend to have greater neocortical α -syn depositions, earlier onset and a higher risk of developing dementia (Sidransky et al., 2009; Neumann et al., 2009; Parkkinen et al., 2011; Oeda et al., 2015).

GBA1 encodes glucocerebrosidase (GCase), a lysosomal enzyme which participates in the turnover of membrane lipids by catalyzing the final step of glycosphingolipid and ganglioside catabolism, breaking down glucosylceramide (GlcCer) to glucose and ceramide. Ceramide serves as the backbone for the synthesis of more complex sphingolipids, including glycosphingolipids and sphingomyelin, in which neuronal membranes are particularly enriched, thus making GCase a critical player in the maintenance of lipid homeostasis.

Following translation, GCase folds in the endoplasmic reticulum and then is transported to the Golgi, where it undergoes glycosylation steps before being trafficked to endosomes and lysosomes.

Homozygous GCase insufficiency, either due to loss of enzymatic activity or reduction in GCase protein levels, causes Gaucher's disease (GD), the most common lysosomal storage disorder (Grabowski et al., 2008). The phenotypic spectrum of GD encompasses primary visceral disease (GD type 1) and neuronopathic forms with central nervous system (CNS)

involvement with varying degrees of severity (GD type 2 and 3).

More than 300 mutations in the *GBA1* gene have been described to cause a detrimental effect on GCase catalytic function, intracellular stability or subcellular trafficking, or both (Jonsson et al., 1987; Liou et al., 2006; Ohashi et al., 1991). Homozygote or compound heterozygote *GBA1* variants causing neuronopathic GD result in the accumulation of GlcCer and glucosylsphingosine (GlcSph) (Nilsson and Svennerholm, 1982; Orvisky et al., 2002).

The two variants most commonly associated with GD and PD are the c.1226A>G (N370S) and the c.1448T>C (L444P) substitutions, representing mild and severe mutations, respectively. Several *GBA1* mutations (*i.e.*, E326K, T369M, K7E or D443N) have been identified in *GBA1*-PD patients, but not in subjects with GD, and are therefore considered risk factors for PD but not causative variants for GD (Davis et al., 2016; Mallet et al., 2016; Gan-Or et al., 2009).

The implications of GCase deficiency in the pathogenesis of PD is still a matter of debate (Futerman and Hardy, 2016; Smith and Schapira, 2022). GCase activity is reduced in the brains of patients with PD with and without *GBA1* mutations (Gegg et al., 2012). Therefore, *GBA1*-PD patients may accumulate a small but sufficient amount of GlcCer, which enhances the progress of PD pathology and impacts the conformation and aggregation status of α -syn (Henderson et al., 2020). However, the few existing studies that investigated GCase substrate accumulation in Lewy body disease brains with heterozygous *GBA1* variants or sporadic PD brains did not find neither GlcCer nor GlcSph accumulation (Gegg et al., 2015; Kurzawa-Akanbi et al., 2021; Clark et al., 2015), with the exception of two studies in which GlcCer and GlcSph have been found to be significantly increased in the substantia nigra of sporadic PD brains (Huebecker et al., 2019; Rocha et al., 2015).

Reduced GCase activity in the brain is associated with increased levels of α -syn (Murphy et

al., 2014). Knock-out GBA1 models (Jo et al., 2021; Kim et al., 2018) or the use of the GCase irreversible inhibitor CBE (Magalhaes et al., 2016; Rocha et al., 2015; Zunke et al., 2018) suggests that GCase loss of function is sufficient for α -synuclein accumulation. Of relevance to Lewy body pathology, proteinase K-resistant α -syn aggregates have been reported in brain sections of mutant GCase mouse models (Rocha et al., 2015b; Sardi et al., 2011, Sardi et al., 2013). Insoluble α -syn has also been described in cellular models treated with CBE (Zunke et al., 2018), but not in all cases (Gegg et al., 2020).

A bidirectional pathogenic loop has been proposed to explain the relationship between GCase reduced activity, α -syn and the lysosome, involving the accumulation of glycosphingolipids and α -syn and prevention of lysosomal trafficking of newly synthesized GCase from the endoplasmic reticulum/Golgi which further exacerbates lysosomal dysfunction (Mazzulli et al., 2011). Furthermore, aberrant lipid accumulation can affect lipid membrane composition and may promote α -syn aggregation by stabilizing toxic α -syn oligomers and enhancing its propagation (Mazzulli et al., 2011; Zunke et al., 2018).

It has been hypothesized that *GBA1* mutation may lead to the production of a misfolded GCase protein, which can be retained in the endoplasmic reticulum and activate pathways associated with endoplasmic reticulum stress, including ERAD and the UPR (Fernandes et al., 2016; Maor et al., 2013; Ron and Horowitz, 2005).

Defective mitophagy may also occur as a consequence of a GCase deficiency and reduced autophagy/lysosomal pathway function, which can lead to the accumulation of defective mitochondria, oxidative stress, reduced ATP production and abnormal mitochondrial morphology (Cleeter et al., 2013; Osellame et al., 2013).

1.1.5 Clinical features

PD is only diagnosed when the first signs and symptoms of motor parkinsonism become overt, and oral dopaminomimetics are found to consistently reduce such symptoms. In PD, synucleinopathic degeneration not only involves midbrain dopaminergic structures, but also the peripheral autonomic nervous system, dorsal motor vagal nucleus, olfactory bulb, brainstem sensory relay centers, and the neocortex. The clinical phenotype, thus, not only comprises the typical motor symptoms, but also a range of non-motor manifestations.

Pending the characteristic chronological spreading of this pathological condition, motor symptoms may be heralded or accompanied by the primary clinical manifestations of the various sites of degeneration located outside of the SN. Characteristically, autonomic nervous system disorders, as well as subtle cognitive dysexecutive impairment, may precede the first motor manifestations in PD. As a rule, more extensive cognitive dysfunction and/or dementia might accompany motor parkinsonism in the later phase, though sometimes cognitive failure with attentional deficits may precede the first signs of motor parkinsonism.

Clinically relevant dysautonomia, variably including cardiovascular (*e.g.*, cardiac sympathetic denervation with orthostatic hypotension and baroreflex failure, and cardiovascular dysregulation), gastrointestinal (*e.g.*, dysphagia, delayed gastric emptying, constipation), urogenital (*e.g.*, sphincter-related bladder dysfunction due to anticholinergic but not levodopa-responsive detrusor muscle hypertrophy, leading to urgency and nocturia, sexual disturbances with the loss of arousal, erectile dysfunction, and premature ejaculation), sudomotor, sympathetic thermoregulatory (*e.g.*, drenching sweats), pupillomotor, sleep and respiratory disorders are present in the majority of PD patients already in the premotor phase.

Sleep-wake disorders mainly manifest as insomnia, sleep fragmentation, excessive daytime

sleepiness, and rapid eye movement (REM) sleep behavior disorder (RBD). RBD is characterized by the lack of motor inhibition during REM sleep, leading to loss of REM-related atonia and a vigorous and potentially harmful dream-enacting behavior. Patients with RBD without overt parkinsonism may concomitantly suffer progressive cognitive dysfunction with impaired executive and visuospatial functions and may score significantly worse on olfactory threshold, odor discrimination, and odor identification tests. These same patients are at risk for developing PD up to 20 years later.

Sensory disorders comprise pain, visual color discrimination problems, and hyposmia with issues in detection, discrimination, and identification of smells. Hyposmia is a well-recognized symptom in premotor PD, with a prevalence of about 80% of all PD cases. About 70% of PD patients suffer neuropathic or nociceptive pain, in over 25% of cases already in the premotor phase.

Neuropsychiatric disorders, including dopamine deficiency-related anhedonia, apathy and psychomotor retardation, anxiety and panic attacks, and noradrenalin and serotonin deficiency-related depression, are also frequently bothering patients, often already in the premotor phase.

In the initial phase of the disease, most, if not all, PD patients already suffer subtle cognitive loss: executive impairments including a deficit of behavioral regulation in sorting or planning tasks in combination with mild mnemonic dysfunction due to defective storage and impaired manipulation of internal representation of visuospatial stimuli. In about 80% of patients, these mild problems may proceed to dementia. Dementia in PD (PDD) is typically characterized by a dysexecutive syndrome with attentional deficits and fluctuating cognition. PDD interferes with everyday life activities and often requires nursing home placement, especially when accompanied by delusions and hallucinations. In a small number of patients, dementia with attentional deficits and additional psychotic symptoms might occur in the premotor phase.

Clinical symptoms in PDD are comparable to those in Dementia with Lewy Bodies (DLB), except for the fact that DLB is a typical premotor feature, whereas PDD, as a rule, becomes only overt in the later stages of PD motor phase or, at least, one year after the onset of motor parkinsonism.

As of yet, clinical hallmarks of PD, enabling the diagnosis, comprise only motor parkinsonism, manifesting with typical striatal dysfunction-related appendicular and axial motor abnormalities. These include bradykinesia/hypokinesia, tremor, rigidity, gait disturbances, and postural instability. Some of these symptoms, mainly the appendicular ones, are responsive to levodopa. Severity of motor parkinsonism is expressed in the modified Hoehn and Yahr score and the Unified PD Rating Scale (UPDRS) adapted by the Movement Disorders Society (MDS).

In the long run (5-10 years after the onset of motor symptoms), the majority of patients suffer iatrogenic purposeless motor abnormalities – so called “dyskinesias” – due to the progressive dopaminergic denervation with the increasing loss of striatal dopamine storage, underlying a progressively more pulsatile stimulation of the post-synaptic dopaminergic receptors.

A subset of patients, due to the treatment with dopamine agonists and irrespective of the duration of disease, might suffer from other iatrogenic treatment complications, such as impulse control disorder (ICD). These pathological behaviors are characterized by excessive or poorly controlled preoccupations, urges, or stereotyped behaviors. ICDs, such as binge eating, compulsive shopping, pathological gambling, and hypersexuality, are considered the expression of a homeostatic hedonistic dysregulation, a class effect of dopamine agonists. Punding, a stereotypy normally seen in amphetamine addiction and characterized by an intense fascination with the purposeless repetitive manipulation, examination, cataloguing, and endless sorting of objects of common use, is seen in about 15% of PD patients.

1.1.6 Diagnosis

The diagnosis of PD is based upon distinctive clinical features discerned from the history and neurologic examination. Bradykinesia, accompanied either by tremor or rigidity, is necessary in order to consider the diagnosis of PD (Table 1.2). In addition, an unequivocal, beneficial response to dopaminergic therapy is a strong supportive feature of the diagnosis, while the absence of an observable response to high-dose levodopa therapy (> 1 g/die) makes the diagnosis of PD very unlikely. Other supportive clinical criteria include unilateral onset, presence of rest tremor, and a persistent asymmetry throughout the course of the disease, with the side of onset being most affected. Postural instability is also a feature of PD, but usually becomes apparent in later stages of the disease.

While the gold standard for a definitive diagnosis is the neuropathologic examination, the standard for a clinical diagnosis according to criteria from the MDS is an expert clinician trained in movement disorders. Referral to a specialist or center with expertise in movement disorders is advised for patients with parkinsonian signs and symptoms when the diagnosis is uncertain.

Table 1.2 – Movement Disorder Society clinical diagnostic criteria for PD.

The first essential criterion is parkinsonism, which is defined as bradykinesia, in combination with at least one of rest tremor or rigidity. Examination of all cardinal manifestations should be carried out as described in the MDS-Unified Parkinson Disease Rating Scale.^[1] Once parkinsonism has been diagnosed:		
Diagnosis of clinically established PD requires:		
1. Absence of absolute exclusion criteria 2. At least two supportive criteria, and 3. No red flags		
Diagnosis of clinically probable PD requires:		
1. Absence of absolute exclusion criteria 2. Presence of red flags counterbalanced by supportive criteria If one red flag is present, there must also be at least one supportive criterion If two red flags, at least two supportive criteria are needed No more than two red flags are allowed for this category		
Supportive criteria (check box if criteria met)		
☐ 1. Clear and dramatic beneficial response to dopaminergic therapy. During initial treatment, patient returned to normal or near-normal level of function. In the absence of clear documentation of initial response a dramatic response can be classified as: a. Marked improvement with dose increases or marked worsening with dose decreases. Mild changes do not qualify. Document this either objectively (>30% in UPDRS III with change in treatment), or subjectively (clearly-documented history of marked changes from a reliable patient or caregiver) b. Unequivocal and marked on/off fluctuations, which must have at some point included predictable end-of-dose wearing off		
☐ 2. Presence of levodopa-induced dyskinesia		
☐ 3. Rest tremor of a limb, documented on clinical examination (in past, or on current examination)		
☐ 4. The presence of either olfactory loss or cardiac sympathetic denervation on MIBG scintigraphy		
Absolute exclusion criteria: The presence of any of these features rules out PD:		
☐ 1. Unequivocal cerebellar abnormalities, such as cerebellar gait, limb ataxia, or cerebellar oculomotor abnormalities (eg, sustained gaze evoked nystagmus, macro square wave jerks, hypermetric saccades)		
☐ 2. Downward vertical supranuclear gaze palsy, or selective slowing of downward vertical saccades		
☐ 3. Diagnosis of probable behavioral variant frontotemporal dementia or primary progressive aphasia, defined according to consensus criteria ^[2] within the first five years of disease		
☐ 4. Parkinsonian features restricted to the lower limbs for more than three years		
☐ 5. Treatment with a dopamine receptor blocker or a dopamine-depleting agent in a dose and time-course consistent with drug-induced parkinsonism		
☐ 6. Absence of observable response to high-dose levodopa despite at least moderate severity of disease		
☐ 7. Unequivocal cortical sensory loss (ie, graphesthesia, stereognosis with intact primary sensory modalities), clear limb ideomotor apraxia, or progressive aphasia		
☐ 8. Normal functional neuroimaging of the presynaptic dopaminergic system		
☐ 9. Documentation of an alternative condition known to produce parkinsonism and plausibly connected to the patient's symptoms, or, the expert evaluating physician, based on the full diagnostic assessment feels that an alternative syndrome is more likely than PD		
Red flags		
☐ 1. Rapid progression of gait impairment requiring regular use of wheelchair within five years of onset		
☐ 2. A complete absence of progression of motor symptoms or signs over five or more years unless stability is related to treatment		
☐ 3. Early bulbar dysfunction: severe dysphonia or dysarthria (speech unintelligible most of the time) or severe dysphagia (requiring soft food, NG tube, or gastrostomy feeding) within first five years		
☐ 4. Inspiratory respiratory dysfunction: either diurnal or nocturnal inspiratory stridor or frequent inspiratory sighs		
☐ 5. Severe autonomic failure in the first five years of disease. This can include: a. Orthostatic hypotension^[3]-orthostatic decrease of blood pressure within three minutes of standing by at least 30 mmHg systolic or 15 mmHg diastolic, in the absence of dehydration, medication, or other diseases that could plausibly explain autonomic dysfunction, or b. Severe urinary retention or urinary incontinence in the first five years of disease (excluding long-standing or small amount stress incontinence in women), that is not simply functional incontinence. In men, urinary retention must not be attributable to prostate disease, and must be associated with erectile dysfunction		
☐ 6. Recurrent (>1/year) falls because of impaired balance within three years of onset		
☐ 7. Disproportionate anterocollis (dystonic) or contractures of hand or feet within the first 10 years		
☐ 8. Absence of any of the common nonmotor features of disease despite five years disease duration. These include sleep dysfunction (sleep-maintenance insomnia, excessive daytime somnolence, symptoms of REM sleep behavior disorder), autonomic dysfunction (constipation, daytime urinary urgency, symptomatic orthostasis), hyposmia, or psychiatric dysfunction (depression, anxiety, or hallucinations)		
☐ 9. Otherwise-unexplained pyramidal tract signs, defined as pyramidal weakness or clear pathologic hyperreflexia (excluding mild reflex asymmetry and isolated extensor plantar response)		
☐ 10. Bilateral symmetric parkinsonism. The patient or caregiver reports bilateral symptom onset with no side predominance, and no side predominance is observed on objective examination		
Criteria application:		
1. Does the patient have parkinsonism, as defined by the MDS criteria?	Yes	No
If no, neither probable PD nor clinically established PD can be diagnosed. If yes:	☐	☐
2. Are any absolute exclusion criteria present?	Yes	No
If yes, neither probable PD nor clinically established PD can be diagnosed. If no:	☐	☐
3. Number of red flags present _____		
4. Number of supportive criteria present _____		
5. Are there at least two supportive criteria and no red flags?	Yes	No
If yes, patient meets criteria for clinically established PD. If no:	☐	☐
6. Are there more than two red flags?	Yes	No
If yes, probable PD cannot be diagnosed. If no:	☐	☐
7. Is the number of red flags equal to, or less than, the number of supportive criteria?	Yes	No
If yes, patient meets criteria for probable PD	☐	☐

from Postuma et al., 2015

1.1.7 Treatment

The symptoms of PD can be improved by different treatment strategies. Seminal observations have indicated that PD is associated with dopamine deficiency in the striatum, secondary to the loss of dopaminergic input from the SNc. Therefore, early targeted treatment involves oral dopamine replenishment in the form of levodopa plus a dopa-decarboxylase inhibitor. This therapy has enabled patients with PD to have impressive, sustained improvement in bradykinesia, rigidity, tremor, and quality of life for over 40 years. Furthermore, the recognition that PD is not only merely a disorder affecting dopaminergic neurons and motor functions has widened the focus of PD treatment to include autonomic dysfunction, sleep-wake disorders, mood and perceptual disturbances, and cognitive impairment. Yet, despite this progress, neuroprotective or even curative therapies remain an elusive goal. However, accumulating evidence indicates that physical and cognitive exercise can directly affect the progression of PD.

Levodopa is the precursor of dopamine, norepinephrine, and epinephrine, and is the most effective pharmacological treatment overall for motor impairment of PD. Many non-motor symptoms of PD are “off” phenomena and readily respond to dopaminergic therapy. After oral intake and gastric passage, levodopa is absorbed from the duodenum. Unlike dopamine, levodopa crosses the blood-brain barrier (BBB) and utilizes large, neutral amino acid transporters. It is then transported to the nigrostriatal nerve terminals where it is converted into dopamine.

To prevent the conversion of levodopa into dopamine in the periphery, it is combined with a decarboxylase inhibitor (i.e., carbidopa, benserazide). The use of the decarboxylase inhibitor greatly increases the amount of levodopa that crosses the BBB. By reducing serum dopamine

levels, this minimizes the nausea that is produced by dopaminergic stimulation of the area postrema in the medulla, which lacks a BBB. A robust, sustained response to levodopa is highly indicative of a diagnosis of PD, and the converse is also true.

Most of levodopa is peripherally metabolized by catechol-O-methyl transferase (COMT). Thus, even when combined with decarboxylase inhibitors, only a low percentage of orally administered levodopa gets into the brain. By inhibiting levodopa degradation, COMT inhibitors (i.e., entacapone, tolcapone, opicapone) increase the half-life of levodopa by up to 50%, and pharmacokinetically, they decrease the low trough levels seen with regular levodopa. Clinically, these compounds increase “on” times by up to 25% over placebo.

Dopamine agonists (DA) are divided into ergot-derived (bromocriptine, pergolide, cabergoline, and lisuride) and non-ergot-derived (pramipexole, rotigotine, ropinirole, and apomorphine). Ergot-derived DA have been shown to induce life-threatening inflammatory-fibrotic reactions of tissues with serosal membranes (e.g., heart valves, pleura), hence their use is discouraged. The major motor effects of DA are thought to arise from D2 receptors stimulation; however, D1 receptor stimulation works in concert with D2 stimulation to mediate motor activity. DA are less likely to induce dyskinesias than levodopa, possibly because of less pulsatile dopamine receptor stimulation. On the other hand, DA are less effective on PD motor symptoms. In addition, DA are more commonly associated with troublesome neuropsychiatric side effects including hallucinations, psychosis, and ICD than levodopa. ICD includes pathological gambling, binge eating, compulsive shopping, and hypersexuality, and related behaviors including punding and dopamine dysregulation syndrome. Furthermore, DA may also induce excessive daytime sleepiness including sleep attacks, orthostatic hypotension, and perimalleolar edema.

A DA may be used as the initial symptomatic therapy in PS, particularly in younger and

otherwise healthy patients. Use of DA as adjunctive therapy to levodopa may be considered when patients begin to suffer from wearing-off symptoms. Injectable apomorphine may be used to relieve severe motor symptoms in advanced PD patients with disabling and protracted wearing-off episodes.

Another class of drugs used to increase the bioavailability of levodopa is monoamine oxidase-type B (MAO-B) inhibitors (i.e., selegiline, rasagiline, and safinamide). These can be used to prolong the effects of dopaminergic agents, while, when used as monotherapy, MAO-B inhibitors are strikingly less effective than levodopa and DA.

Amantadine is used in the therapy of PD mainly for reducing levodopa-induced dyskinesia. The pharmacology of amantadine is complex, given its multiple putative actions; however, its anti-dyskinetic effects may stem from inhibition of glutamate receptors of the N-methyl-D-aspartate (NMDA) subtype.

Chapter 2 Aims of the Study

Parkinson's disease (PD), the most common neurodegenerative movement disorder, is characterized by aggregates of α -synuclein (α -syn) in dopaminergic neurons in the midbrain substantia nigra (SN). Mutations in the *GBA1* gene, encoding the lysosomal enzyme glucocerebrosidase (GCase), cause Gaucher's disease (GD) when present on both alleles, and are the most common genetic risk factor for PD. A pathogenic bidirectional loop between accumulation of glucosylceramide (GlcCer) due to GCase deficiency and the stabilization of toxic α -syn oligomeric intermediates has been proposed. However, the mechanistic relationship between GCase dysfunction and substrate accumulation, α -syn aggregation, and neurodegeneration remains to be clarified.

Establishing reliable and comprehensive experimental models is a critical step to dissect the molecular underpinnings of PD and develop therapies. Genetic models in rodents and human cell-based studies have so far failed to recapitulate fundamental hallmarks of the disease, including neurodegeneration and α -syn aggregation. Consequently, our understanding of the pathological mechanisms underlying disease progression remains limited, and therapeutic interventions that halt these processes have yet to be discovered.

The overall aim of this project is to establish a novel cell culture system of midbrain organoids (MOs) enriched in dopaminergic neurons derived from induced pluripotent stem cells (iPSCs) of PD and GD patients to recapitulate *GBA1*-related pathology and test compounds for the rescue of disease phenotypes.

In details, the specific aims of this project are:

Aim 1: To generate and characterize iPSC-derived MOs from control subjects and PD patients carrying the *GBA1* L444P mutation.

- 1.1 To generate iPSC lines from fibroblasts of one PD patient carrying a heterozygous L444P *GBA1* mutation, a GD/PD patient carrying homozygous L444P *GBA1* mutations, and two age- and gender-matched healthy subjects.
- 1.2 To differentiate obtained iPSC lines and one additional control iPSC line (purchased from ATCC) into MOs with a newly developed protocol.
- 1.3 To characterize the MO model by assessing the expression of nigral dopaminergic markers, presence of neuromelanin, neuronal membrane lipid composition, and intrinsic membrane properties.

Aim 2: To analyze *GBA1*-related PD pathology in patient-derived MOs.

- 2.1 To study GCase enzymatic activity and protein levels and the effect of *GBA1* deficiency on lipid metabolism (e.g., substrate accumulation).
- 2.2 To study α -syn expression in its physiological and pathological forms, seeding properties, and interaction with synapsin III.

Aim 3: To test compounds for the rescue of pathological phenotypes observed in *GBA1*-mutated MOs.

- 3.1 To assess the ability of ambroxol to increase GCase activity and modulate α -syn pathology.
- 3.2 To assess the ability of GZ667161 to inhibit GlcCer accumulation and rescue α -syn pathology.

Chapter 3 Materials and Methods

3.1 Selection of human subjects

We selected one PD patient carrying a heterozygous *GBA1* L444P mutation (PD^{L444P}), one patient affected by both GD and PD carrying the *GBA* L444P homozygous mutation (GD/PD^{L444P}), and two age- and gender-matched healthy donors with wild type *GBA* (CTR1^{wt} and CTR2^{wt}). One additional control iPSC line (CTR3^{wt}) was purchased from the ATCC cell bank (#ACS-1023; American Type Culture Collection, Manassas, VA, USA).

3.2 Clinical data

The *GBA1* heterozygous PD patient was a Caucasian male with no family history of PD, who developed an akinetic-rigid form of PD at the age of 51. 3 years after the disease onset, the clinical presentation was accompanied by an incomplete response to levodopa treatment, eyelid apraxia, freezing of gait, marked dysautonomia (*i.e.*, orthostatic hypotension, urinary incontinence, constipation, sweating dysfunction), severe REM-sleep behavior disorder, and cataplexy. At the age of 56, PD was complicated by dementia and hallucinations. The patient died at 64 years of aspiration pneumonia. This patient was the donor of skin fibroblasts used to generate the PD^{L444P} iPSC line and of the brain autoptic sample SNc_PD^{L444P}.

The *GBA1* homozygous patient is a Caucasian woman with positive family history of PD (maternal uncle). She is affected by GD type 3 since the age of 4 and receives enzymatic replacement therapy since the age of 29. The visceral GD phenotype included hepatosplenomegaly, thrombocytopenia, pulmonary hypertension, autoimmune thyroiditis, and

history of multiple bone fractures. The neurological involvement consisted of oculomotor apraxia and gait ataxia. At the age of 52, she developed parkinsonism (*i.e.*, right hand bradykinesia, rigidity in the upper limbs, postural and kinetic hand tremor, upward gaze limitation, and postural instability), and dysautonomia with orthostatic hypotension. The DaTSCAN SPECT displayed reduced signal of the left putamen, indicative of nigrostriatal degeneration.

CTR1^{wt} and CTR2^{wt} donors were a Caucasian male and a Caucasian female subject, respectively, with a normal neurological examination. They did not have a family history of PD or other neurodegenerative disorders. CTR3^{wt} was a 36-year-old white female healthy donor from whom the KYOU-DXR0109B cell line was generated at the University of Kyoto (purchased through the ATCC cell bank, #ACS-1023).

Specifics of subjects included in the study are summarized in Results – Table 4.1.

3.3 Human tissues

Fibroblasts were isolated from skin samples obtained from 3 mm punch biopsies performed on the arm's ventral surface of subjects included in the study (*i.e.*, PD^{L444P}, GD/PD^{L444P}, CTR1^{wt}, and CTR2^{wt}). Fibroblasts of all four lines were cultured in DMEM supplemented with 15% FBS, 2 mM glutamine, 0.1 M non-essential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin until 70-90% confluency was reached.

Paraffin-embedded sections and fresh frozen tissue collected from SN *pars compacta* of the PD^{L444P} patient (SNc_PD^{L444P}), as well as of three healthy donors (SNc_CTR1^{wt}, SNc_CTR2^{wt}, and SNc_CTR3^{wt}) kindly supplied by the Parkinson's UK Brain Bank and used in Longhena et al. (2018), were used for immunostaining and biochemical analyses (Table 4.1).

3.4 Generation and maintenance of human iPSC lines

Fibroblasts were reprogrammed following manufacturer's instructions. Specifically, the day before transfection, cells were harvested by aspirating medium, washing twice with PBS 1X, and incubating with 0.05% trypsin for 5 minutes at 37°C and 5% CO₂. When fibroblasts were detached, trypsin was inactivated with basal culture medium and cells were collected in a 15 mL conical tube. Cells were centrifuged at 1800 rpm for 6 minutes at room temperature and the supernatant was discarded. Cells were counted with a hemocytometer so that 1×10^5 fibroblasts were plated in each well of a 6-well plate.

Following overnight incubation at 37°C and 5% CO₂, spent medium was removed and Sendai viruses carrying human *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* (CytoTune Reprogramming kit®) were added to each well of a 6-well plate at a multiplicity of infection of 5 in basal culture medium. For each cell line, two wells of a 6-well plate were transfected.

The day after transfection, supernatant was removed and replaced with 2 ml fresh medium. The same day, irradiated human foreskin fibroblasts were plated at 1×10^6 in 6-well plates and incubated overnight.

The following day, transfected cells were trypsinized and plated onto human foreskin fibroblasts at 1×10^4 - 10^5 cell density.

Two days later, medium was aspirated and replaced with E8 medium® (DMEM/F12 containing 20% knockout serum replacement, 2 mM glutamine, 0.1 M non-essential amino acids, 55 µM 2-mercaptoethanol, 10 ng/mL bFGF, and 50 U and 50 µg/mL penicillin and streptomycin). Medium was changed every day.

About 3-4 weeks later, colonies with iPSC-like morphology started to become visible and were manually picked and plated into Cultrex®(R&D)-coated 6-well plates. After 2-3 consecutive

passages, colonies were transferred to Cultrex®-coated 6 well plates.

For each cell line, at least 3 independent clones were selected and expanded in order to represent biological variability. iPSCs were cultured with E8 medium (Thermo Fisher Scientific) in Cultrex®(R&D)-coated plates and split with EDTA 5 mM every 3-5 days. Cells were periodically tested for Mycoplasma and maintained Mycoplasma free for the whole duration of culturing.

3.5 Genetic analyses of iPSCs

All iPSC lines were screened for mutations in genes associated with PD (*i.e.*, *GBA*, *LRRK2*, *SNCA* point mutations and multiplications, *PRKN* and *PINK1* point mutations and rearrangements, *DJ-1*, *FBXO7*, *DNAJC6*, *SYNJ1*, *RAB39B*, *VPS35*, *VPS13C*, *PTRHD1*, *LRP10*, *DNAJC13*). The PD^{L444P} and GD/PD^{L444P} lines were confirmed to harbor the L444P *GBA* variants in heterozygosity and homozygosity, respectively, and no other mutations in genes associated with PD. Genetic screening in CTR1^{wt}, CTR2^{wt}, and CTR3^{wt} lines ruled out mutations in PD-associated genes.

Cytogenetic analysis of iPSC colonies was carried out according to the standard laboratory procedure. Briefly, iPSCs were detached to obtain single-cell dissociation and treated with colchicine for 3 hours. iPSCs were then incubated with a hypotonic solution (0.6% sodium citrate and 0.13% potassium chloride) and fixed with methanol/acetic acid solution. at 1:3 ratio. Fixed cells were immersed in Quinacrine solution to obtain Q-banding (Caspersson et al., 1999). Karyograms of iPSCs were prepared from microphotographs of metaphase chromosomes acquired with a fluorescence microscope at 100X magnification and analyzed with MetaSyste-Ikaros analytical system. Conventional karyotyping was performed at 400

banding level, according to the ISCN (Simons et al., 2013) and the European General Guidelines and Quality Assurance for Cytogenetics (Hastings et al., 2007).

3.6 Immunocytochemistry of iPSCs

Cells grown on Cultrex-coated borosilicate glass coverslips were washed with PBS 1X, fixed in 4% paraformaldehyde for 10 minutes, washed three times with PBS 1X, and incubated with 0.1% Triton-X100 in PBS 1X with 5% normal donkey serum for one hour at room temperature. Cells were then incubated with 0.1% Triton-X100, 1% normal donkey serum, and primary antibodies at proper dilution in PBS 1X overnight at 4°C (Table 3.1). The following day, cells were washed three times with 0.1% Triton-X100 in PBS 1X and incubated with 0.1% Triton-X100, 1% normal donkey serum, and secondary antibodies at proper dilution in PBS 1X for one hour at room temperature.

Cells were mounted on Fisherbrand™ Superfrost™ Plus Microscope Slides using DAPI Fluoromount-G® (SouthernBiotech). For image acquisition, a ZEISS LSM 800 confocal laser scanning microscope was used. Images were analyzed using ImageJ.

The following primary antibodies were used: Sox2 (D6D9) Rabbit mAb (1:200, Cell Signaling); TRA-1-60 (S) Mouse mAb (1:200, Cell Signaling); Oct-4A (C30A3) Rabbit mAb (1:200, Cell Signaling); and SSEA4 (MC813) Mouse Ab (1:200, Cell Signaling).

3.7 Generation of midbrain organoids

All iPSC lines included in the study were differentiated into MOs with a dopaminergic-patterning protocol devised by this PhD applicant and adapted from published methods (Kriks

et al., 2011; Lancaster and Knoblich, 2014; Tieng et al., 2014; Zhang et al., 2014).

The following media were used:

hES medium: 75% DMEM-F12, 20% KOSR, 3% ES-quality FBS, 1% 100X Glutamax supplement, 1% MEM-NEAA, 96 μ M 2-Mercaptoethanol, and 4 ng/ml bFGF.

Neural induction medium: 97% DMEM-F12, 1% 100X N2 supplement, 1% 100X Glutamax supplement, 1% MEM-NEAA, and 1 μ g/ml Heparin.

Cerebral organoid differentiation medium: 48% DMEM-F12, 48% Neurobasal, 0.5% 100X N2 supplement, 0.4 μ M Insulin, 1% 100X Glutamax supplement, 0.5% MEM-NEAA, 1% 100X penicillin/streptomycin stock solution, 48 μ M 2-Mercaptoethanol, and 1% 50X B27 supplement without vitamin A.

KSR differentiation medium: 83% DMEM, 15% 75 ml KSR, 1% 100X MEM-NEAA, 1% 100X penicillin/ampicillin stock solution, and 10 μ M 2-Mercaptoethanol.

N2 differentiation medium: 98% DMEM, 1% 100X N2 supplement, and 1% 100X penicillin/ampicillin stock solution.

B27 differentiation medium: 96% Neurobasal, 2% 50X B27 supplement, 1% 100X Glutamax supplement, and 1% 100X penicillin/ampicillin stock solution.

iPSC colonies were grown in one well of a 6-well plate until 70-80% confluency was reached.

On day 0, cells were washed with 1 ml D-PBS without calcium and magnesium and incubated with 600 μ l 0.5mM EDTA solution in D-PBS without calcium and magnesium for each well of 6-well plate at 37°C and 5% CO₂ for 4 minutes. EDTA solution was aspirated, and cells were incubated with 1 ml Accutase® (Thermo Fisher) at 37°C and 5% CO₂ for 4 minutes. Using a 1 ml pipette tip, colonies were detached with 1 ml E8 medium® (Thermo Fisher), transferred to a 15 ml conical tube, and triturated. 2 repetitions of 5 μ l were taken for cell counting using a hemocytometer. Cells were centrifuged at 270 g for 5 minutes. Cells were resuspended in hES

medium with 50 μ M ROCK inhibitor and plated at 9,000 live cells/150 μ l in each well of a low attachment 96-well U-bottom plate. Plates were kept in the incubator for 48 hours.

On day 2 and 4, formed embryoid bodies (EBs) were fed by gently aspirating approximately half of the medium and addition of 150 μ l of fresh media. 50 μ M ROCK inhibitor and 4 ng/ml bFGF were included until EBs began to show brighter edges or were larger than 350-400 μ m in diameter.

On day 6, each EB was transferred to one well of a low attachment 24-well plate containing 500 μ l Neural induction medium using a cut 200 μ l pipette tip.

On day 8, 500 μ l of Neural induction medium were added to each well.

On day 9, dimpled Parafilm sheets were prepared by layering a square of Parafilm over an empty tip tray for size 200 μ l tips and by pressing over each hole in the tip tray to create a grid of 4×4 dimples in the Parafilm. Each square of Parafilm was placed into a 60 mm tissue culture dish and sterilized overnight under UV light.

On day 10, neuroepithelial tissues were transferred one by one to each dimple in the Parafilm with a cut 200 μ l tip. After removing excess medium from each tissue, a 30 μ l droplet of Cultrex[®] (R&D) was added to each aggregate, which was positioned in the center of the droplet using a 10 μ l pipette tip. The 60 mm dish containing droplets on Parafilm was placed back into the 37°C incubator and incubated for 30 minutes. 5 ml of Cerebral organoid differentiation medium without vitamin A were added to the 60 mm dish. Cultrex[®] droplets containing neuroepithelia were removed from Parafilm by gently pipetting medium under each droplet and placed back into the incubator.

On day 12, medium was aspirated and replaced with 5 ml fresh Cerebral organoid differentiation medium without vitamin A.

On day 14, embedded organoids were transferred to either a 125 ml size (96 EBs total) or

a 250 ml size (192 EBs total) spinning bioreactor (Corning) by using a cut 1 ml pipette tip with an opening of approximately 3 mm. Organoids were cultured in 80 ml (for the 125 ml spinner flask) or 110 ml (for the 250 ml spinner flask) of appropriate medium in spinning bioreactors placed on a magnetic stir plate installed in the incubator at an initial speed of 25 rpm.

Dopaminergic patterning was obtained by culturing organoids in the following supplemented media:

- day 14: KSR differentiation medium supplemented with 10 μ M SB431542 and 100 nM LDN-193189;

- day 15-16: KSR differentiation medium supplemented with 10 μ M SB431542, 100 nM LDN-193189, 0.25 μ M SAG, 2 μ M purmorphamine, and 50 ng/ml FGF8b;

- day 17-18: KSR differentiation medium supplemented with 10 μ M SB431542, 100 nM LDN-193189, 0.25 μ M SAG, 2 μ M purmorphamine, 50 ng/ml FGF8b, and 3 μ M CHIR99021;

- day 19-20: 75% KSR differentiation medium plus 25% N2 differentiation medium supplemented with 100 nM LDN-193189, 0.25 μ M SAG, 2 μ M purmorphamine, 50 ng/ml FGF8b, and 3 μ M CHIR99021;

- day 21-22: 50% KSR differentiation medium plus 50% N2 differentiation medium supplemented with 100 nM LDN-193189 and 3 μ M CHIR99021;

- day 23-24: 25% KSR differentiation medium plus 75% N2 differentiation medium supplemented with 100 nM LDN-193189 and 3 μ M CHIR99021;

- day 25-26: B27 differentiation medium supplemented with 3 μ M CHIR99021, 10 ng/ml BDNF, 10 ng/ml GDNF, 1 ng/ml TGF3, 0.2 mM ascorbic acid, and 0.1 mM cAMP;

- day 27-day 200: B27 differentiation medium supplemented with 10 ng/ml BDNF, 10 ng/ml GDNF, 1 ng/ml TGF3, 0.2 mM ascorbic acid, and 0.1 mM cAMP.

3.8 RNA sequencing analysis of midbrain organoids

RNAseq was performed on PD^{L444P}, GD/PD^{L444P}, CTR1^{wt}, and CTR2^{wt} MOs collected at three time points during neural differentiation (6, 35, and 80 DIV). For each organoid and time point, experiments were performed in triplicate. RNA was isolated using the Eurogold Trifast kit (Euroclone) following the manufacturer's instructions. Concentrations were determined using a Nanodrop spectrophotometer (Wilmington, DE). RNA quality was assessed on an Agilent 4200 TapeStation, obtaining a mean RNA integrity number (RIN) of 8.5. Libraries were prepared starting from 15 ng of total RNA with the low input SMARTer Stranded RNA-Seq Kit for Illumina (Clontech-Takara), following manufacturer's instructions, and sequenced as 75-bp paired-end reads on a NextSeq500 platform (Illumina).

Reads were mapped to the hg19 reference genome and to the *Homo sapiens* transcriptome [Illumina's iGenomes reference annotation, http://support.illumina.com/sequencing/sequencing_software/igenome.html] with Star v.2.7.1a (Dobin et al., 2013). Quality control (QC) was performed on raw data (.fastq files) using FastQC v.0.11.8 (Andrews, 2010) as well as BBtools (<https://sourceforge.net/projects/bbmap/>), and on aligned data (.bam files) using RSeQC v.2.6.4 (Wang et al, 2012). Time course analysis of differential gene expression was performed using a likelihood ratio test (LRT) for significance using the DeSeq2 Bioconductor package (Love et al., 2014). Differentially expressed (DE) genes were selected using a false discovery rate (FDR) of ≤ 0.01 , a fold change of ≥ 2 or ≤ -2 , and a mean expression > 30 counts. Gene ontology (GO) enrichment analyses were performed using Metascape (Zhou et al., 2019) with default parameters, independently on upregulated and downregulated genes.

To estimate the relative proportion of different brain cell types (neurons and astrocytes)

present in bulk RNAseq data based on the expression of sets of well-validated cell type-specific marker genes in differentiating MOs, we used the “brainCells” function within the BRETIGEA (BRain cEll Type specific Gene Expression Analysis) R package, setting the species to human (McKenzie et al., 2018).

3.9 Immunohistochemistry of midbrain organoids

3.9.1 Immunostaining of paraffin-embedded midbrain organoids

MOs were transferred to a standard 24-well plate by gently pipetting with a cut 1 ml pipette tip. Medium was removed and samples were washed with 1 ml PBS 1X. MOs were fixed for 48 hours at room temperature in 10% neutral buffered formalin. Subsequently, organoids were processed on a Leica ASP300 and embedded in paraffin. Given the small diameter of organoids (1-3 mm), a cytoblock procedure was performed with Bio-Agar (Bio-Optica 05-9803S) prior to paraffin embedding in order to avoid the possibility of tissue damage. Paraffin-embedded specimens were cut into 5 µm sections on charged slides and allowed to air dry at room temperature. Slides were hematoxylin (Richard-Allan Scientific 7211) and eosin (Leica 3801619) stained using routine laboratory method (Carson et al., 2009). Images were collected on an Olympus BX43F brightfield microscope using Olympus cellSens Entry (version 1.18).

3.9.2 Schmorl's method

For Schmorl's reaction for neuromelanin, sections were brought to distilled water via xylene and ethanol and subsequently treated with ferric-cyanide solution (10% freshly prepared 0.4%

aqueous potassium ferricyanide, 75% freshly prepared 1% aqueous ferric chloride, 15% distilled water) for 10 minutes. Sections were then washed with water for 10 minutes and counterstained with Kernechtrot solution (0.1% nuclear fast red, 5% aluminum sulfate in distilled water) for 5 minutes.

3.9.3 Immunostaining of cryosections

For immunofluorescence staining, MOs were transferred to a 24-well plate by gently pipetting with a cut 1 ml pipette tip. Medium was removed and samples were washed with 1 ml PBS 1X. Samples were incubated with 1 ml 4% PFA at 4°C for 15 minutes in the dark. 3 washes with 1 ml PBS 1X were performed. Final PBS wash was replaced with 1 ml 30% sucrose solution and tissues were placed at 4°C overnight. The next day, sucrose solution was replaced with 1 ml of warmed gelatin/sucrose solution. Tissues were placed at 37°C for 15 minutes. Using a cut 1 ml pipette tip, MOs were transferred from the 24-well plate to a thin polymerized gelatin/sucrose layer in a cryomold, covered with warm gelatin/sucrose solution, and placed at 4°C for 30 min. A small block containing each sample was cut out of the polymerized gelatin block and immersed for 1 minute into a freezing bath previously prepared by dropping small pieces of dry ice into a bath of isopentane until a final temperature of - 50°C was reached. Frozen blocks were sectioned in 30 µm sections with a cryostat. After permeabilization in 20% methanol and 0.1% Triton X-100 in PBS 0.1 M, slides were incubated for 1 hour at room temperature in blocking solution (2% v/v NGS, 3% w/v Bovine Serum Albumin (BSA), 0.1% Triton X-100 in PBS 0.1 M) and with the primary antibody diluted in blocking solution overnight at 4°C (Table 3.1). The following day, slides were washed with 0.1% Triton X-100 PBS 0.1 M and incubated with the fluorochrome-conjugated secondary antibody in 0.1% Triton X-100 PBS

0.1 M plus 1 mg/ml BSA for 1 hour at room temperature. For double immunofluorescence staining, after three washes in 0.1% Triton X-100 PBS 0.1 M, slides were incubated for 2 hours at room temperature with the second primary antibody, followed by incubation for 1 hour at room temperature with the optimal secondary antibody. Cell nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich) and then slices were mounted onto glass slides using Vectashield (Vector Laboratories). Images were taken on a ZEISS LSM 800 confocal laser scanning microscope.

Primary antibodies used are summarized in Table 3.1.

3.9.4 Double 3D immunofluorescence staining

MOs at 50, 100, and 150 DIV were fixed for 1 hour at room temperature in Immunofix and washed 3 times with PBS 0.1 M. Fixed MOs were permeabilized for 2 hours in PBS 0.1 M supplemented with 20% methanol and 0.1% Triton X-100, washed and incubated overnight at room temperature in blocking solution (10% normal goat serum (NGS), 0.1% Triton-X100 in PBS 0.1M) and then with primary antibodies at proper dilution (Table 3.2) in blocking solution overnight for 5 days at room temperature. MOs were washed 3 times with 0.1% Triton-X100 PBS 0.1M for 24 hours and incubated with the fluorochrome-conjugated secondary antibodies (goat anti-mouse cy3 and goat anti-rabbit Alexa Fluor 488, Jackson ImmunoResearch) in 0.1% Triton-X100 PBS 0.1 M plus 3 % NGS for 5 days at room temperature. After three washes in 0.1% Triton X-100 PBS for 24 h, cells were clarified overnight in 47% 2,2'-thiodiethanol (TDE) and observed by means of a Zeiss confocal laser microscope LSM 880 with tile scan and z-stack reconstructions. Images (512 × 512 pixels) were then reconstructed using Zen lite 2.3.

ANTIBODY	CATALOG #	MANUFACTURER	HOST SPECIES	WORKING DILUTION		
				WB	IF	IHC
α -synuclein	610787	BD	Mouse	1:1000	1:100	
α -synuclein	847902	BioLegend	Mouse		1:100	
β -actin	251011	Synaptic System	Mouse	1:5000		
CNPase	GTX103954	Gentex	Rabbit		1:100	
DAT	AB2231	Millipore	Rabbit		1:600	
GAPDH	G8795	Sigma-Aldrich	Mouse	1:5000		
GBA	G4171	Sigma-Aldrich	Rabbit	1:1000		
GFAP	G3893	Sigma-Aldrich	Mouse		1:400	1:400
GIRK2	ab65096	Abcam	Goat		1:100	
HOECHST	33258	Sigma-Aldrich			1:2000	
MAP2	AB5622	Millipore	Rabbit		1:200	
NeuN	MAB377	Millipore	Mouse		1:100	
Oct-4	2840	Cell Signaling	Rabbit		1:200	
OLIG2	AB9610	Millipore	Rabbit		1:400	
S100b	HPA015768	Sigma	Rabbit		1:1000	
Sox2	3579	Cell Signaling	Rabbit		1:200	
SSEA4	4755	Cell Signaling	Mouse		1:200	
Syn III	106303	Synaptic Systems	Rabbit	1:1000		
TH	AB152	Millipore	Rabbit		1:700	
TRA-1-60	4746	Cell Signaling	Mouse		1:200	
VMAT2	138313	Synaptic System	Rabbit		1:500	

Table 3.1 – Specifics of antibodies.

List of primary antibodies and working dilution used in Western Blots (WB), immunofluorescence (IF), and immunohistochemistry (IHC).

3.9.5 Thioflavin-S staining

Fixed MOs were incubated with 0.05% thioflavin-S (Sigma-Aldrich) dissolved in ethanol 50% and subsequently washed in 80% ethanol to eliminate unspecific dye residues. Sections were then blocked for subsequent immunostaining with the antibody against α -syn and TH, mounted on slides and analyzed by confocal microscopy. For thioflavin-S detection, images were acquired with laser excitation set between 350-400 nm.

3.9.6 Immunohistochemistry of proteinase K-resistant α -synuclein

Proteinase K-resistant α -syn was visualized using chromogenic or fluorescent immunohistochemistry using previously described protocols (Drummond et al., 2017; Drummond et al., 2018). Briefly, 8 μ m thick sections were deparaffinized and rehydrated through a series of xylene and ethanol washes. Sections were incubated with a working solution of proteinase K (1:800; Enzo Life Sciences, catalog #ENZ-33801) for 20 minutes at 37°C. Further antigen retrieval was performed by treatment with 88% formic acid for 7 minutes, followed by boiling in citrate buffer (10mM sodium citrate, 0.05% Tween-20; pH6). For chromogenic IHC only, endogenous peroxidase activity was blocked using 0.3% H₂O₂ for 20 minutes. Sections were blocked with 10% normal goat serum and incubated overnight at 4°C with an anti-aggregated α -syn primary antibody (1:100; BioLegend, catalog #847902). Sections were then incubated with an anti-mouse IgG fluorescent or biotinylated secondary antibody (1:500, from Jackson ImmunoResearch, 2h at RT or 1:1000, 1hr at RT). For chromogenic IHC, sections were incubated with ABC solution (1hr at RT) followed by DAB. Sections were coverslipped using ProLong™ Diamond Antifade Mountant (Invitrogen).

3.9.7 Analysis of Hoechst-, TH- and NeuN-positive neurons

The number of neurons was counted by using Imaris Software 9.2.0 (BitPlane, UK). The whole tile scan reconstruction was used to quantify the number of Hoechst-, NeuN- and TH-positive cells by creating new spot surface and setting the minimum diameter at 15, 20 and 23.7 μm , respectively. The number of spots was then automatically measured for each channel. Twelve organoids per condition were analyzed by examining whole organoid volume.

3.9.8 Analysis of α -synuclein-positive area in TH-positive cells

The optical density of α -syn- and TH-positive area was examined in blind from every z-stack digitized image acquired by confocal microscopy by using FIJI Software. The α -syn-positive area in TH-positive neurons was calculated by extruding α -syn from TH immunoreactivity to avoid bias due to the different distribution of neurons in whole organoids. The ratio between α -syn- and TH-positive areas was analyzed for single image and averaged for every z-stack. The acquisition parameters during confocal imaging were maintained constant for all the image settings used for the analysis. Two organoids per patient were analyzed by examining an average of 5 fields per condition.

3.10 Lipid analyses

At selected timepoints for specific experiments, MOs were treated for four days with 36 nM radioactive sphingosine ([1-3H] sphingosine, specific radioactivity 1.06 Ci/ mmol) solubilized in the complete culturing medium in order to metabolically label the sphingolipids. At the end of

the incubation, organoids were lysed in water containing protease inhibitors and lyophilized. Lipids were extracted using chloroform:methanol:water 2:1:0.1 (v:v:v). The total lipid extracts were then partitioned into two phases: the aqueous phase containing gangliosides and the organic phase containing the residual lipids. The aqueous phase underwent water dialysis to remove possible salts contamination. The organic phases were subjected to alkaline methanolysis to remove glycerophospholipids (Schöndorf et al., 2014; Samarani et al., 2018). The lipids contained in the aqueous phases and in the alkaline-stable organic phases were separated by high-performance thin-layer chromatography (HPTLC) loading the same amount of radioactivity for each sample. The neutral sphingolipids were separated using the solvent system chloroform:methanol:water 110:40:6 (v:v:v), whereas gangliosides contained in the aqueous phases were separated using the solvent system chloroform:methanol:0.2% CaCl_2 50:42:11 (v:v:v). Sphingolipids were identified by comigration with authentic radioactive lipid standards. Radioactive lipids were detected by digital autoradiography (Beta-Imager TRacer, BioSpace lab) and quantified with M3 Vision software.

3.11 Calcium imaging

For experiments shown in Figure 4.14c, MOs were loaded with Fluo-4 AM (5 μM ; Thermo Fisher) for 45 minutes at 37°C prior to live Ca^{2+} imaging through 20X and 4X long working distance air objectives (Edmund Optics; #58373) on an epifluorescence microscope (Thorlabs; Bergamo-II) using 470 nm excitation light. Full-field images of emitted light (band-passed at 500-550 nm) were acquired at 10 Hz with a monochrome CCD camera (1501M; Thorlabs) and analyzed offline with ImageJ (NIH). Regions of interest (ROIs) were manually selected, and mean fluorescence was calculated for each frame.

For experiments shown in Figure 4.41d-g, MOs were loaded with 10 μ M FluoForte-AM (Enzo) for 1 hour at 37 °C in culture solution supplemented with 25 μ M sulfinpyrazone and Pluronic F-127 (0.1%). For de-esterification, the MOs were transferred to a chamber mounted on the stage of an upright two-photon microscope (Bergamo Thorlabs) incorporating a Chameleon (Coherent) tunable pulsed laser and equipped with a water immersion objective (25X, 1.05 NA, Olympus). Intracellular FluoForte fluorescence was excited at 800 nm and selected around 525 nm by a 525/40m narrow-band interference filter. At recording time, each MO was held in the center of the perfusion chamber by gentle suction of a small tube below and super-perfused with an extracellular solution containing (in mM) NaCl 140, KCl 5, CaCl₂ 2, MgCl₂ 1, HEPES 10, Glucose 10, pH 7.4. Image sequences were processed off-line using a lab software written in Matlab 2019b (The MathWorks, Inc., Natick, MA, USA).

For all Ca²⁺ imaging experiments, changes in fluorescence were calculated as follows:

$$\Delta F / F = (F - F_{\text{basal}}) / F_{\text{basal}}$$

where F_{basal} is the mean fluorescence across all frames.

For pharmacological experiments, we imaged Ca²⁺ from MO neurons dissociated using the NeuroCult™ Enzymatic Dissociation Kit for Adult CNS Tissue (Stemcells Technologies, according to manufacturer's instructions) and loaded with Fluo-4 AM as previously described. TTX (1 μ M, Alomone Labs) was applied to the perfusate.

3.12 GCase enzymatic activity assay

Enzyme activities of β -glucocerebrosidase (GCase) was determined in total MOs lysates using fluorogenic substrates (Glycosynth, Warrington, United Kingdom) as previously described (Samarani et al., 2018). Briefly, organoids were washed with PBS, harvested, and resuspended in water containing a protease inhibitor cocktail (Roche). Aliquots of the lysates corresponding to 20 μ g of proteins were pre-incubated for 30 minutes at room temperature in 96-well plates with 75 μ l of a reaction mixture composed by: 25 μ l of Mcllvaine buffer 4X (0.4 M citric acid, 0.8 M Na_2HPO_4) pH 5.2, AMP-DNM (N-(5-adamantane-1-yl-methoxy-pentyl)-Deoxynojirimycin) at a final concentration of 5 nM, which is a specific inhibitor of non-lysosomal β -glucosylceramidase, and water to a final volume of 100 μ l. At the end of the incubation, the assay was started adding 25 μ l of the specific fluorogenic substrate 4-Methylumbelliferyl- β -D-glucopyranoside at a final concentration of 6 mM. Each reaction mixture was incubated for one hour at 37°C under gentle agitation. Finally, 10 μ l of the reaction mixture were transferred to black 96-well plates adding 190 μ l of 0.25 M glycine pH 10.7. Plates were analyzed by Victor microplate reader (Perkin Elmer). Specific activity was expressed as nanomoles of converted substrate/mg of proteins/hours.

3.13 Western blot analysis

Total proteins were extracted with Radioimmunoprecipitation Assay (RIPA) buffer made up by 50 mM Tris-HCl pH 7.4, 150 mM NaCl, NP-40 1%, sodium deoxycholate 0.1%, sodium dodecyl sulfate (SDS) 0.1%, 1 mM NaF, 1 mM NaVO_4 plus complete protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) after sonication. Protein concentration in the

samples was measured by using the Bio-Rad DCTM protein assay kit (Bio-Rad Laboratories, Milan, Italy). Equal amounts of proteins (30 µg) were run on 10% polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membrane. Densitometric analysis of the bands were performed by using ImageJ Software and all bands were normalized to GAPDH or β -actin levels as a control of equal loading of samples in the total protein extracts. List of primary antibodies is reported in Table 3.2. Secondary antibodies used were goat anti-mouse or goat anti-rabbit HRP (Promega).

3.14 Detergent-insoluble α -synuclein extraction for western blot analysis

α -syn insoluble aggregates were extracted from MOs pellets after homogenization in Tris Buffer Saline + (TBS+) solution and centrifugation at 4°C at 50,000 g for 30 minutes, as previously described (Faustini et al., 2018). Briefly, pellets were re-suspended in TBS+ solution plus 1% Triton X-100 and centrifuged at 4 °C at 50,000 g for 30 minutes and then in RIPA buffer and subjected to a further centrifugation. The final pellets were resuspended in urea 8 M plus SDS 5% and loaded on 10% homemade polyacrylamide gel. For densitometry analysis of bands, each experimental condition was performed in triplicate and the resulting data were averaged subjected to statistical analysis. All bands were normalized to β -actin levels as a control of equal loading of samples in the UREA/SDS protein extracts.

3.15 Detergent-insoluble α -synuclein extraction for TEM analysis

100 µL of 10% MOs homogenate was diluted in 400 µL of PBS and incubated with 2% sodium lauroyl sarcosinate under shaking (550 rpm) for 30 minutes at 37°C and then

centrifuged at 10,000 g for 10 minutes at room temperature. The supernatants were transferred into new tubes and centrifuged at 100,000 g for 20 minutes at 4°C. Resulting pellets were re-suspended in 25 µL of buffer A (10 mM Tris-HCl (pH 7.4), 0.8 M NaCl, 10% sucrose, and 1 mM EDTA) and subsequently supplemented with 75 µL of buffer B (50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 10% sucrose, and 0.2% sodium lauroyl sarcosinate). Samples were centrifuged at 166,000 g for 30 minutes at 4°C. Final pellets, containing insoluble α-synuclein, were re-suspended in 20 µL of 30 mM Tris-HCl (pH 7.4) and subjected to transmission electron microscopy analysis.

3.16 TEM analysis of insoluble α-synuclein extracts

Insoluble α-syn extracts obtained from MOs were properly diluted in water. 10 µL of samples was adsorbed onto 200-mesh Formvar-carbon coated nickel grids for 30 minutes. The remaining sample was then dried using filter paper and the grids were stained with 25% Uranyl Acetate Replacement (UAR) for 10 minutes. The remaining staining solution was removed with filter paper and the grids were air-dried for 15 minutes. Samples were finally analyzed with a FEI Tecnai Spirit equipped with an Olympus Megaview G2 camera.

3.17 α-synuclein RT-QuIC analysis of brains and midbrain organoids

Brains and MOs were homogenized at 10% (weight/volume in PBS) and diluted until 10^{-10} and 10^{-2} (volume/volume in PBS), respectively, before α-syn RT-QuIC analysis that was performed as previously described⁹⁰. Briefly, the RT-QuIC mix was composed by 0.1 mg/mL recombinant α-syn (rec-αS, rPeptide), 40 mM HEPES (pH 8.0), 170 mM sodium citrate and 20

μ M Thioflavin-T (ThT). All reactions were performed in quadruplicate in a black 384-well optical flat bottom plate (Thermo Fisher Scientific). Each well was supplemented with 2 silica beads (0.8 mm OPS Diagnostics) and 48 μ L of reaction mix. 2 μ L of sample was then added to each well. The plate was sealed with a sealing film (Thermo Fisher Scientific) and subjected to cycles of shaking (1 minute at 600 rpm, single orbital) and incubation (1 minute) at 42 °C using a fluorescence microplate reader (BMG Labtech FluoStar OMEGA). Fluorescence intensities, expressed as arbitrary units (AU), were taken every 30 minutes (excitation 450 ± 10 nm; emission 480 ± 10 nm) with a bottom read. To distinguish samples with or without seeding activities, thresholds of time (15 hours) and fluorescence (45,000 AU) were set and the seeding effect was considered when at least two replicates out of four emitted fluorescent signals before these thresholds.

3.18 Pharmacological treatments

CTR^{wt} MOs at 65 DIV were treated with 500 μ M CBE (Merck Millipore) for 14 days.

MOs of all lines at 100 DIV were cultured in B27 medium supplemented with either 100 μ M ambroxol (Sigma-Aldrich) for 10 days or 100 nM GZ667161 (Sanofi) for 14 days. For all treatments, medium was changed every other day and replaced with fresh medium supplemented with either CBE, ambroxol, or GZ667161 at working concentration.

3.19 Statistical analyses

A two-tailed Student's t test was used for statistical analyses for all experiments, unless otherwise specified. The number of replicates used for each experiment and P values are indicated in the figure legends. Graphs in figures represent mean $\pm$ s.e.m.

3.20 Ethics statement

The study on human brain samples obtained approval from the Ethics Committee of Brescia District. The generation and use of iPSCs obtained approval from the Institutional Review Board (IRB) of “Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico”. IRB-approved written informed consent was collected from all subjects donating samples to the study.

Chapter 4 Results

4.1 Generation of iPSC lines

Induced pluripotent stem cell (iPSC) lines were generated from skin fibroblasts of one PD patient carrying the *GBA1* L444P heterozygous mutation (PD^{L444P}); one patient affected by both GD type 3 and PD carrying the *GBA1* L444P homozygous mutation (GD/PD^{L444P}); and two age- and gender-matched healthy donors with a wild type *GBA1* (CTR1^{wt} and CTR2^{wt}). Of each line, ten clones were selected for immunocytochemistry characterization and karyotyping (see Chapter 4.3). One additional control iPSC line (CTR3^{wt}) was purchased from the ATCC cell bank (#ACS-1023; American Type Culture Collection, Manassas, VA, USA) (Table 4.1).

4.2 Collection of human tissues

Paraffin-embedded sections as well as fresh frozen tissue from SN *pars compacta* (SNc) of the PD patient carrying the heterozygous L444P *GBA1* mutation from whom the PD^{L444P} line was derived (SNc_PD^{L444P}) and of three unaffected donors (SNc_CTR1^{wt}, SNc_CTR2^{wt}, and SNc_CTR3^{wt}) (kindly supplied by the Parkinson's UK Brain Bank and used in Longhena et al., 2018) were collected from *post-mortem* brains and consequently used for immunostainings and biochemical studies (Table 4.1).

ID #	TYPE OF SAMPLE	GBA GENOTYPE	PHENOTYPE	GENDER	AGE AT DISEASE ONSET	AGE AT SAMPLING
CTR1 ^{wt}	iPSCs	wt	CTR	M	-	52 yo
CTR2 ^{wt}	iPSCs	wt	CTR	F	-	44 yo
CTR3 ^{wt}	iPSCs	wt	CTR	F	-	36 yo
PD ^{L444P}	iPSCs	GBA1 L444P het	PD	M	51 yo*	59 yo
SNC_PD ^{L444P}	Brain					64 yo
GD/PD ^{L444P}	iPSCs	GBA1 L444P hom	GD + PD	F	4 yo** 52 yo*	49 yo
SNC_CTR1 ^{wt}	Brain	wt	CTR	M	-	88 yo
SNC_CTR2 ^{wt}	Brain	wt	CTR	M	-	68 yo
SNC_CTR3 ^{wt}	Brain	wt	CTR	M	-	65 yo

Table 4.1 – Demographic data of donor subjects of iPSC lines and brain samples.

Abbreviations: CTR, control subject; F, female; *GBA1*, glucocerebrosidase gene; GD, Gaucher's disease; het, heterozygous; hom, homozygous; iPSCs, induced pluripotent stem cells; M, male; PD, Parkinson's disease; wt, wild type. *Onset of PD. **Onset of GD.

4.3 Characterization of iPSC lines

All iPSC lines used in the study were screened for mutations in genes associated with PD (*i.e.*, *SNCA*, *LRRK2*, *GBA1*, *VPS35*, *PRKN*, *PINK1*, *DJ1*, *FBXO7*, *ATP13A2*, *PLA2G6*, *DNAJC6*, *SYNJ1*, *PTRHD1*, *RAB39B*, and *VPS13*), confirming the presence of *GBA1* variants in PD^{L444P} and GD/PD^{L444P} and absence of mutations in PD-associated genes in CTR1^{wt}, CTR2^{wt}, and CTR3^{wt}.

All iPSC lines showed typical stem cell-like morphology and immunoreactivity for stem cell markers (*i.e.*, SOX2, OCT4, SSEA4, and TRA-1-60) (Figure 4.1a, b).

Karyotype analysis of iPSC lines was normal and ruled out major chromosomal rearrangements during reprogramming (Figure 4.1c).

Three clones of each generated line showing expression of stem markers and with a normal karyotype were consequently differentiated into midbrain organoids.

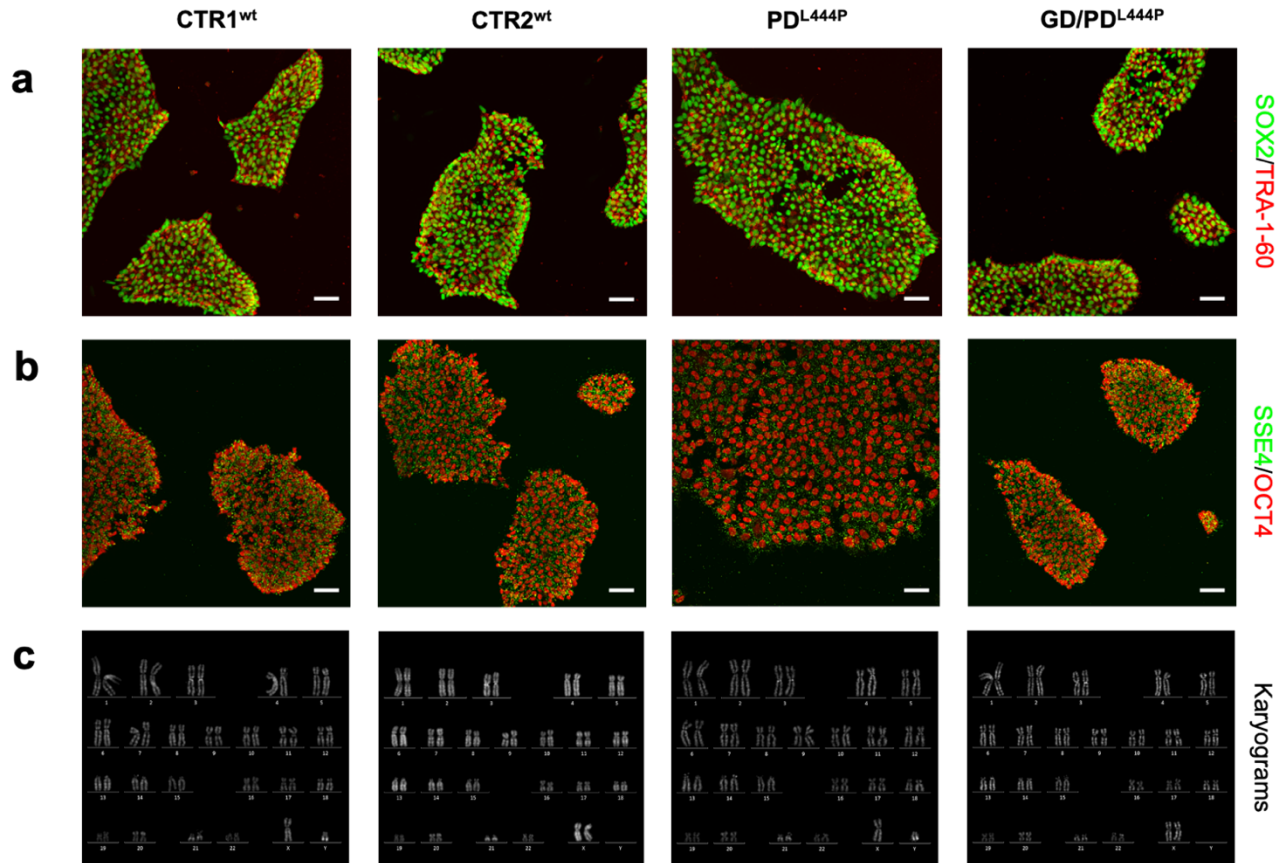


Figure 4.1 – Generation of iPSC lines from control subjects and *GBA1*-mutated patients.

a, b. Representative immunocytochemistry images of one clone of each generated iPSC line showing immunoreactivity for stem cell markers SOX2/TRA-1-60 (a) and SSEA4/OCT4 (b). Scale bars, 50 μm.

c. Karyotype analysis of generated iPSC lines ruled out chromosomal rearrangements. 40 metaphase chromosome spreads were analyzed for each line.

Abbreviations: CTR^{wt}, control subject; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; SOX2, sex determining region Y-box 2; TRA-1-60, T cell receptor alpha locus-1-60; SSEA4, stage-specific embryonic antigen-4; OCT4, octamer-binding transcription factor 4.

4.4 Generation of iPSC-derived midbrain organoids

Three independent clones of the PD^{L444P}, GD/PD^{L444P}, CTR1^{wt}, and CTR2^{wt} lines and one clone of the CTR3^{wt} line were differentiated into midbrain organoids with a dopaminergic-patterning protocol developed in our lab and adapted from published methods (Kriks et al., 2011; Lancaster and Knoblich, 2014; Tieng et al., 2014; Zhang et al., 2014) (Figure 4.2).

Our protocol specifically addresses two main goals: 1) the induction and specification of key adult human midbrain markers and 2) the recapitulation of a three-dimensional spatial organization. First, embryoid bodies (EBs) were generated by aggregating iPSCs in low attachment 96-well plates and grown in a medium supplemented with low bFGF and high-dose ROCK inhibitor. EBs that reached a larger size (*i.e.*, 400-600 μ m) and developed defined margins and brighter edges (*i.e.*, radial neuroepithelium) were selected for further culturing, transferred into 24-well plates and cultured in a minimal medium to allow neuroectodermal differentiation.

Selected samples were embedded into hydrogel droplets (Cultrex[®]) to provide a scaffold for ordered spatial organization of cells and to promote outgrowth of neuroepithelium. Finally, tissues were transferred to spinning bioreactors for extensive growth via improved nutrient and oxygen exchange. Once in spinner flasks, timed exposure to selected small molecules driving midbrain development *in vivo* (*i.e.*, SB431542, LDN193189, purmorphamine, SAG, FGF8b, and CHIR99021) and neurotrophic factors for maturation and maintenance of neurons (*i.e.*, BDNF, GDNF, TGF β 3, cAMP, and ascorbic acid) allowed proper maturation and specification of midbrain identity (Figure 4.2). Midbrain organoids were cultured for up to 150 days *in vitro* (DIV) and collected at various time points of differentiation for analyses.

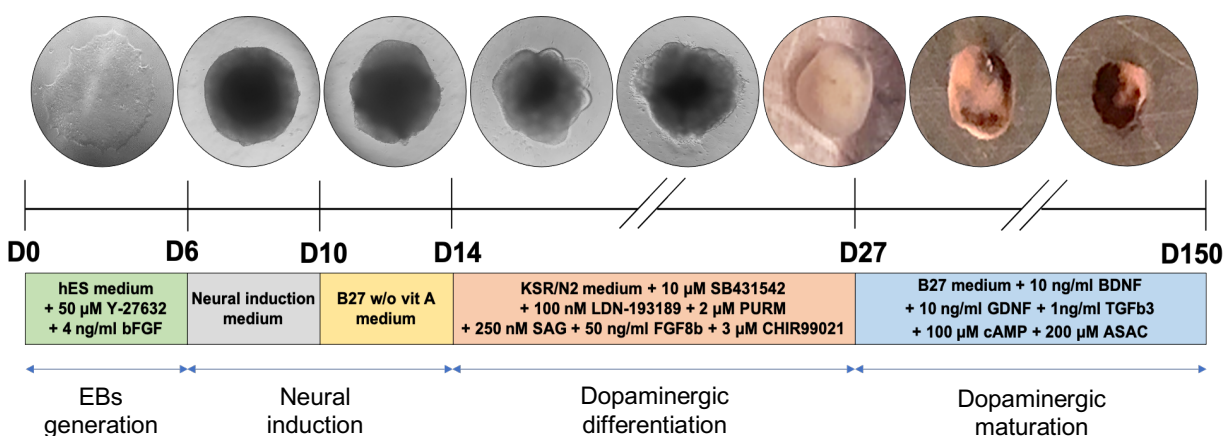


Figure 4.2 – Schematic overview of the differentiation protocol for the generation of midbrain organoids.

On day 0 (D0), iPSCs are seeded into ultra-low attachment plates to allow embryoid body formation (D0 – D6). Following neural induction (D6 – D10), embryoid bodies are embedded in Cultrex® (R&D) (D10) and cultured in static conditions for another 4 days (D10 – D14). On D14, tissues are transferred to spinning bioreactors, where they are subdued to timed exposure to dopaminergic patterning factors. Trophic factors are added on D27 to aid dopaminergic maturation and survival. MOs are cultured for up to 150 DIV.

Abbreviations: ASAC, ascorbic acid; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; cAMP, cyclic adenosine monophosphate; DIV, days *in vitro*; EBs, embryoid bodies; FGF8b, fibroblast growth factor 8b; GDNF, glial cell line-derived neurotrophic factor; hES, human embryonic stem cells; KSR, knock-out serum replacement; PURM, purmorphamine; SAG, smoothened agonist; TGFb3, transforming growth factor beta-3.

4.5 Characterization of iPSC-derived midbrain organoids

To assess the proper region-specificity and progressive maturation of generated midbrain organoids, samples were collected at various time points during differentiation (*i.e.*, 6 DIV, 10 DIV, 14 DIV, 25 DIV, 35 DIV, 50 DIV, 65 DIV, 80 DIV, 100 DIV, 120 DIV, and 150 DIV) for extensive characterization (*i.e.*, gene expression analysis, neuromelanin characterization, membrane lipid composition, and electrophysiological properties).

4.5.1 Transcriptional profiling of midbrain organoids

Bulk RNA-sequencing analysis of MOs at an early (*i.e.*, 6 DIV), intermediate (*i.e.*, 35 DIV), and late time point (*i.e.*, 80 DIV) demonstrated progressive enrichment in neuronal and glial transcripts, as well as in key dopaminergic markers expressed during midbrain development (Sloan et al., 2017; McKenzie et al., 2018) (Figure 4.3a).

Gene ontology enrichment analyses of differentially expressed genes across differentiation (*i.e.*, 6 DIV versus 35 DIV; 6 DIV versus 80 DIV) revealed upregulation of neuronal clusters (*i.e.*, synapse organization, regulation of membrane potential, synaptic vesicle cycle, trans-synaptic signaling, neuron projection morphogenesis) and downregulation of cell proliferation markers (*i.e.*, cell cycle G2/M phase transition, regulation of DNA metabolic process, regulation of chromosome organization, DNA replication, cell division, cell cycle phase transition, cell cycle DNA replication, signal transduction by p53 class mediator, telomere organization, chordate embryonic development) (Figure 4.3b).

The estimated fraction of neurons and astrocytes within MOs based on the expression of cell-specific marker genes (according to the BBrain cell Type specific Gene Expression Analysis

- BRETIGEA described by McKenzie et al., 2018) increased at later time points of analysis (Figure 4.3c).

In differentiating MOs, the level of expression of well-established neuronal-specific and astrocyte-specific genes (15 and 10 genes, respectively) (Cahoy et al., 2008; Sloan et al., 2017) progressively increased at later *in vitro* stages (Figure 4.3d, e).

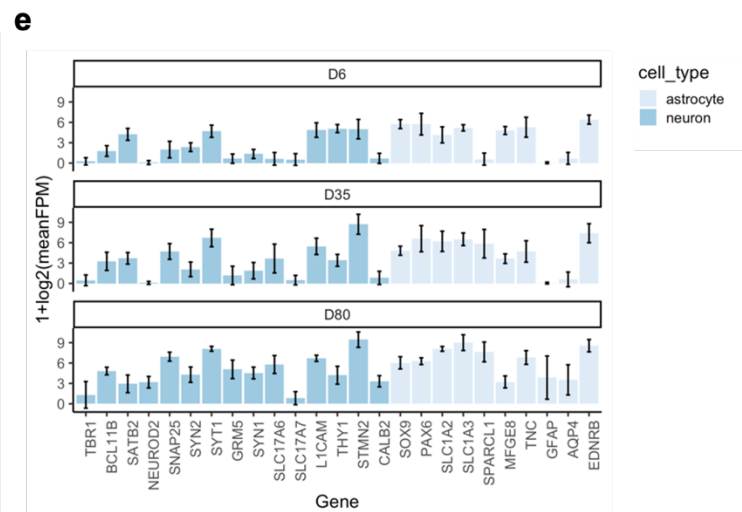
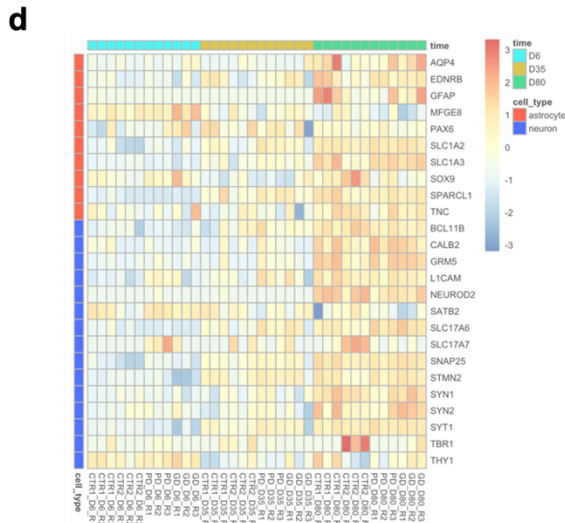
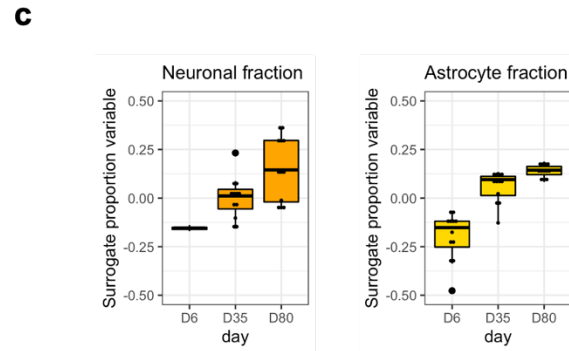
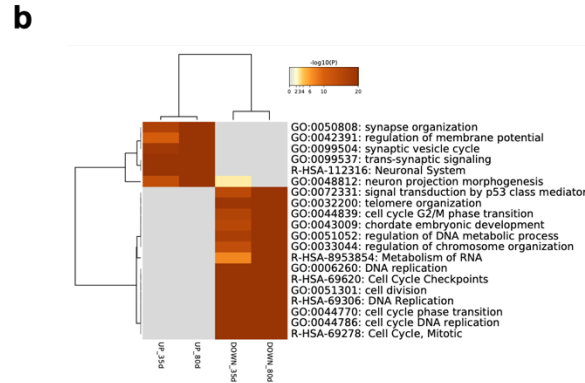
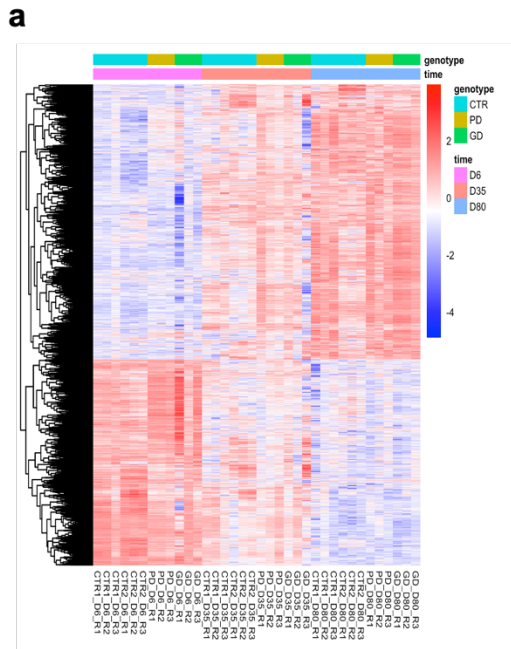


Figure 4.3 – Transcriptional profile of midbrain organoids across differentiation.

- a. Heatmap showing significantly deregulated genes ($FDR \leq 0.01$) in MOs during neuronal differentiation at 6, 35, and 80 DIV. Each row was normalized by z score.
- b. Metascape bar graph showing top non-redundant enrichment clusters for genes differentially expressed ($FDR \leq 0.01$) in MOs at 35 and 80 DIV compared to baseline (*i.e.*, 6 DIV). Enrichment was evaluated separately for upregulated and downregulated genes.
- c. Boxplots representing the estimated fraction of neurons (orange) and astrocytes (gold) within differentiating MOs at 6, 35, and 80 DIV, based on the expression of cell-specific marker genes. Neuronal and astrocyte fractions are expressed as the surrogate proportion variable obtained with the BRETIGEA (BRain cEll Type specific Gene Expression Analysis) R package (McKenzie et al., 2018).
- d. Heatmap showing gene expression of 15 neuronal-specific and 10 astrocyte-specific markers (Sloan et al., 2017) in MOs at 6, 35, and 80 DIV. Each row was normalized by z score.
- e. Histograms showing the mean FPM across all samples for the selected markers. Data are presented as mean $\pm$ s.d.m.

For all experiments shown, $n = 3$ biological replicates per line for each time point.

Abbreviations: CTR^{wt}, control subject; DIV, days *in vitro*; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; MO, midbrain organoid; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation.

More specifically, expression levels of key dopaminergic genes of interest (*i.e.*, tyrosine hydroxylase – TH, dopamine receptor D2 – DRD2, and G-protein-regulated inward-rectifier potassium channel 2 – GIRK2) and of the astrocytic gene glial fibrillary protein (GFAP) increased linearly over time (Figure 4.4a).

Gene expression profiles associated with neurons of the SN and the ventral tegmental area (VTA) were found to be expressed in mature MOs at 80 DIV (Poulin et al., 2014) (Figure 4.4b), indicating that dopaminergic populations of diverse midbrain areas co-exist within mature MOs.

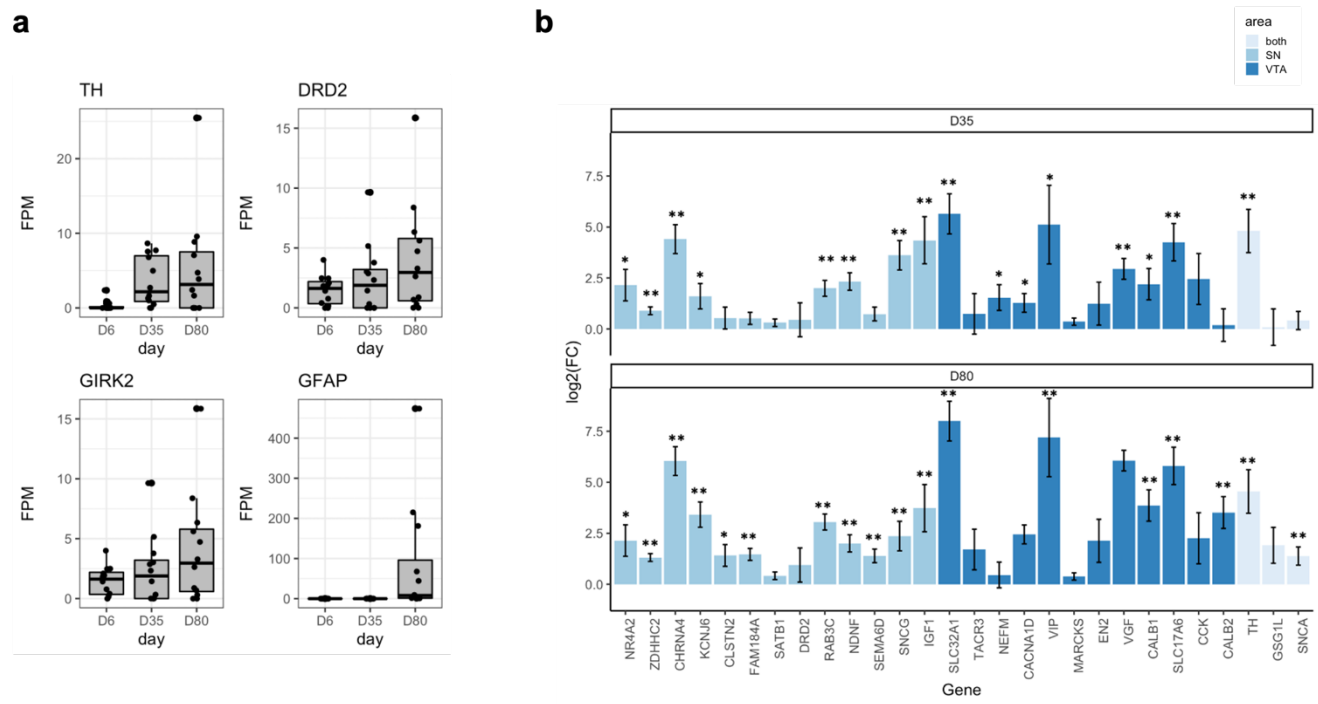


Figure 4.4 – Expression analysis of transcripts of interest during differentiation.

- a. Boxplots showing the gene expression level (expressed in FPM) of selected dopaminergic markers (TH, DRD2, and GIRK2) and astrocytic marker GFAP at 6, 35, and 80 DIV.
- b. Expression of dopaminergic markers in MOs. Histograms represent the log2 fold change difference in gene expression at 35vs6 DIV (top) and 80vs6 DIV (bottom) for markers previously reported to be overexpressed in dopaminergic neurons (Poulin et al., 2014). Differential expression between conditions was performed using contrasts to specify the comparison between the two groups of interest and a Wald test of significance. Error bars represent standard errors of the log2 fold change, as calculated by Deseq2 R package. Asterisks indicate significant p-value for differential gene expression at $FDR \leq 0.05$ (*) or ≤ 0.01 (**), respectively.

For all experiments shown, $n = 3$ biological replicates per line for each time point were analysed.

Abbreviations: DRD2, dopamine receptor D2; GFAP, glial fibrillary acid protein; GIRK2, G-protein-regulated inward-rectifier potassium channel 2; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

4.5.2 Identification of neuronal, dopaminergic, and glial populations

Cells showing neuronal morphology and immunoreactivity for tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthetic pathway of dopamine and other catecholamines, exhibited widespread and extended axonal projections and appeared to preferentially localize in the outer layers of MOs (Figure 4.5a). Immunohistochemical staining of TH showed that the content of dopaminergic neurons in MOs increased during differentiation. At 65 DIV, the calculated mean differentiation efficiency of our protocol was ~ 30% as measured by the co-expression of TH and Neuronal Nuclei (NeuN) (Figure 4.5b, c).

Moreover, MAP2-positive neurons also exhibited immunoreactivity for the G-protein-regulated inward-rectifier potassium channel 2 (GIRK2), which is peculiar to dopaminergic neurons (Reyes et al., 2012) (Figure 4.5d). The TH-positive fibers were found to be partially co-localized by the dopamine transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2)-positive terminals, supporting proper dopaminergic differentiation and synaptic maturation (Figure 4.5e, f).

Non-dopaminergic neurons were detected by immunolabelling for the GABAergic markers GAD65 and GAD67 (Figure 4.5f).

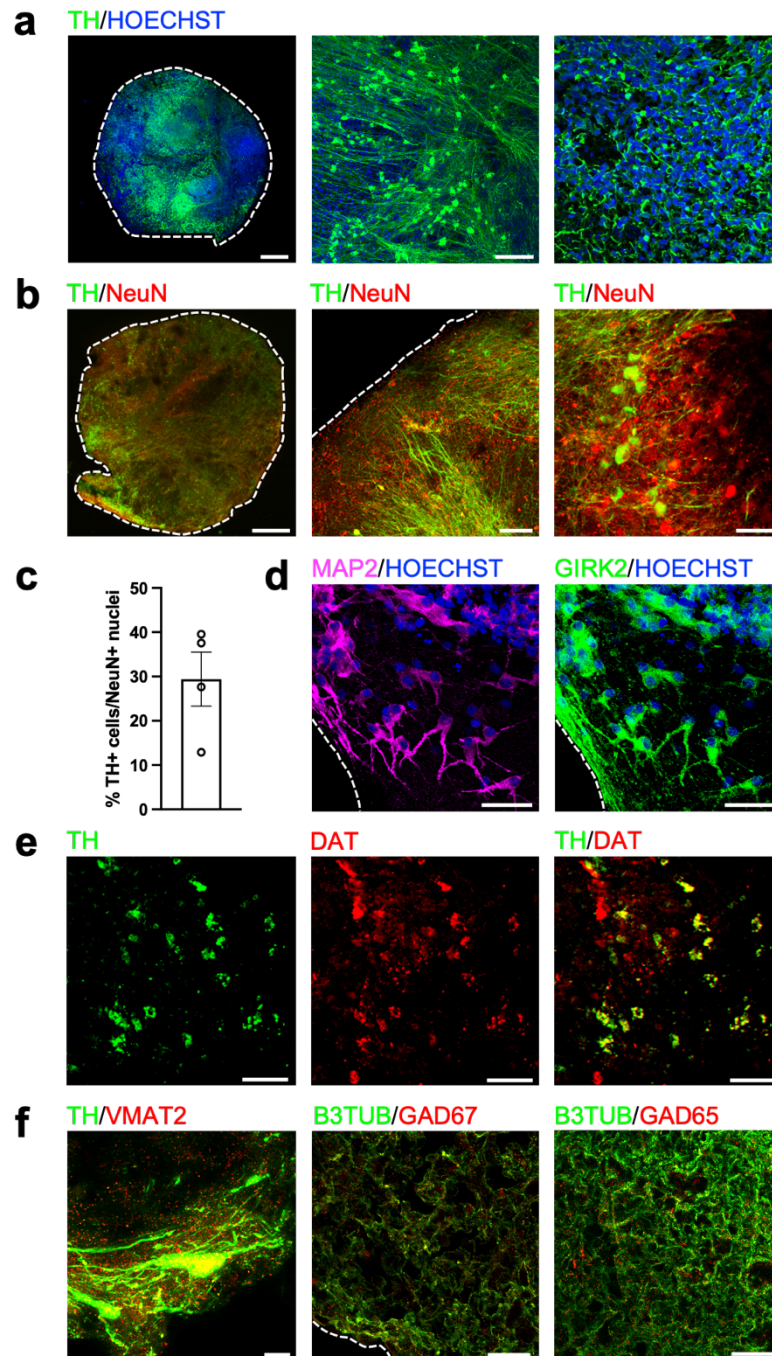


Figure 4.5 – Characterization of neuronal populations in midbrain organoids.

- a. Representative images of TH immunoreactivity in MOs at 65 DIV.
- b. Representative IHC images of MOs showing TH and NeuN expression at different magnifications.
- c. Differentiation protocol efficiency measured as quantification of TH-positive cells over NeuN-positive nuclei in MOs at 65 DIV. Data are presented as mean $\pm$ s.e.m. (n = 4 organoids).
- d-f. Representative IHC images showing co-expression of post-mitotic neuronal marker MAP2 and dopaminergic marker GIRK2 (d), TH and DAT (e), TH and VMAT2 (f left), and immunoreactivity for GABAergic proteins GAD67 and GAD65 in B3TUB-positive neurons (f middle and right).

Scale bars, 10 μ m (f left), 25 μ m (f middle and right), 50 μ m (e, b right), 100 μ m (b middle, a middle), and 500 μ m (a left, b left).

Abbreviations: DAT, dopamine transporter; DIV, days *in vitro*; GAD65/67, glutamic acid decarboxylase 65/67; GIRK2, G-protein-regulated inward-rectifier potassium channel 2; MAP2, microtubule-associated protein 2; NeuN, neuronal nuclei; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2.

Along with neuronal cells, glial elements were progressively specified in MOs during differentiation. Astrocytes were detected by positive staining for GFAP and calcium-binding protein B (S100B) and were abundantly represented, particularly at later time points (Figure 4.6a-c). Sparse oligodendroglial elements showed immunoreactivity for oligodendrocyte transcription factor 2 (OLIG2) and the myelin-associated enzyme 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) (Figure 4.6d, e).

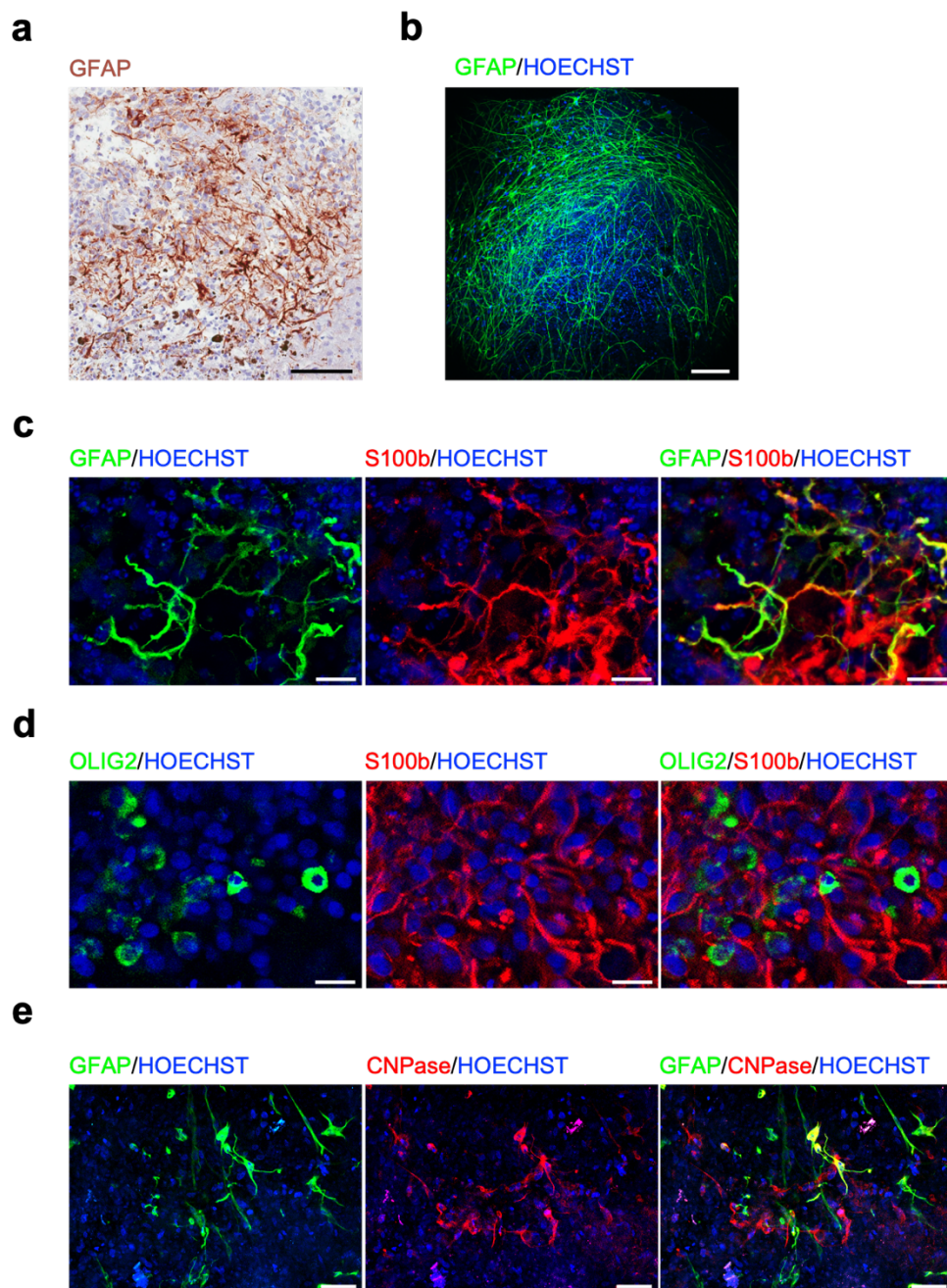


Figure 4.6 – Characterization of glial populations in midbrain organoids.

a, b. Expression of GFAP in glial cells within MOs with DAB (a) and fluorescent (b) immunostaining.

c-e. Representative images demonstrating the expression of astrocytic (GFAP, S100B) and oligodendroglial (OLIG2, CNPase) proteins.

Scale bars, 20 μm (c, d), 50 μm (e), and 100 μm (a, b).

Abbreviations: CNPase, 2',3'-cyclic-nucleotide 3'-phosphodiesterase; DIV, days *in vitro*; GFAP, glial fibrillary protein; OLIG2, oligodendrocyte transcription factor 2; S100b, S100 calcium-binding protein B.

4.5.3 Presence of neuromelanin

As early as 35 DIV, we observed the appearance of pigmentation in the form of granular spots of variable size on the surface of MOs, which progressively increased over time (Figure 4.7a, b). At the last time point analyzed (*i.e.*, 150 DIV), the pigmented area covered ~ 70% of the total surface area of MOs (Figure 4.7c).

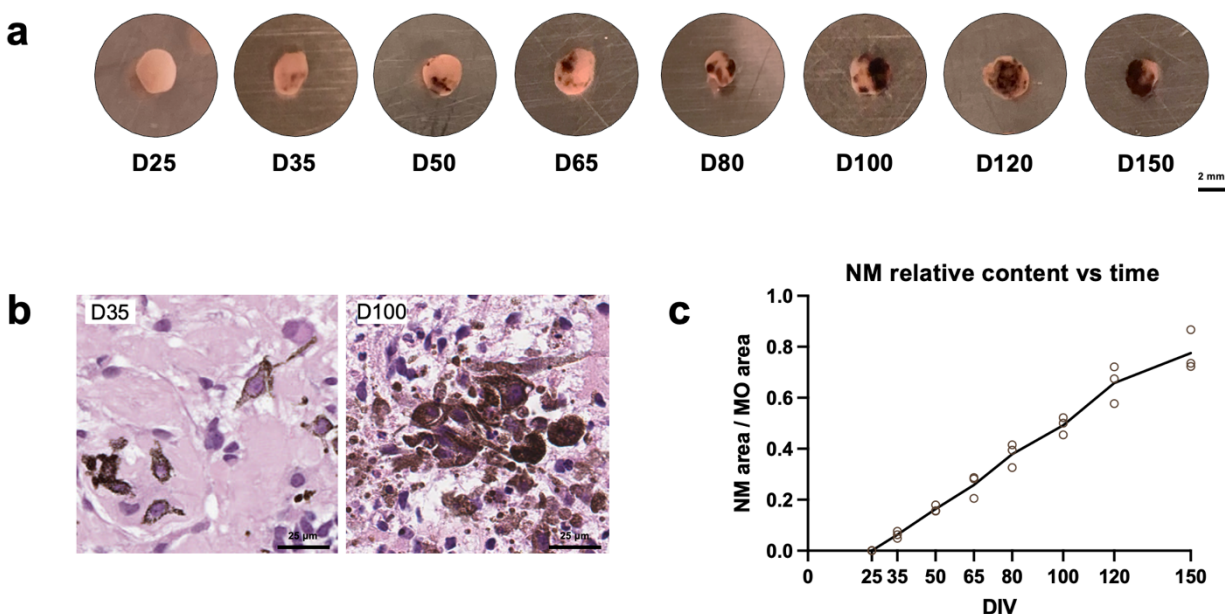


Figure 4.7 – Characterization of neuromelanin (I).

- Representative images of MOs at various maturation stages showing macroscopic evidence of progressive increase in neuromelanin pigmentation during differentiation.
- High magnification images of neurons containing neuromelanin at 35 DIV and 100 DIV.
- Quantification of neuromelanin content measured as ratio of pigmented area over total surface area of MOs at selected time points (n = 3 organoids for each time point).

Scale bars, 25 µm (b) and 2 mm (a).

Abbreviations: DIV, days *in vitro*; MO, midbrain organoid; NM, neuromelanin.

High-magnification brightfield microscopy imaging of hematoxylin-eosin-stained sections showed that the pigment was contained in cells with neuronal morphology (Figure 4.8).

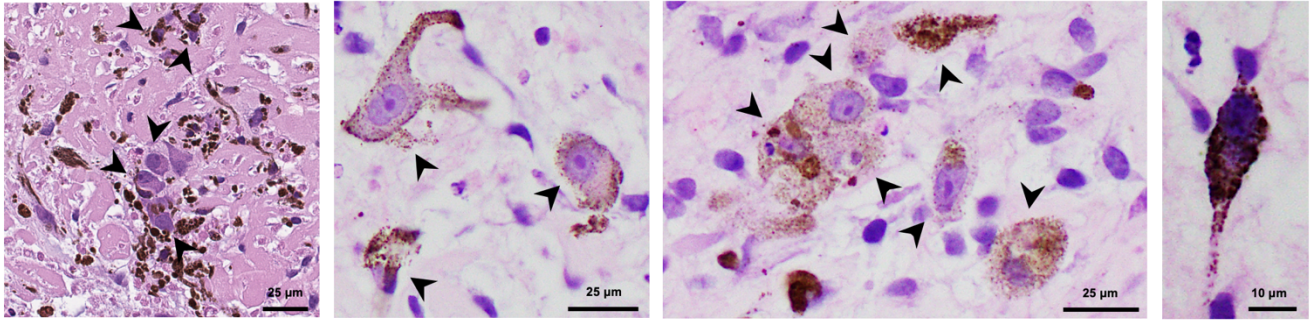


Figure 4.8 – Characterization of neuromelanin (II).

Higher magnification imaging of hematoxylin and eosin-stained sections demonstrates the presence of pigments (*i.e.*, neuromelanin) in neurons at single-cell resolution. Arrowheads indicate neuronal cells with large nuclei and prominent nucleoli and presenting cytoplasmic pigment granules.

Scale bars, 25 μm.

Schmorl's ferricyanide reduction method for melanic pigments detected sites of reduction activity in MOs, which appeared as large, blue-stained granules within the neuronal cytosol, similar to deposits observed in human *post-mortem* SN sections. Melanin sites corresponded to the same pigmented areas already visible in hematoxylin and eosin-stained slides, suggesting that the brown pigment contained within MOs is a type of melanin (Figure 4.9).

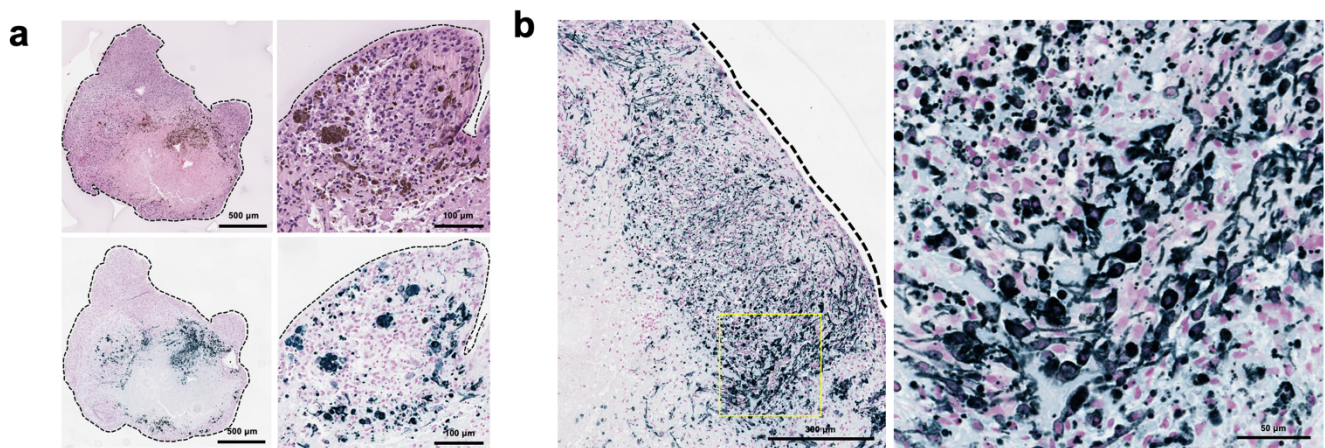


Figure 4.9 – Characterization of neuromelanin (III).

- a. Hematoxylin and eosin staining (upper) and Schmorl's ferricyanide reduction method (lower) highlight the presence of neuromelanin pigmentation within MOs at 120 DIV.
- b. Schmorl's method showing sites of reduction within neuronal cells indicative of melanic pigmentation. Boxed area is expanded in the inset on the right.

Scale bars, 50 µm (b, right), 100 µm (a, right), 300 µm (b, left), 500 µm (a, left) and 2 mm (a).

To differentiate melanin, which already displays a brown pigmentation, from any given staining in DAB immunohistochemistry, we used a red chromogen to label TH. The presence of pigmented granules in the cytoplasm of TH-positive neurons indicated the content of a type of melanin within dopaminergic neurons (Figure 4.10).

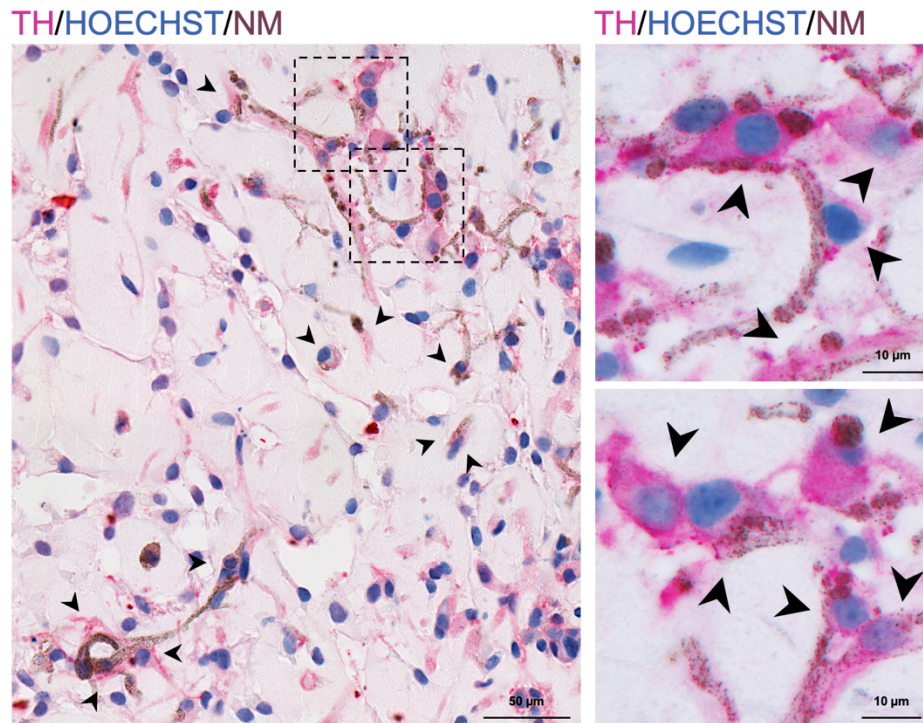


Figure 4.10 – Characterization of neuromelanin (IV).

DAB staining of TH (red). Arrowheads indicate TH-positive cells containing pigmented granules.

Boxed areas are expanded in the insets on the right.

Scale bars, 10 µm (right) and 50 µm (left),).

Abbreviations: NM, neuromelanin; TH, tyrosine hydroxylase.

To better characterize the biosynthetic process that leads to the formation of this type of melanin in dopaminergic neurons within MO, we performed staining for tyrosinase. First, we validated the antibody in a section of a nevus, which showed immunoreactivity for tyrosinase and lack of expression of TH (Figure 4.10). Pigmented neurons in MOs displayed immunostaining of TH, but not of tyrosinase (Figure 4.11), suggesting that the melanin contained in MOs was in fact neuromelanin (Figure 4.11). In line with data reported in the literature (Ikemoto et al., 1997; Tribl et al., 2007) and with our observations in MOs, immunostaining of a substantia nigra section of a healthy donor subject did not detect expression of tyrosinase (Figure 4.11).

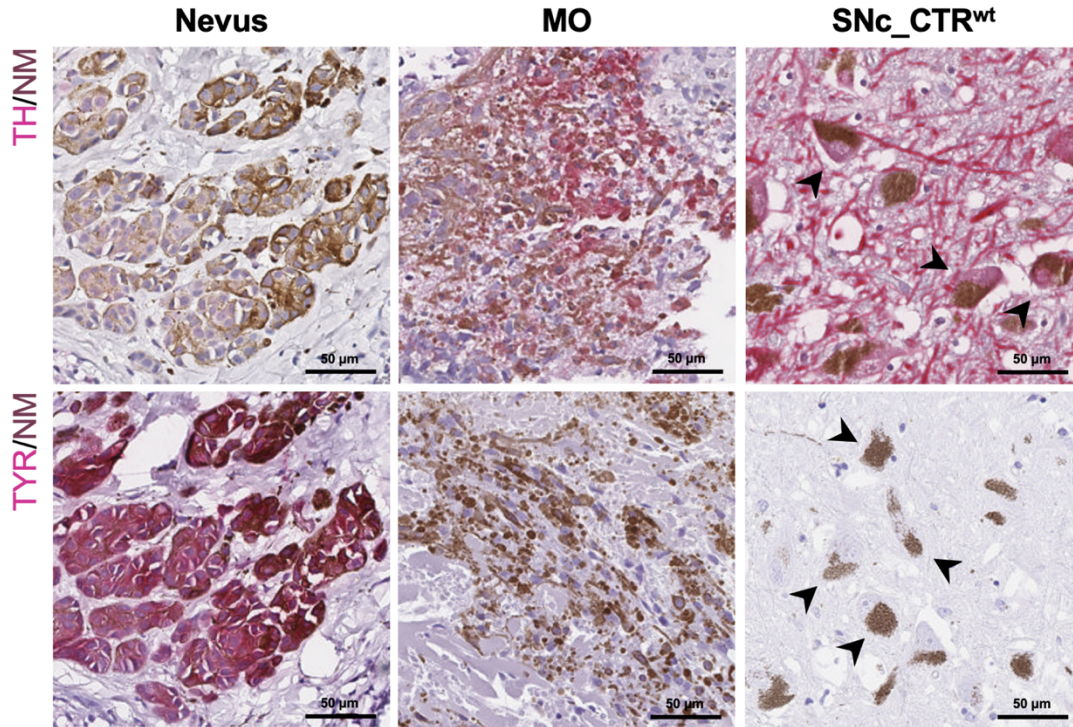


Figure 4.11 – Characterization of neuromelanin (V).

Chromogenic DAB staining of TH (upper) and tyrosinase (lower) in red. The pigment found in MO is abundant in TH-positive neurons and is not stained with tyrosinase, like neuromelanin found in SNc_CTRLwt. Melanin expressed by melanocytes in nevus is immunoreactive for tyrosinase and is expressed in TH-negative cells.

Scale bars, 50 μm.

Abbreviations: MO, midbrain organoid; NM, neuromelanin; SNc_CTRLwt, substantia nigra pars compacta of a healthy donor with wild type *GBA1*; TH, tyrosine hydroxylase; TYR, tyrosine.

Electron dense granules of 2-3 μm in size were detected in the perikaryon of pigmented neuronal cells (Figure 4.12). Some of these presented a membrane and a vacuolar lipid body (Figure 4.12), thereby recapitulating the ultrastructural features of naturally occurring neuromelanin observed in human substantia nigra specimens (Duffy and Tennyson, 1965; Moses et al., 1966; Hirose, 1968).

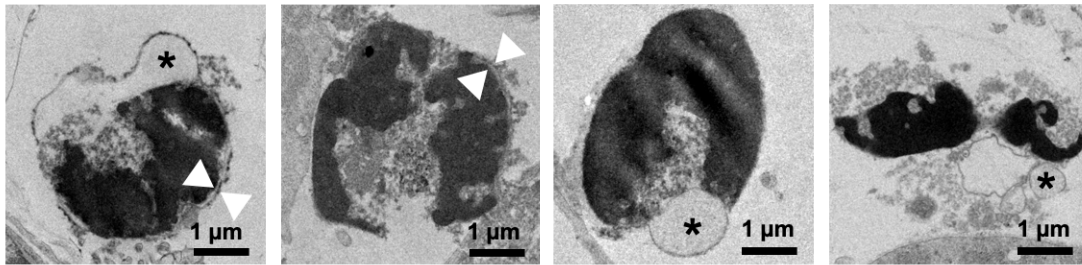


Figure 4.12 – Characterization of neuromelanin (VI).

Transmission electron microscopy images of membrane-bound (arrowheads) pigmented organelles in midbrain organoids containing neuromelanin granules (dark-coloured pigment) and typical vacuolar lipid bodies (asterisks).

Scale bars, 1 μm .

4.5.4 Neuronal membrane lipid profile

Membranes of neurons have a unique lipid composition that differentiates neuronal membranes from all other cell types. Glycosphingolipids (GSLs) – lipid molecules composed of ceramide and one or more carbohydrate units – and gangliosides – sialic acid-containing derivatives of GSLs – are particularly abundant in the nervous system (10 times higher in the brain than in other organs) (Svennerholm et al., 1989). The expression pattern of GSLs, in particular of gangliosides, changes drastically during brain development, increasing in overall content and transitioning from a pattern of neuronal precursors (GD3, GM3) to one of fully mature neurons (GM1, GD1a, GD1b, GT1b) (Ngamukote et al., 2007). As such, changes in membrane lipid composition can be analyzed as a marker of neuronal differentiation.

To investigate whether midbrain organoids recapitulate such developmental changes during *in vitro* differentiation and have a GSL composition of developmentally mature neurons, gangliosides were metabolically labelled at the steady state with the radioactive precursor [1-³H]sphingosine. The GSL pattern was evaluated by high-performance thin-layer chromatography and radioactive lipids were visualized by digital autoradiography.

Over the course of differentiation, the expression pattern of GSLs changed significantly, resembling the profile observed during neuronal development *in vivo*. Specifically, early-stage MOs presented high content of GM3 and lower amounts of GM2, GM1 and GD1a components, whereas fully differentiated MOs at 65 DIV were enriched in all the polysialogangliosides found in mature neurons of the adult brain (*i.e.*, GM1, GD1a, GD1b, GQ, and GT1b) as well as in sphingomyelin, signifying their mature identity (Figure 4.13). Levels of GM3 and GD3 were still detected, suggesting the presence of non-neuronal cell types and proliferating neural stem cells, respectively.

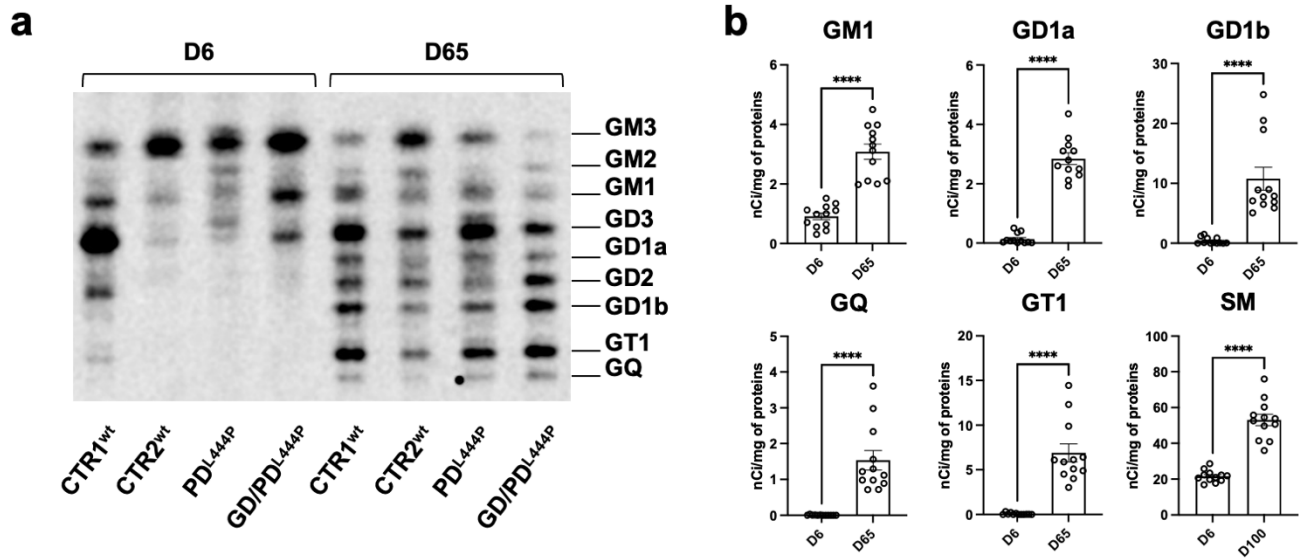


Figure 4.13 – Membrane lipid composition.

- Representative digital autoradiography of the ganglioside composition of MOs. Ganglioside pattern was evaluated at 6 and 65 DIV by the metabolic labelling at the steady state using [1-³H]sphingosine.
- Sphingolipid composition of MOs at early (6 DIV) and mature (65 or 100 DIV) time points. Fully differentiated MOs have a higher content of gangliosides and sphingomyelin enriched in human adult brain. Data are presented as mean $\pm$ s.e.m. ($n = 12$ organoids for each time point; **** $P < 0.0001$, two-tailed unpaired Student's t-test).

Abbreviations: CTR^{wt}, control subject; DIV, days *in vitro*; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation. PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; SM, sphingomyelin.

4.5.5 Calcium activity imaging

In neurons, intracellular calcium plays a critical role in induction of synaptic activity and activation of signaling pathways. In order to assess the presence of spontaneous activity in neurons within MOs, functional imaging of Ca^{2+} was performed. Samples were loaded with a Ca^{2+} indicator (*i.e.*, a molecule exhibiting an increase in fluorescence upon binding calcium), and Ca^{2+} activity was imaged with one-photon excitation microscopy.

In whole MOs, the presence of fast Ca^{2+} transients (< 1 second in duration) was consistent with cellular activity *in vitro*, which was recorded both in basal conditions or upon incubation with either glutamic acid or dopamine (Figure 4.14). In order to image Ca^{2+} events at a single-cell resolution, MOs were enzymatically dissociated into bidimensional cultures. Individual cells with typical neuronal morphology exhibited spontaneous Ca^{2+} surges of longer duration (~ 5 seconds) and lower firing rate, suggesting that a three-dimensional organization may contribute to neural activity complexity. Action potential blockade by administration of tetrodotoxin resulted in suppression of Ca^{2+} spikes, indicating that Ca^{2+} events observed in MOs depend on Na^+ channel functional integrity.

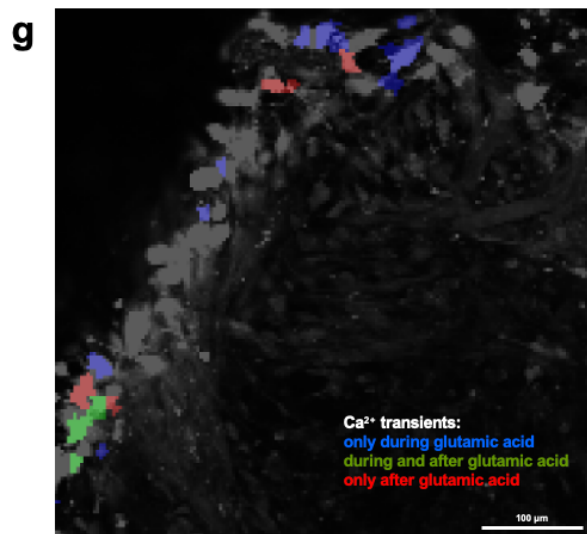
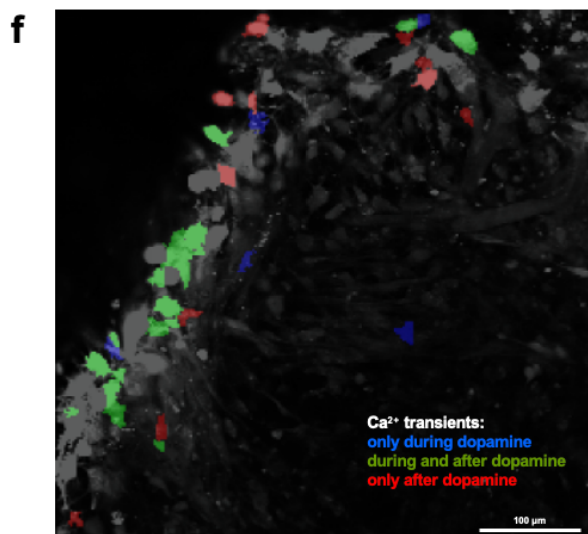
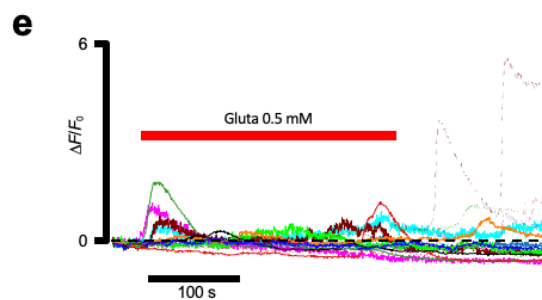
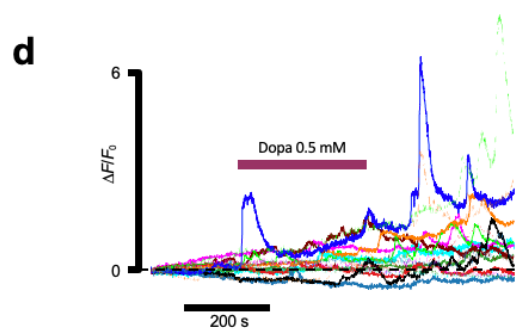
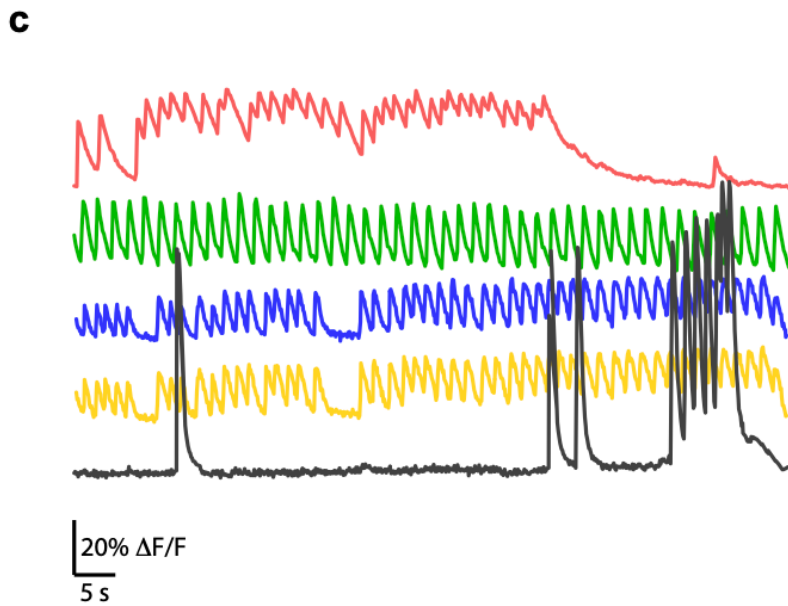
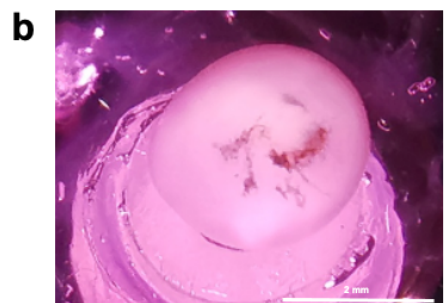


Figure 4.14 – Ca²⁺ imaging in living organoids.

- a, b. Experimental setup for calcium imaging. (a) One CTR^{wt} MO at 147 DIV is held at the center of the perfusion chamber by gentle suction of a small tube positioned below. A two-photon upright microscope equipped with a 25X objective (WI, NA 1.05) is utilized for imaging experiments. (b) Abundant neuromelanin pigmentation is visible on the outer surface of the sample.
- c. Ca²⁺ imaging traces of cells expressing Fluo-4 AM showing spontaneous calcium transients indicative of intrinsic cellular activity in MOs.
- d, e. Representative FluoForte $\Delta F/F_0$ traces indicate a heterogeneous Ca²⁺ dynamics among cells within MOs perfused with extracellular saline solution containing either 0.5 mM dopamine (d) or 0.5 mM glutamic acid (e).
- f, g. Cells exhibiting Ca²⁺ transients during the experiments in (d) and (e) were divided into three color-mapped categories, according to whether they showed Ca²⁺ activity only during incubation with dopamine/glutamic acid (blue), only after incubation with dopamine/glutamic acid (red), or both during and after incubation with dopamine/glutamate (green).

Scale bars, 100 μ m (f, g) and 2 mm (b).

Abbreviations: CTR^{wt}, control subject; DIV, days *in vitro*; MO, midbrain organoid.

4.6 Study of glucocerebrosidase and glycosphingolipid accumulation

In Gaucher disease, the presence of *GBA1* mutations is invariably associated with a reduction in GCase enzyme activity and subsequent intracellular accumulation of GlcCer in macrophages (*i.e.*, Gaucher cells) and other cell types, including neurons (Liou et al., 2006; Grabowski, 2008). Reduced GCase activity has been reported in brains of *GBA1*-related PD and sporadic PD patients without *GBA1* mutations (Gegg et al., 2012; Rocha et al., 2015; Murphy et al., 2014; Huebecker et al., 2019). However, whether GCase deficiency leads to substrate accumulation in PD is controversial, with contrasting evidence on GlcCer levels in brains from patients (Huebecker et al., 2019; Gegg et al., 2015; Boutin et al., 2016).

Along with primary accumulation of GlcCer, GD type 2 brains also show increased amount of its lysolipid form, glucosylsphingosine (GlcSph), derived from enzymatic deacetylation of GlcCer (Orvisky et al., 2000). GlcSph has also been implicated in mediating a toxic effect and to be directly involved in α -synuclein pathology in *GBA1*-related PD (Taguchi et al., 2007). However, the evidence of accumulation of GlcSph in *GBA1*-mutated PD is less clear.

4.6.1 Glucocerebrosidase enzymatic activity assay

In order to establish a pharmacological model of GCase deficiency, CTR^{wt} MOs were treated with the GCase irreversible inhibitor Conduritol B Epoxide (CBE), which resulted in suppression of GCase activity (Figure 4.15a). Similarly, an almost undetectable GCase enzymatic activity was observed in GD/PD^{L444P} MOs (Figure 4.15b). In PD^{L444P} MOs, we observed a reduction of approximately 50% in GCase activity (Figure 4.15b), in line with that of the SNc_PD^{L444P} (Figure 4.15c).

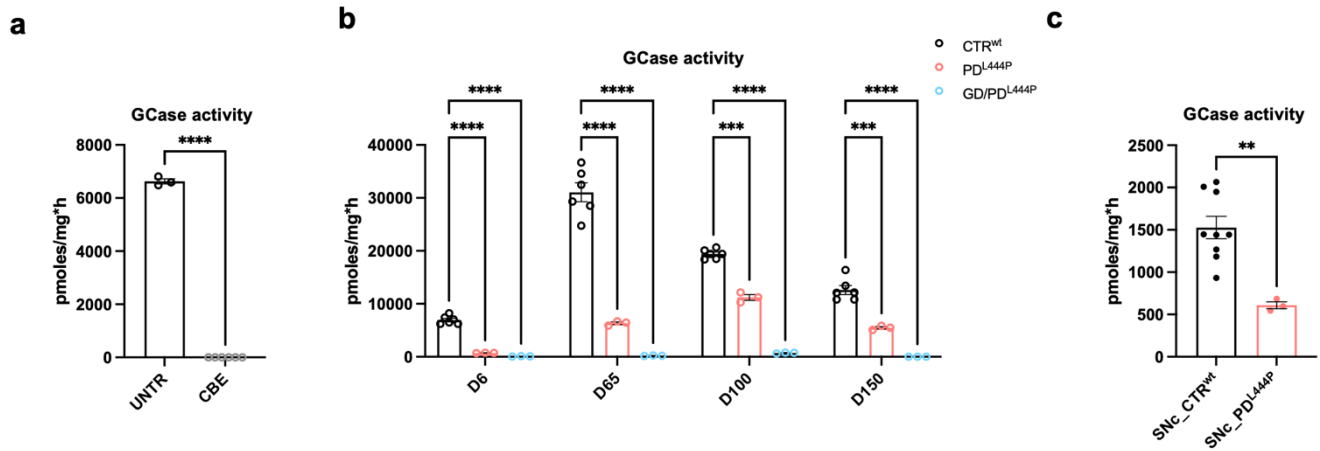


Figure 4.15 – Assessment of glucocerebrosidase activity.

- GCase enzymatic activity in CTR^{wt} MOs at 65 DIV with and without treatment with 500 μ M CBE for 14 days. Data are presented as mean $\pm$ s.e.m. ($n = 9$ organoids; **** $P < 0.0001$, two-tailed unpaired Student's t-test).
- GCase enzymatic activity in MOs at 6, 65, 100, and 150 DIV. Data are presented as mean $\pm$ s.e.m. ($n = 3$ biological replicates per line for each time point; **** $P < 0.0001$, *** $P < 0.001$, two-way ANOVA with Tukey's post hoc test).
- GCase enzymatic activity in SNc_CTR^{wt} and SNc_PD^{L444P} brain extracts. Data are presented as mean $\pm$ s.e.m. ($n = 12$ samples; ** $P < 0.01$, two-tailed unpaired Student's t-test).

Abbreviations: CBE, conduritol B epoxide; CTR^{wt}, control subject; DIV, days *in vitro*; GCase, glucocerebrosidase; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; SNc_CTR^{wt}, substantia nigra from *post-mortem* brain of a healthy donor; SNc_PD^{L444P}, substantia nigra from *post-mortem* brain of Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; UNTR, untreated.

4.6.2 Glucocerebrosidase protein amount

SNc_PD^{L444P} displayed a reduction in GCase protein amount, which was reproduced in PD^{L444P} MOs at 100 and 150 DIV (Figure 4.16a-d). GCase protein content was also reduced in GD/PD^{L444P} MOs and in the CTR^{wt} line treated with CBE (Figure 4.16a, b, e, f).

4.6.3 Glucosylceramide accumulation

We explored whether the deficiency of GCase may lead to substrate accumulation. By metabolically labelling glycosphingolipids with [1-³H]sphingosine, we observed a significant accumulation of GlcCer in CBE-treated CTR^{wt} and in GD/PD^{L444P} MOs starting from 65 DIV (Figure 4.17). Of particular interest, PD^{L444P} MOs also accumulated GlcCer starting from 100 DIV.

4.6.4 Glucosylsphingosine accumulation

We performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify the levels of GlcSph, the metabolic intermediate that results from the deacetylation of GlcCer, which is known to be a primary storage lipid in the context of GCase depletion (Nillsen and Svennerholm, 1982) and has been implicated in α -synuclein pathology in mutant *GBA1*-associated PD (Taguchi et al., 2007). We found GlcSph accumulation in MOs treated with CBE and in GD/PD^{L444P} MOs (Figure 4.18).

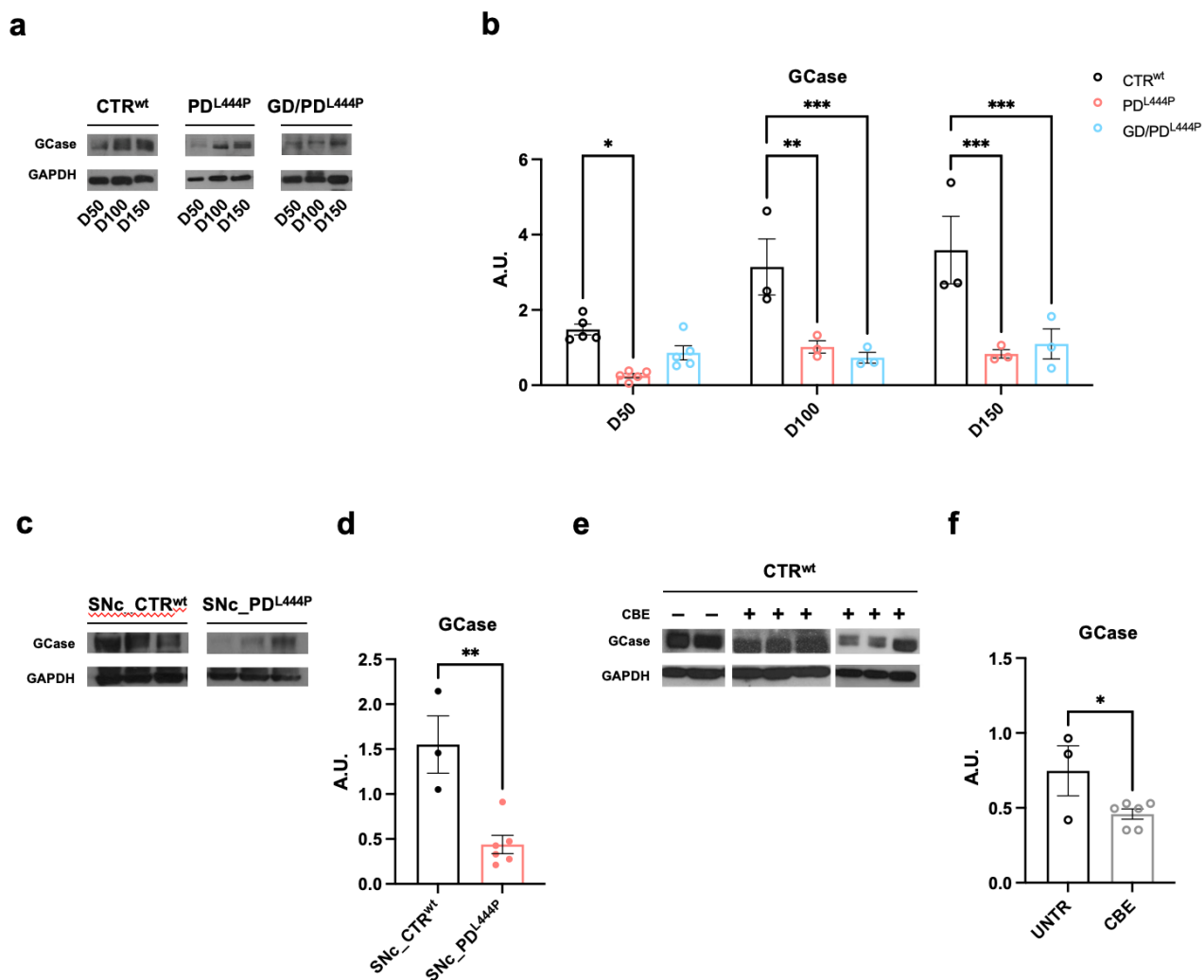


Figure 4.16 – Assessment of glucocerebrosidase protein levels.

a, b. Representative western blots and relative densitometries showing GCase in MOs at 50, 100, and 150 DIV. Data are presented as mean $\pm$ s.e.m. (n = 33 organoids; ***P < 0.001, **P < 0.01, *P < 0.05, two-way ANOVA with Tukey's post hoc test).

c, d. Western blots and relative densitometry of GCase and GAPDH amount in CTR^{wt} MOs at 65 DIV with and without treatment with CBE 500 μ M for 14 days. Data are presented as mean $\pm$ s.e.m. (n = 9 organoids; *P < 0.05, two-tailed unpaired Student's t-test).

e, f. Representative western blots and relative densitometries showing GCase in patients' brain extracts. Data are presented as mean $\pm$ s.e.m. (n = 9 samples; **P < 0.01, two-tailed unpaired Student's t-test).

Abbreviations: see Figure 4.15.

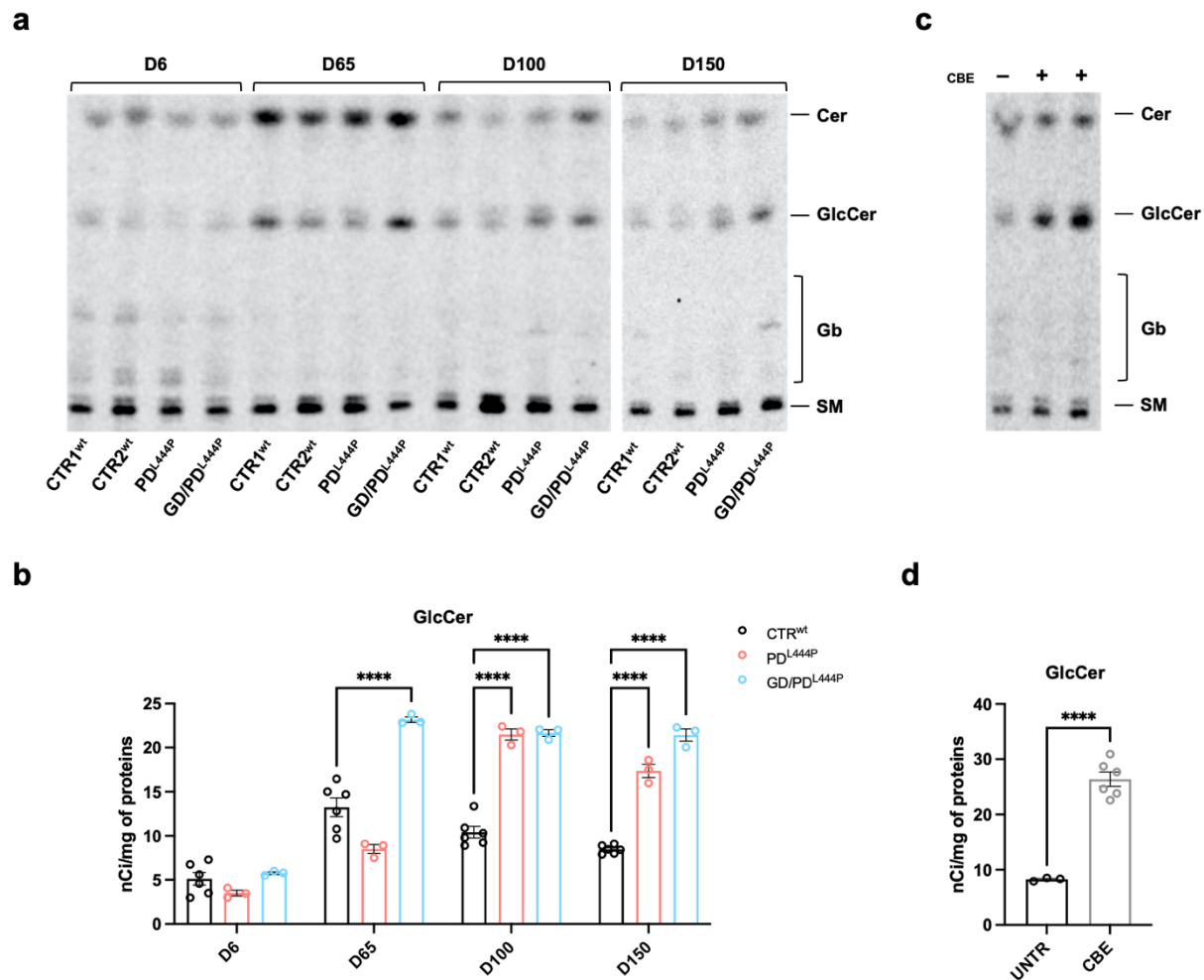


Figure 4.17 – Assessment of glucosylceramide levels.

a-d. Representative digital autoradiography (a, c) and relative densitometry (b, d) of the neutral sphingolipids' composition of MOs at 6, 65, 100, and 150 DIV (a, b) and following CBE treatment (c, d) evaluated by the metabolic labelling at the steady state using radioactive sphingosine. In (b), data are presented as mean $\pm$ s.e.m. ($n = 3$ biological replicates per line for each time point; **** $P < 0.0001$, two-way ANOVA with Tukey's post hoc test). In (d), data are presented as mean $\pm$ s.e.m. ($n = 9$ organoids; **** $P < 0.0001$, two-tailed unpaired Student's t -test).

Abbreviations: CBE, conduritol B epoxide; Cer, ceramide; CTR^{wt}, control subject; DIV, days *in vitro*; Gb, globosides; GlcCer, glucosylceramide; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; SM: Sphingomyelin; UNTR, untreated.

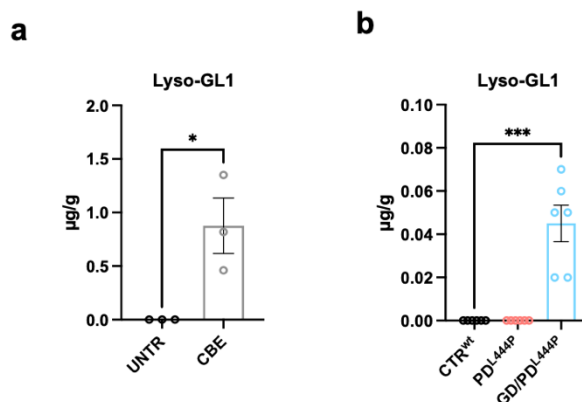


Figure 4.18 – Assessment of glucosylsphingosine levels.

- Lyso-GL1 content in CTR^{wt} MOs at 65 DIV with and without treatment with 500 μM CBE for 14 days evaluated by LC-MS/MS. Data are presented as mean $\pm$ s.e.m. ($n = 3$ organoids for each condition; * $P < 0.05$, two-tailed unpaired Student's t-test).
- Lyso-GL1 content in MOs at 65 DIV evaluated by LC-MS/MS. Data are presented as mean $\pm$ s.e.m. ($n = 6$ organoids for each line; *** $P < 0.001$, two-way ANOVA with Dunnett's post hoc test).

Abbreviations: CBE, conduritol B epoxide; CTR^{wt}, control subject; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; LC-MS/MS, liquid chromatography and tandem mass spectrometry; Lyso-GL1, glucosylsphingosine; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; UNTR, untreated.

4.7 Study of α -synuclein pathology

Along with the degeneration of dopaminergic neurons in the substantia nigra, the accumulation of insoluble α -syn in LBs and LNs constitutes the neuropathological signature of PD. α -syn pathology has also been found in brains of GD patients with or without PD (Tayebi et al., 2003; Wong et al., 2004) and in experimental models of GCase deficiency (Sardi et al., 2011; Xu et al., 2011; Rocha et al., 2015; Sardi et al., 2013; Migdalska-Richards et al., 2017), suggesting a relationship between GCase insufficiency and protein aggregation.

In *GBA1*-mutated PD, it has been hypothesized that GlcCer accumulation in the lysosome, due to GCase deficiency, accelerates and stabilizes soluble α -syn oligomers, which eventually convert into amyloid fibrils (Mazzulli et al., 2011). Conversely, accumulation of α -syn blocks the trafficking of GCase from the endoplasmic reticulum to the Golgi and, from here, to the lysosome, further amplifying GlcCer accumulation and aggregation of α -syn oligomers (Mazzulli et al., 2011).

4.7.1 Assessment of total α -synuclein levels

In line with data on *post-mortem* brains linking GCase deficiency with α -syn pathology, we observed that CBE-induced inhibition of GCase activity led to accumulation of total α -syn in CTR^{wt} MOs (Figure 4.19a, b). PD^{L444P} MOs exhibited a progressive increase in total α -syn content (Figure 4.19e, f), thus reproducing the accumulation of α -syn in SNc_PDL444P compared with SNc_CTRwt (Figure 4.19c, d). Moreover, the analysis of α -syn immunolabelling in TH-positive cells of PD^{L444P} and GD/PD^{L444P} MOs showed an incremental accumulation of the protein over time specifically in dopaminergic neurons (Figure 4.19g, h).

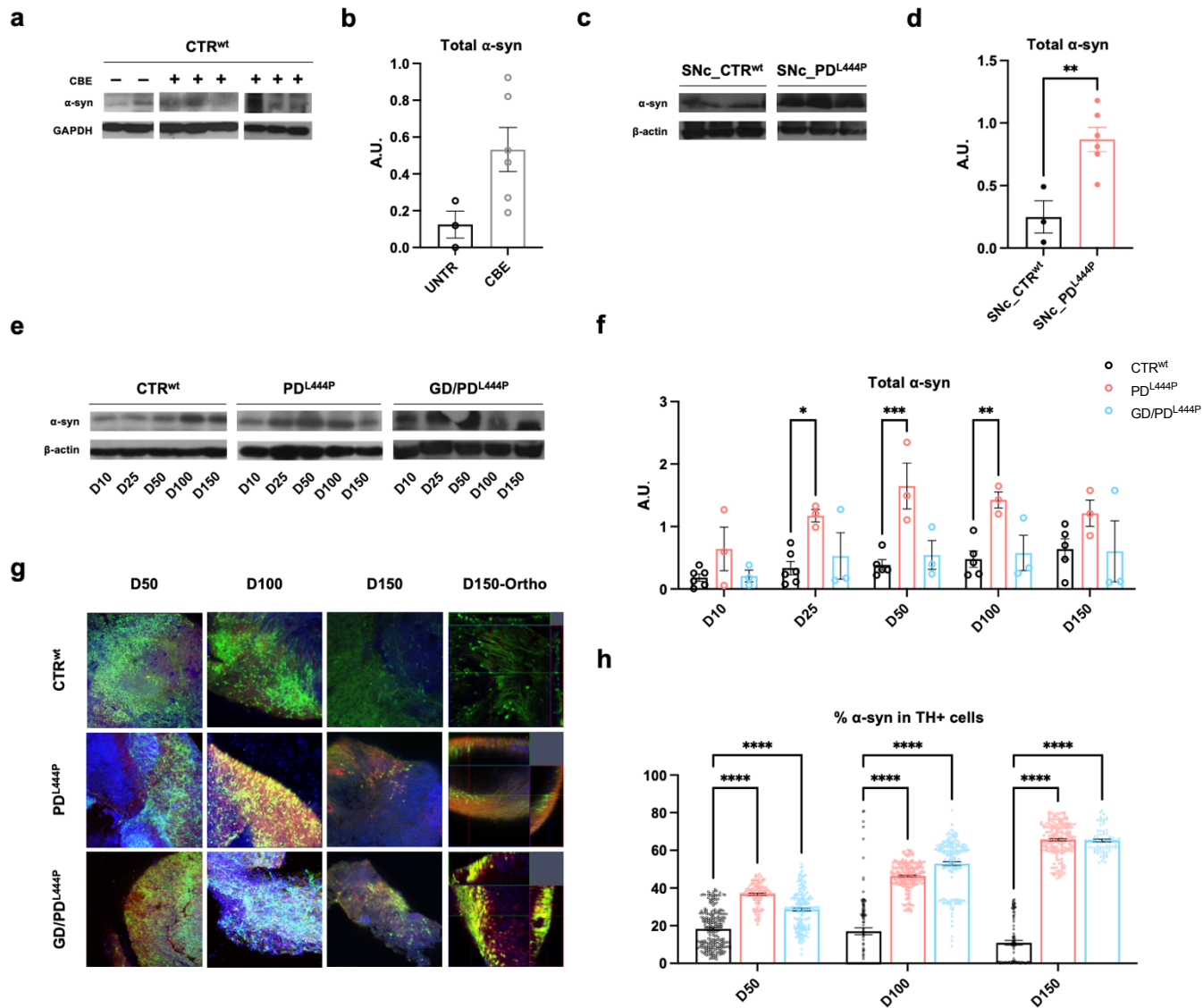


Figure 4.19 – Evaluation of total α -synuclein.

- a, b. Western blots and relative densitometry of total α -syn and GAPDH amount in CTR^{wt} MOs with and without treatment with CBE (500 μ M for 14 days). Data are presented as mean $\pm$ s.e.m. (n = 3 organoids per condition; *P < 0.05, two-tailed unpaired Student's t-test).
- c, d. Western blots and relative densitometry of total α -syn and GAPDH levels in SNc_CTR^{wt} and in SNc_PD^{L444P} substantia nigra from *post-mortem* brains. Data are presented as mean $\pm$ s.e.m. (n = 9 organoids; **P < 0.01, two-tailed unpaired Student's t-test).
- e, f. Representative western blots and relative densitometry of total α -syn and GAPDH in CTR^{wt}, PD^{L444P}, and GD/PD^{L444P} MOs at 10, 25, 50, 100, and 150 DIV. Data are presented as mean $\pm$ s.e.m. (n = 57 organoids; *P < 0.05, **P < 0.01, ***P < 0.001, two-way ANOVA).
- g, h. Representative IHC images showing the expression of α -syn and TH in MOs at 50, 100, and 150 DIV (g) and relative analysis of percentage of α -syn in TH-positive neurons showing progressive accumulation over time in *GBA1* mutant MOs (h). Data are presented as mean $\pm$ s.e.m. (****P < 0.0001, one-way ANOVA).

Abbreviations: α -syn, α -synuclein; CTR^{wt}, control subject; DIV, days *in vitro*; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; SNc_CTR^{wt}, substantia nigra from *post-mortem* brain of a healthy donor; SNc_PD^{L444P}, substantia nigra from *post-mortem* brain of Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; TH, tyrosine hydroxylase.

4.7.2 Study of insoluble α -synuclein

Detergent-insoluble α -syn was detected in PD^{L444P} and GD/PD^{L444P} MOs, as well as in CTR^{wt} MOs exclusively after exposure to CBE (Figure 4.20a-d).

Transmission electron microscopy analysis of the insoluble α -syn fraction extracted from MOs confirmed the presence of amyloid fibrils in PD^{L444P} and GD/PD^{L444P} MOs, that were not found in CTR^{wt} MOs (Figure 4.20e, f).

4.7.3 Immunostaining of α -synuclein aggregates

Following proteinase K-mediated digestion of soluble proteins, fluorescent immunolabelling of α -syn with an antibody developed against fibrillary components of LBs (BioLegend, 847902) showed the presence of several insoluble Lewy-like deposits with different morphology in patients' MOs (Figure 4.21a). Similar structures were detected in MOs with DAB immunohistochemistry, resembling the neuropathology of the donor patient's brain (Figure 4.21b). The observation of α -syn-immunopositive deposits at higher magnification showed morphological differences of aggregates that may be ascribed to diverse maturation stages, ranging from early diffuse cytoplasmic staining to discrete and packed inclusions, reminiscent of mature LBs (Figure 4.21a, c). Remarkably, both PD^{L444P} and GD/PD^{L444P} MOs also exhibited aggregated α -syn-immunoreactive neuronal processes, which were suggestive of LNs (Figure 4.21d).

In line with these observations, Thioflavin-S staining confirmed the presence of fibrillary aggregated α -syn within TH-positive cells in CTR^{wt} MOs treated with CBE and in patients' MOs (Figure 4.21e-g).

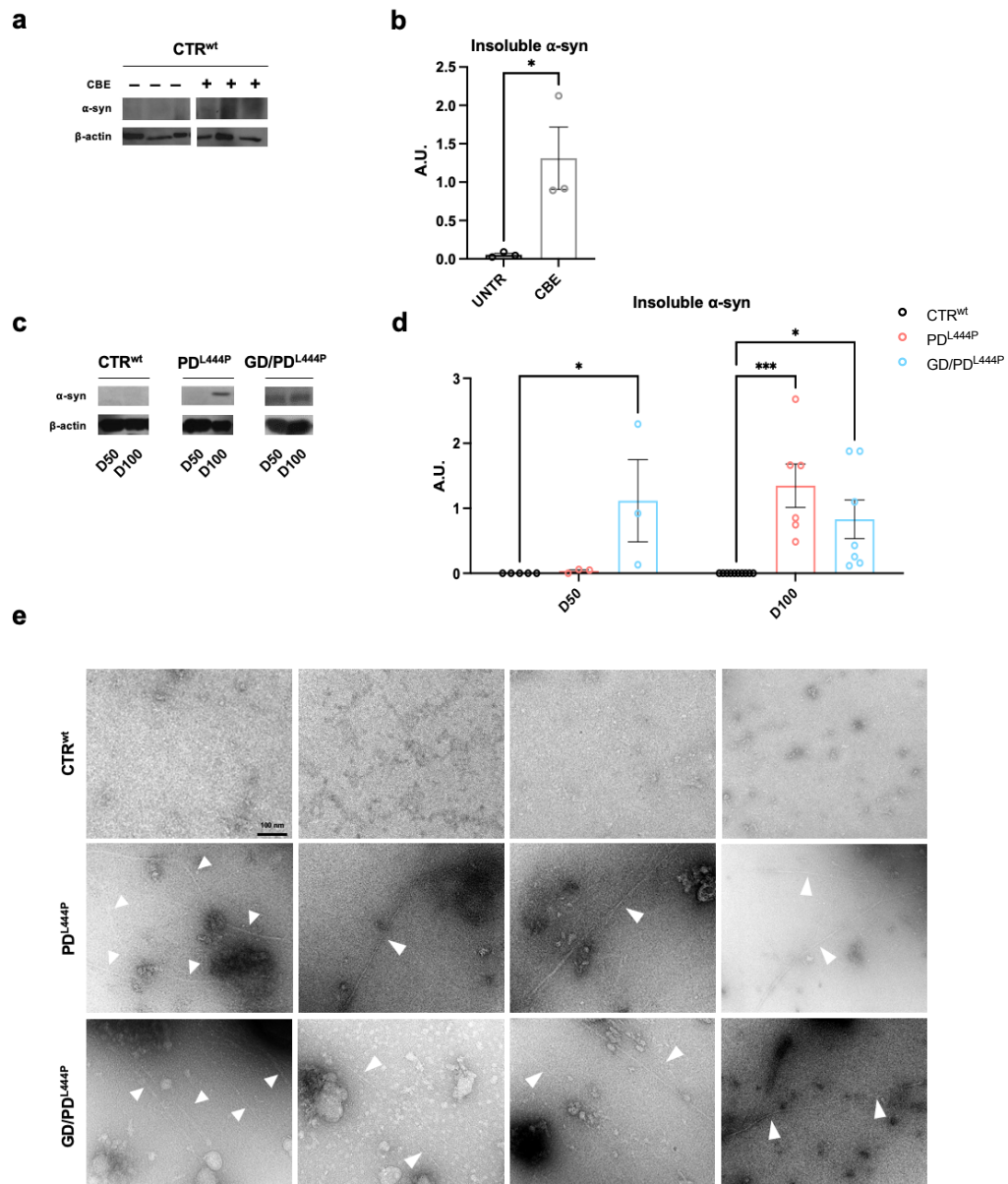


Figure 4.20 – Evaluation of insoluble α -synuclein.

- a, b. Western blots and relative densitometry of detergent-resistant α -syn and β -actin amount in CTR^{wt} MOs with and without treatment with CBE (500 μ M for 14 days). Data are presented as mean $\pm$ s.e.m. (n = 3 organoids per condition; *P < 0.05, two-tailed unpaired Student's t-test).
- c, d. Representative western blots and relative densitometry of detergent-resistant insoluble α -syn fractions in CTR^{wt}, PD^{L444P}, and GD/PD^{L444P} MOs at 50 and 100 DIV. Data are presented as mean $\pm$ s.e.m. (n = 33 organoids; *P < 0.05, ***P < 0.001, two-way ANOVA).
- e, f. Representative transmission electron microscopy images of α -syn amyloid fibrils extracted from PD^{L444P} and GD/PD^{L444P} MOs (white arrowheads). In contrast, no fibrils were detected in CTR^{wt} MOs.

Scale bar, 100 nm.

Abbreviations: α -syn, α -synuclein; CTR^{wt}, control subject; DIV, days *in vitro*; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation.

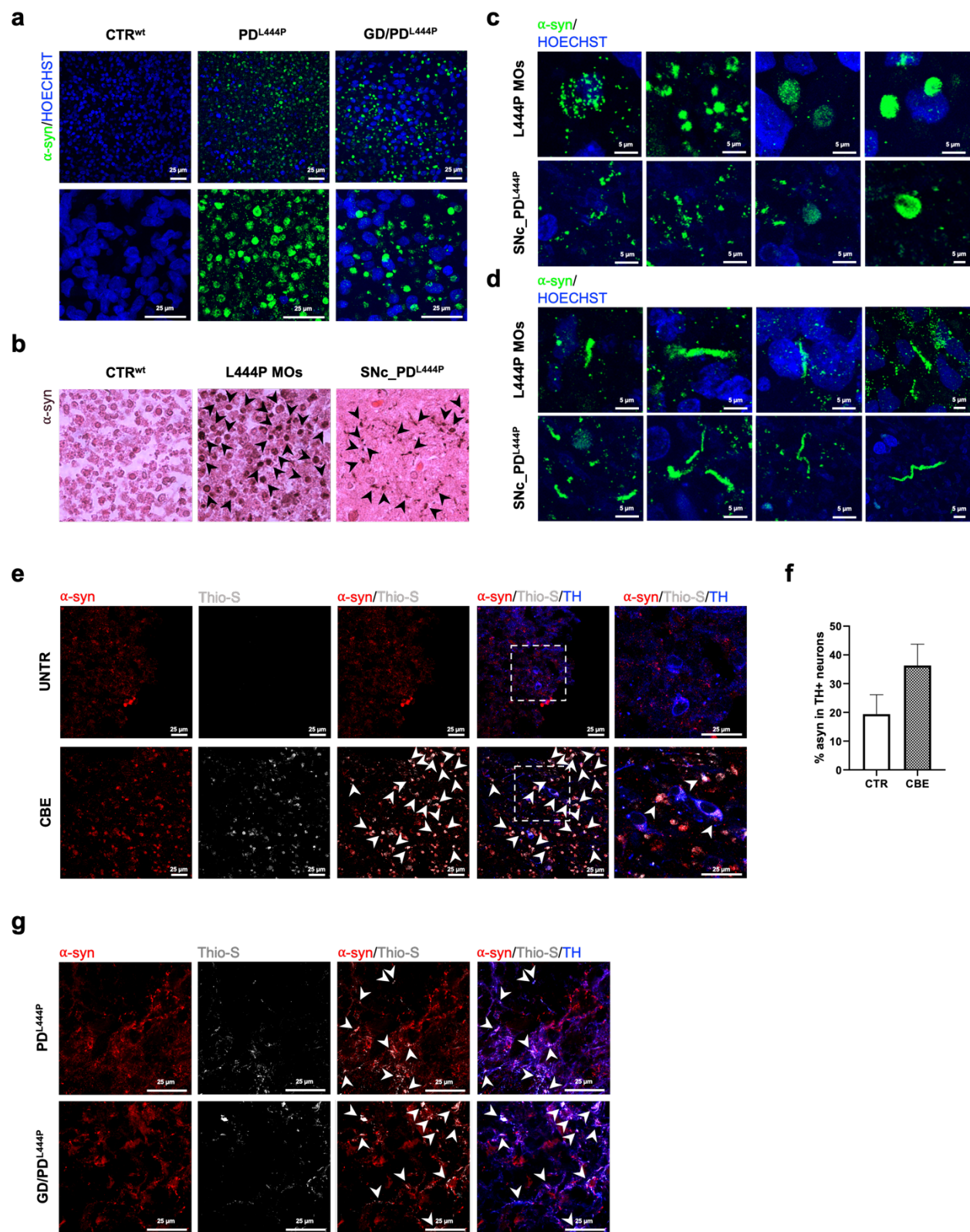


Figure 4.21 – Immunostaining of Lewy-like pathology.

- a. Representative IHC images of proteinase K-resistant insoluble α -syn immunoreactivity in MOs at 100 DIV.
- b. DAB immunoreactivity of α -syn in eosin-stained MOs at 100 DIV following treatment with proteinase K. Arrowheads indicate proteinase K-resistant inclusions of insoluble α -syn.
- c, d. High magnification images of insoluble α -syn organization in MOs from subjects carrying L444P *GBA1* mutations at 100 DIV and in SNc_PD^{L444P} substantia nigra from *post-mortem* brain: heterogeneous morphology of α -syn inclusions may be indicative of various stages of maturation of LB-like aggregates (c) and LN-like processes (d).
- e. Representative IHC images of α -syn and Thioflavin S-labelled aggregates in TH-positive neurons in CTR^{wt} MOs with and without treatment with CBE. Arrowheads indicate inclusions of fibrillary α -syn with double staining for α -syn and Thioflavin-S.
- f. Analysis of the percentage of α -syn in TH-positive neurons in CTR^{wt} MOs following CBE treatment from data shown in (e).
- g. Representative IHC images of α -syn and Thioflavin S-labelled aggregates in TH-positive neurons in PD^{L444P} and GD/PD^{L444P} MOs at 120 DIV. Arrowheads indicate inclusions of fibrillary α -syn with double staining for α -syn and Thioflavin-S.

Scale bars, 5 μ m (c, d) and 25 μ m (a, e, g).

Abbreviations: α -syn, α -synuclein; CTR^{wt}, control subject; DIV, days *in vitro*; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; LB, Lewy body; LN, Lewy neurite; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; SNc_CTR^{wt}, substantia nigra from *post-mortem* brains of healthy donor; SNc_PD^{L444P}, substantia nigra from *post-mortem* brain of Parkinson's disease patient carrying heterozygous L444P *GBA* mutation; TH, tyrosine hydroxylase; Thio-S, Thioflavin-S.

4.7.4 Study of α -synuclein fibrils seeding properties

The real-time quaking induced conversion (RT-QuIC) assay for ultrasensitive detection of aggregated α -syn demonstrated an efficient seeding activity for recombinant α -syn induced by PD^{L444P} and GD/PD^{L444P} MOs (Figure 4.22a). Similar results were obtained from RT-QuIC in SNc_PD^{L444P} and brain homogenates of subjects with α -synucleinopathy (Figure 4.22b).

We then investigated the ability of α -syn fibrils to seed the pathology in unaffected tissues. We extracted the α -syn insoluble fraction from PD^{L444P} MOs and injected it into CTR3^{wt} MOs. Two weeks after the inoculation, we examined α -syn/Thioflavin-S co-staining and found LB-like aggregates similar to those previously observed in the donor PD^{L444P} MOs (Figure 4.22c, d).

4.7.5 Study of synapsin III

We explored whether the interaction of α -syn with other proteins at the pre-synaptic terminal may favor its aggregation. Synapsin III (Syn III), a synaptic phosphoprotein that regulates dopamine release in cooperation with α -syn, has been shown to be a component of LBs, where it co-localizes with α -syn insoluble fibrils (Longhena et al., 2018). Previous evidence showed that Syn III acts as a key mediator of α -syn aggregation and toxicity (Faustini et al., 2018, Faustini et al., 2020).

In *GBA1*-mutant MOs and in SNc_PD^{L444P}, we observed a significantly higher amount of Syn III, that reproduces previous observations on sporadic PD brains (Figure 4.23a-d). Levels of Syn III increased after inhibition of GCase activity with CBE (Figure 4.23e, f). Interestingly, Syn III and α -syn showed a positive linear correlation in PD^{L444P} and GD/PD^{L444P} MOs, supporting data of a possible interplay between these proteins in PD pathology (Figure 4.23g).

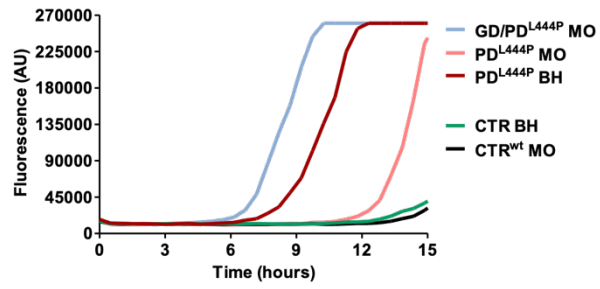
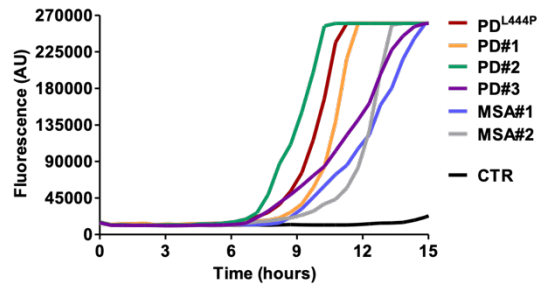
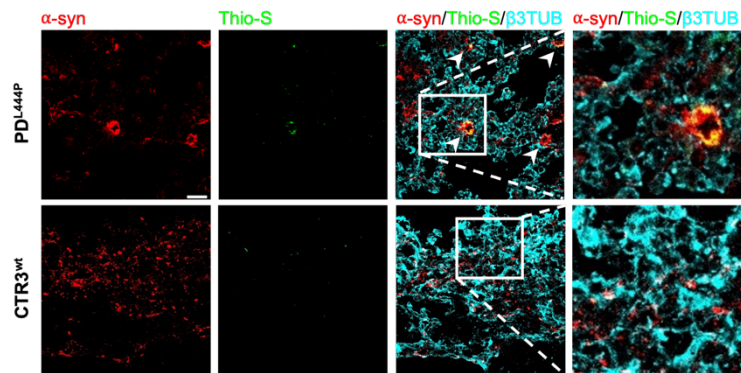
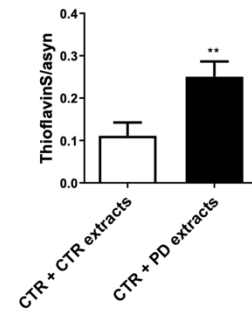
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Figure 4.22 – Seeding properties of α -synuclein.

- a, b. Aggregation kinetics of recombinant α -syn induced by MOs (a) and brain homogenates of patients with PD or MSA (b). In (a), brain homogenates of the PD^{L444P} patient and one control subject were used as internal reaction controls.
- c. Representative IHC images of α -syn and Thioflavin S-labelled aggregates in β -3-tubulin positive neurons in CTR3^{wt} MOs previously injected with α -syn extracted from PD^{L444P} MOs or CTR^{wt} MOs. Arrowheads indicate inclusions of fibrillary α -syn with double staining for α -syn and Thioflavin-S.
- d. Analysis of the overlay area of α -syn and Thioflavin-S in CTR3^{wt} MOs following injection of α -syn from data shown in (c).

Scale bar, 25 μ m (c).

Abbreviations: α -syn, α -synuclein; AU, arbitrary units; CTR^{wt}, control subject; BH, brain homogenate; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; MO, midbrain organoid; MSA, multiple system atrophy; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation.

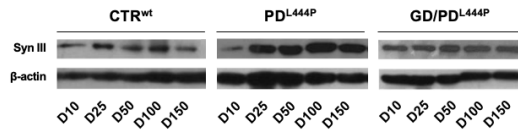
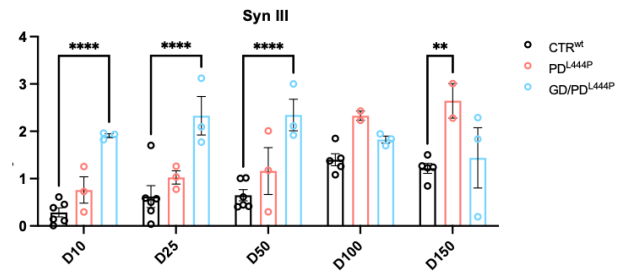
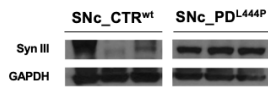
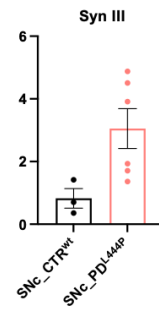
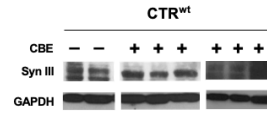
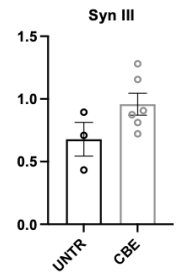
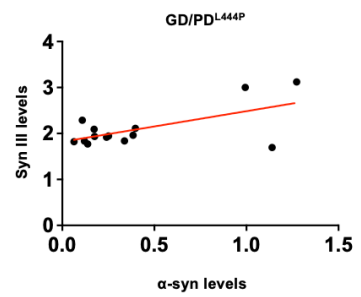
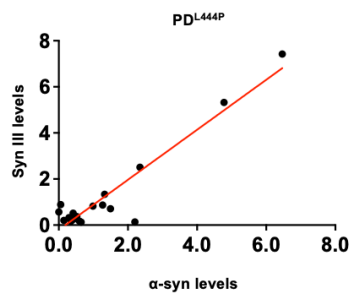
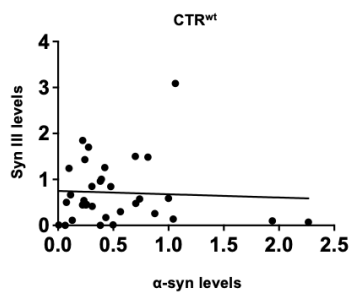
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Figure 4.23 – Study of synapsin III.

- a, b. Western blot and relative densitometry of Syn III and GAPDH levels in MOs at 10, 25, 50, 100, and 150 DIV. Data are presented as mean $\pm$ s.e.m. (n = 84 organoids; *P < 0.05, ***P < 0.001, two-way ANOVA).
- c, d. Western blot and relative densitometry of Syn III and GAPDH levels in SNc_CTRL^{wt} and in SNc_PD^{L444P} substantia nigra from *post-mortem* brains. Data are presented as mean $\pm$ s.e.m. (n = 12; **P < 0.01, two-tailed unpaired Student's t-test).
- e, f. Western blot and relative densitometry of Syn III and GAPDH amount in CTRL^{wt} MOs with and without treatment with CBE (500 μ M for 14 days). Data are presented as mean $\pm$ s.e.m. (n = 15; *P < 0.05, two-tailed unpaired Student's t-test).
- g. Linear regression analysis of Syn III against α -syn in CTRL^{wt} (r^2 = 0.003), PD^{L444P} (r^2 = 0.890), and GD/PD^{L444P} (r^2 = 0.384) MOs at 10, 25, 50, 100, and 150 DIV (n = 45).

Abbreviations: α -syn, α -synuclein; CTRL^{wt}, control subject; DIV, days *in vitro*; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; SNc_CTRL^{wt}, substantia nigra from *post-mortem* brains of healthy donor; SNc_PD^{L444P}, substantia nigra from *post-mortem* brain of Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; Syn III, synapsin III.

4.8 Pharmacological treatment of midbrain organoids

GCase activity is reduced in the brains and cerebrospinal fluid of PD patients with and without mutations in *GBA1* (Gegg et al., 2012; Parnetti et al., 2014) and is associated with increased levels of α -syn in brains of PD subjects (Rocha et al., 2015). Moving from this evidence and from our observations on the link between GCase insufficiency and α -syn pathology, we tested the capability of two compounds engaging with the GCase pathway to counteract α -syn pathological accumulation in *GBA1*-mutated MOs.

4.8.1 Ambroxol

We treated MOs with ambroxol, a small molecule chaperone for GCase with proven efficacy in restoring GCase activity *in vitro* and *in vivo* (McNeill et al., 2014; Yang et al., 2017; Migdalska-Richards et al., 2016; Migdalska-Richards et al., 2017; Magalhaes et al., 2018; Mazzulli et al., 2016).

Upon treatment of MOs at 100 DIV with 100 μ M ambroxol for 10 days, GCase enzymatic activity in PD^{L444P} MOs was restored to normal levels and was comparable to untreated CTR^{wt} MOs (Figure 4.22a). GCase activity was increased also in CTR^{wt} samples, whereas it remained undetectable in GD/PD^{L444P} MOs (Figure 4.24a). Ambroxol treatment led to an increase in the amount of GCase protein in MOs of all lines (1.57, 2.37, 1.71 mean increase relative to basal levels in CTR^{wt}, PD^{L444P}, and GD/PD^{L444P}, respectively) (Figure 4.24b, c). The amount of [1-³H]sphingosine-labelled GlcCer was not affected by ambroxol treatment in PD^{L444P} and GD/PD^{L444P} MOs (Figure 4.24d, e).

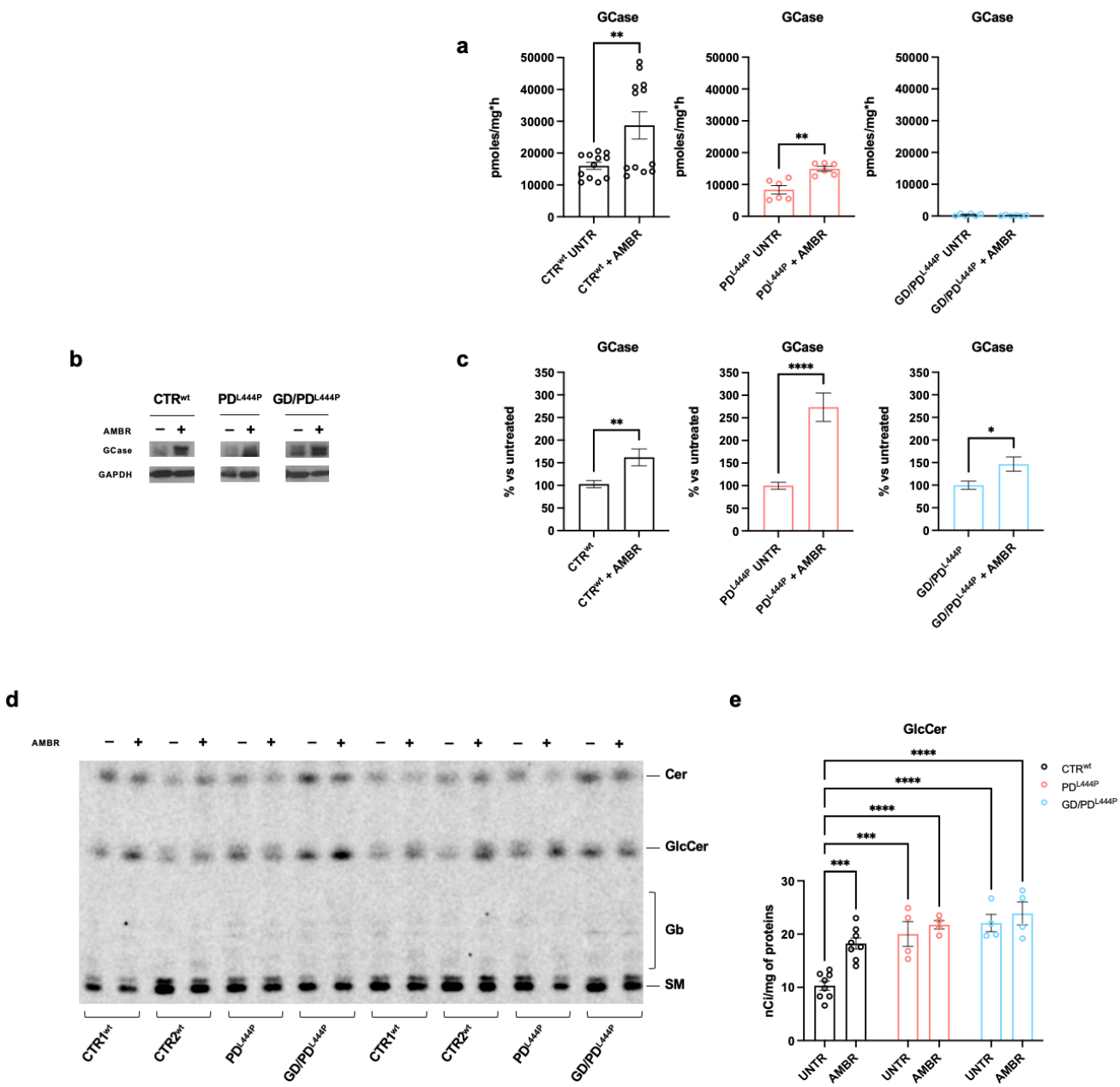


Figure 4.24 – Effect of ambroxol treatment (I)

- a. GCase enzymatic activity in MOs at 100 DIV with and without treatment with ambroxol 100 μ m for 10 days. Data are presented as mean $\pm$ s.e.m. (n = 48 organoids; **P < 0.01, two-way ANOVA).
- b, c. Representative western blots and relative densitometry of GCase protein amount in MOs at 100 DIV with and without treatment with ambroxol 100 μ m for 10 days. Data are presented as mean $\pm$ s.e.m. (*P < 0.05, ***P < 0.001, Student's t-test).
- d, e. Representative digital autoradiography of the neutral sphingolipids' composition and glucosylceramide levels of MOs with and without treatment with ambroxol (100 μ M for 10 days) evaluated by the metabolic labelling at the steady state using [1-3H]sphingosine. Data are presented as mean $\pm$ s.e.m. (n = 32 organoids; ****P < 0.0001, two-way ANOVA).

Abbreviations: AMBR, ambroxol; Cer, ceramide; CTR^{wt}, control subject; DIV, days *in vitro*; Gb, globosides; GCase, glucocerebrosidase; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; GlcCer, glucosylceramide; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; SM, sphingomyelin; UNTR, untreated.

We then explored whether changes in GCase activity and protein levels had an effect on pathological protein accumulation. Ambroxol significantly reduced the amount of total α -syn in CTR^{wt}, PD^{L444P}, and GD/PD^{L444P} MOs (-75.28%, -71.36%, and -43.86% mean decrease relative to basal condition, respectively) (Figure 4.25a, b). Interestingly, we also found that ambroxol treatment reduced Syn III levels in CTR^{wt} MOs as well as in both PD^{L444P} and GD/PD^{L444P} MOs (-45.92%, -46.68%, and -61.05% mean reduction relative to basal levels, respectively) (Figure 4.25c, d). Notably, proteinase K-resistant α -syn aggregates were reduced in *GBA1* MOs, suggesting a potential therapeutic effect of ambroxol on pathological forms of α -syn (Figure 4.25e, f).

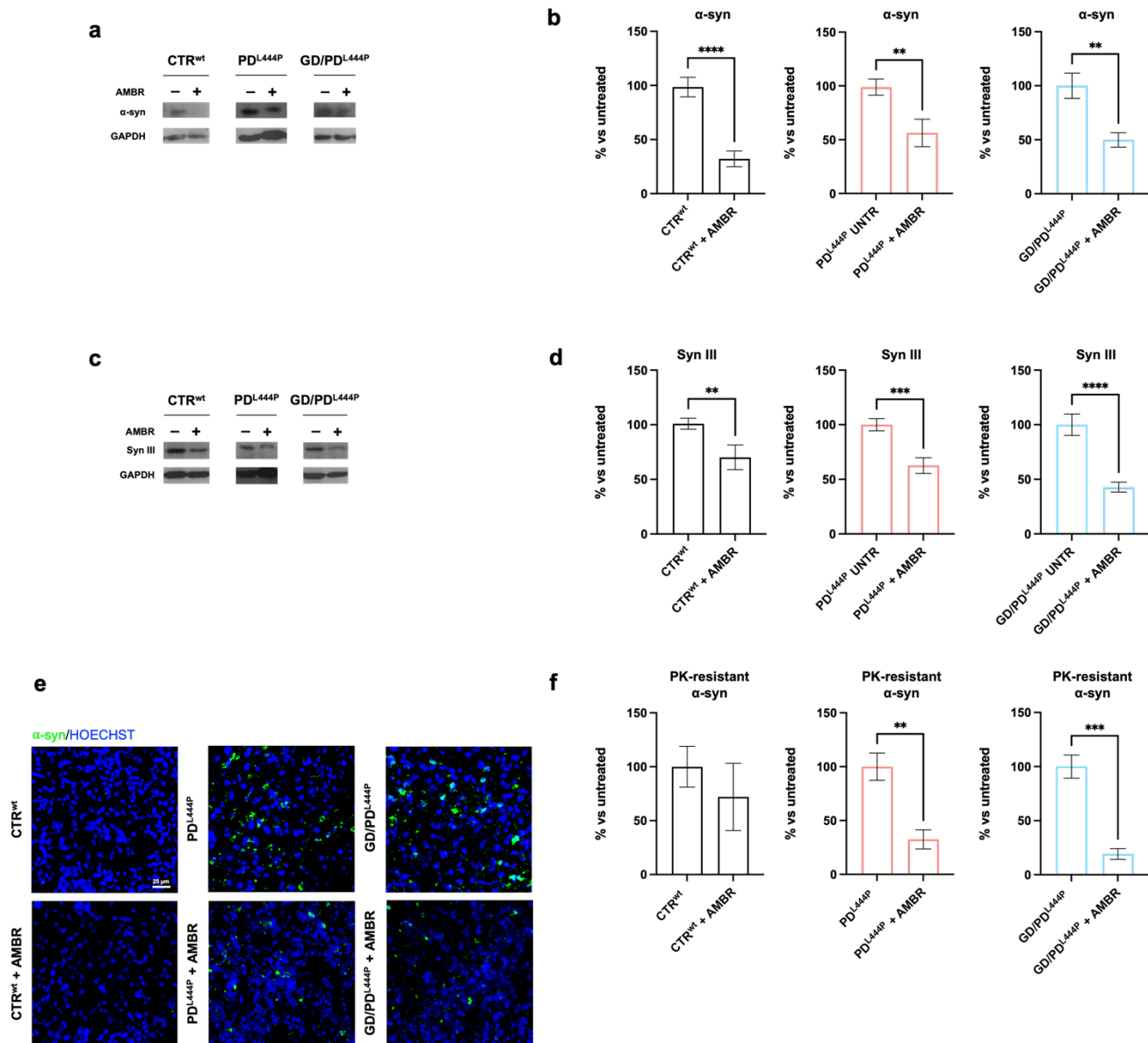


Figure 4.25 – Effect of ambroxol treatment (II)

- a, b. Representative western blots and relative densitometry of α -syn amount in MOs at 100 DIV with and without treatment with ambroxol (100 μ M for 10 days). Data are presented as mean $\pm$ s.e.m. (**P < 0.01, ***P < 0.001, ****P < 0.0001, Student's t-test).
- c, d. Representative western blots and relative densitometry of Syn III and GAPDH amount in MOs with and without treatment with ambroxol (100 μ M for 10 days). Data are presented as mean $\pm$ s.e.m. (***P < 0.001, ****P < 0.0001, Student's t-test).
- e, f. Representative IHC images of proteinase K-resistant insoluble α -syn immunoreactivity in MOs with and without treatment with ambroxol (100 μ M for 10 days) and relative analysis of the area of α -syn signal. Data are presented as mean $\pm$ s.e.m. (**P < 0.01, ***P < 0.001, Student's t-test).

Abbreviations: α -syn, α -synuclein; AMBR, ambroxol; CTR^{wt}, control subject; DIV, days *in vitro*; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; PK, proteinase K; Syn III, synapsin III; UNTR, untreated.

4.8.2 GZ667161

We tested the effect of GZ667161 (Sanofi), an experimental GlcCer synthase inhibitor which has been shown to decrease the levels of GlcCer and α -syn aggregates in a GD mouse model and in mice expressing human A53T α -syn (Sardi et al., 2017).

After treatment of mature MOs at 100 DIV with 100 nM GZ667161 for 14 days, GlcCer levels measured by metabolic labelling of sphingolipids with [1-³H]sphingosine were undetectable in all samples (Figure 4.26a, b), therefore validating the activity of GZ667161 as a potent GlcCer synthase inhibitor. GCase protein amount was reduced in CTR^{wt} and in GD/PD^{L444P} MOs (-34.69% and -52.23% mean decrease relative to basal condition, respectively) (Figure 4.26c, d).

We then tested the ability of GZ667161 treatment to rescue α -syn pathology. Levels of total α -syn were reduced in CTR^{wt} MOs (-40.92% mean decrease relative to basal condition), but not in *GBA1* MOs (Figure 4.27a, b). Similarly, treatment with GZ667161 lowered the levels of Syn III in CTR^{wt} and in GD/PD^{L444P} (-32.69% and -58.21% mean decrease relative to basal condition, respectively). Syn III was reduced also in PD^{L444P}, although this did not reach statistical significance (Figure 4.27c, d). The area occupied by proteinase K-resistant α -syn aggregates was reduced in all treated MOs (Figure 4.27e, f).

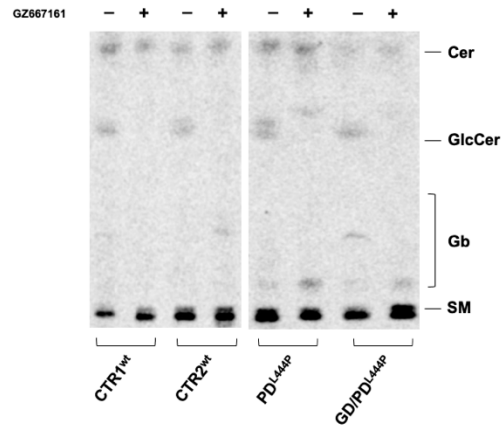
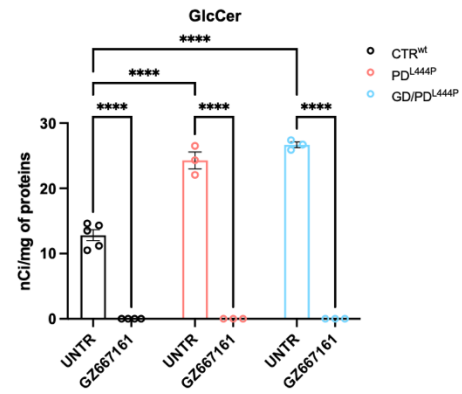
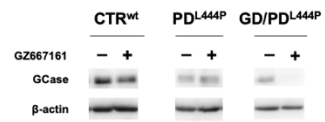
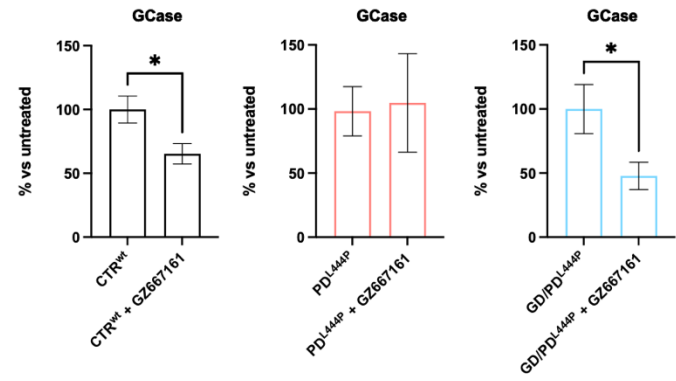
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Figure 4.26 – Effect of GZ667161 treatment (I)

- a, b. Representative digital autoradiography of the neutral sphingolipids' composition and glucosylceramide levels of MOs with and without treatment with GZ667161 (100 nM for 14 days) evaluated by the metabolic labelling at the steady state using [1-³H]sphingosine. Data are presented as mean ± s.e.m. (n = 21 organoids; ****P < 0.0001, two-way ANOVA).
- c, d. Representative western blots and relative densitometry of GCase protein amount in MOs at 100 DIV with and without treatment with GZ667161 (100 nM for 14 days). Data are presented as mean ± s.e.m. (*P < 0.05, Student's t-test).

Abbreviations: Cer, ceramide; CTR^{wt}, control subject; DIV, days *in vitro*; Gb, globosides; GCase, glucocerebrosidase; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; GlcCer, glucosylceramide; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; SM, sphingomyelin; UNTR, untreated.

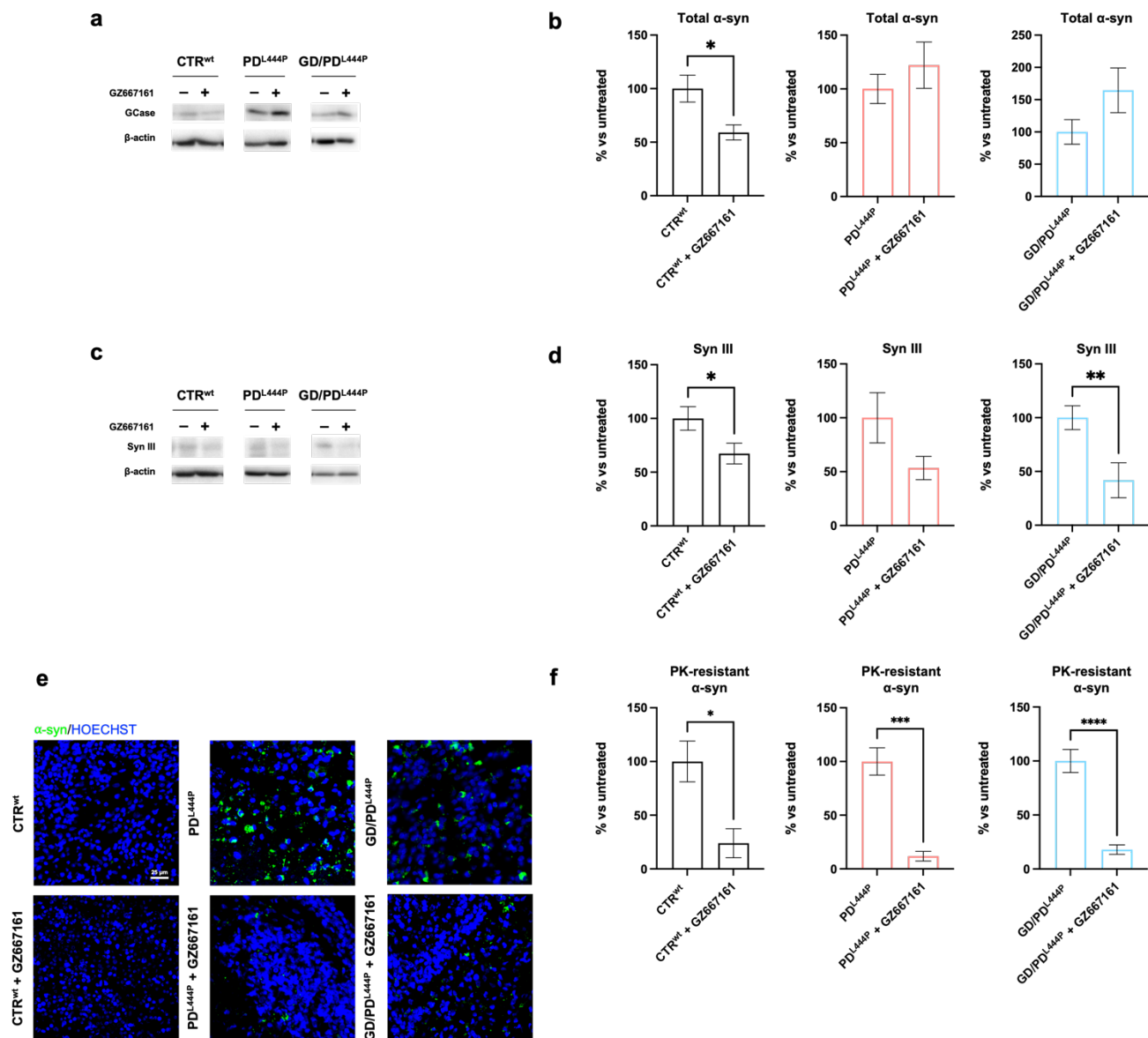


Figure 4.27 – Effect of GZ667161 treatment (II)

- a, b. Representative western blots and relative densitometry of α -syn amount in MOs at 100 DIV with and without treatment with GZ667161 (100 nM for 14 days). Data are presented as mean $\pm$ s.e.m. (*P < 0.05, Student's t-test).
- c, d. Representative western blots and relative densitometry of Syn III and GAPDH amount in MOs with and without treatment with GZ667161 (100 nM for 14 days). Data are presented as mean $\pm$ s.e.m. (*P < 0.05, **P < 0.01, Student's t-test).
- e, f. Representative IHC images of proteinase K-resistant insoluble α -syn immunoreactivity in MOs with and without treatment with GZ667161 (100 nM for 14 days) and relative analysis of the area of α -syn signal. Data are presented as mean $\pm$ s.e.m. (*P < 0.05, ***P < 0.001, ****P < 0.0001, Student's t-test).

Abbreviations: α -syn, α -synuclein; CTR^{wt}, control subject; DIV, days *in vitro*; GD/PD^{L444P}, Gaucher's disease/Parkinson's disease patient carrying homozygous L444P *GBA1* mutation; PD^{L444P}, Parkinson's disease patient carrying heterozygous L444P *GBA1* mutation; PK, proteinase K; Syn III, synapsin III; UNTR, untreated.

Chapter 5 Discussion

Advancements in modern medicine and improvements in public sanitation have added years to global life expectancy (WHO, 2020). Inevitably, the increase in longevity has forced humankind to confront the intrinsic limitations of its own biology. Cells programmed to functionally peak in the first few decades of life, now face challenges with extended survival. In this respect, neurodegeneration, which mainly occurs in ageing brains, has become a major public health concern, with the number of people aged 60 or older predicted to reach almost 2.1 billion by 2050 (United Nations report, 2015). This scenario has been worsened by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, that in the long term is expected to accelerate ageing phenotypes of survivors by affecting multiple organs, including the brain (Lippi et al., 2020; Frontera et al., 2021).

Neurodegenerative disorders share multiple similar pathological features, such as unmet energy demand and inefficient waste product disposal in neuronal cells, which cumulatively promote protein build-up and ultimately lead to cell death. Although *post-mortem* samples and pre-clinical models have been largely used to study the biological bases of such disorders, the molecular underpinnings of protein accumulation and aggregation and of neuronal loss remain largely obscure. This accounts for the dramatic unavailability of disease-modifying therapies for most neurodegenerative conditions.

Parkinson's disease (PD) is the most common movement disorder and the second most prevalent neurodegenerative disorder (Feigin et al., 2019). The neurobiological substrate of PD manifestations is the degeneration of dopaminergic neurons in the substantia nigra (SN) of the midbrain projecting to the striatum. Defining neuropathological hallmarks of PD are insoluble inclusions forming in the cytoplasm and processes of neuronal cells and known as "Lewy

bodies” (LBs) and “Lewy neurites” (LNs), respectively (Lewy et al., 1912; Tretiakoff et al., 1919; Braak and Del Tredici, 2017). A heterogeneous mixture of proteins, lipid membranes, and organelles has been described to accumulate in these deposits, with aggregated α -synuclein (α -syn) being the major component (Wakabayashi et al., 2013; Spillantini et al., 1997; Shahmoradian et al., 2019).

Though most PD cases are deemed sporadic, monogenic traits are found in at least 5% of patients and genetic risk variants account for 16-36% of the heritable risk of PD (Nalls et al., 2019). Mutations in the *GBA1* gene, encoding the lysosomal glucosylceramide (GlcCer)-catalyzing enzyme, glucocerebrosidase (GCase), are the main genetic risk factor for PD, being found in up to 10-15% of Caucasian PD cases, 5% of Chinese patients, and 25% of Ashkenazi Jewish PD patients (Sidransky et al., 2009; Lesage et al., 2011; Neumann et al., 2009; Zhang et al., 2018; Zhao et al., 2020).

GCase insufficiency due to biallelic *GBA1* mutations leads to cellular accumulation of GlcCer and causes Gaucher’s disease (GD), an autosomal recessive lysosomal storage disorder (Grabowski et al., 2008). Over 300 different mutations of the *GBA1* gene have been described. The two most commonly associated with GD and PD are the c.1226A>G (N370S) and the c.1448T>C (L444P), representing mild and severe variants, respectively (Gan-Or et al., 2015; Hruska et al., 2008).

Compelling evidence hints at a link between GCase deficiency, accumulation of glycosphingolipids, α -syn aggregation, and neurodegeneration in *GBA1*-related PD (Mazzulli et al., 2011; Schöndorf et al., 2014; Cullen et al., 2011; Sardi et al., 2011). This notwithstanding, whether loss of GCase activity is involved in α -syn pathological deposition and degeneration of dopaminergic cells in PD still remains to be determined.

A main obstacle in this endeavor is the lack of a comprehensive model recapitulating the pathological processes resulting from GCase dysfunction in the human brain. Elucidating the pathogenic processes that underlie neurodegeneration and developing novel therapies demand an experimental system that recapitulate distinctive features of the human brain and fundamental hallmarks of neuropathology observed in patients.

Previous models generated so far failed to exhaustively reproduce features of mature, post-natal neurons of the human SN and to replicate multiple fundamental neuropathological signatures of PD. iPSC-derived dopaminergic neurons, for example, acquire the ability to produce neuromelanin only when overexpressing a truncated form of lamin A associated with premature ageing (Miller et al., 2013). Genetic rodent models have also failed to recapitulate key hallmarks of the disease, including neurodegeneration and α -syn aggregation. Consequently, our understanding of the molecular mechanisms underlying disease progression remains limited, and therapeutic interventions that halt this process have yet to be discovered.

The advent of patient-derived midbrain organoids (MOs) as three-dimensional cultures enriched in dopaminergic neurons has changed this scenario (Tieng et al., 2014; Jo et al., 2016; Kim et al., 2019; Kwak et al., 2020; Smits et al., 2019; Monzel et al., 2017; Qian et al., 2016; Jo et al., 2021; Smits et al., 2020). Nonetheless, a human model capable of recapitulating the main features of PD pathology is still missing.

Expanding this line of research, we have generated a novel system of MOs containing mature and functional dopaminergic neurons with SN identity and applied it to the study of PD. In this project, we aimed to leverage MOs as an innovative, human-based experimental model to recapitulate cardinal features of *GBA1* disruption in PD and as *in vitro* platforms for the screening of therapeutic compounds.

We showed that MOs are a complex culture enriched in heterogeneous neuronal populations (most importantly dopaminergic neurons) and glial cells. Extensive analyses aimed at characterizing the cell composition of MOs have been carried out at several time points during the differentiation protocol, in order to verify the progressive transition from pseudo-embryonic cell elements to fully developed neurons. To support this statement, we provided evidence of neuronal maturation as assessed by transcriptional profile, expression of dopaminergic markers of interest, presence of neuromelanin, neuronal-like membrane lipidic composition, and spontaneous electrical activity.

To assess the progressive specification of immature cells towards developed neurons, we verified the expression of midbrain-specific transcripts and proteins and found that MOs exhibit a characteristic profile of human midbrain, with both SN and ventral tegmental area identities. The additional presence of cells expressing GABAergic and glial-specific markers strengthens the evidence that MOs are a composite and comprehensive multi-culture system mimicking the *in vivo* conditions.

The presence of neuromelanin (NM) in MOs further evidences the post-natal identity of this model. In human midbrain, NM contained in dopaminergic neurons confers the SN its characteristic pigmentation, becoming apparent after the third year of life and increasing in amount with age (Foley et al., 1958; Cowen et al., 1986; Zecca et al., 2004; Zecca et al., 2008). Previous studies based on three-dimensional dopaminergic cultures reported the presence of a pigment referred to as NM (Jo et al., 2016; Kim et al., 2019; Kwak et al., 2020; Smits et al., 2019; Monzel et al., 2017). Here, we proposed to determine whether the pigment we observed has the defining characteristics of naturally occurring NM of the human midbrain, according to specific structural and immunohistochemical criteria (Foley et al., 1958; Tribl et al., 2007; Ikemoto et al., 1998; Duffy et al., 1965; Moses et al., 1966; Hirose et al., 1968; Zecca et al.,

2008). The unprecedented characterization of a pigment contained in electron-dense organelles exhibiting a typical double membrane and vacuolar lipid bulbs and progressively enriched in TH⁺/TYR⁺ neurons corroborated the interpretation that NM is in fact occurring in our MO model.

Moreover, plasma membranes of neurons within MOs showed a lipid pattern of adult central nervous system tissues (Svennerholm et al., 1989; Ngamukote et al., 2007). Notably, during MO differentiation the expression pattern of glycosphingolipids transitioned from simple species to polysialogangliosides that are typical of fully differentiated neurons. The recapitulation of the lipid profile changes observed during brain development represents an additional element of novelty of MOs as a model of the human brain.

Additionally, neurons within MOs showed functional properties as demonstrated by the presence of spontaneous Na⁺-dependent calcium transients of regular amplitude. Furthermore, the expression of synaptic proteins (e.g., DAT, VMAT2, GIRK2) indicated a proper maturation of electrical and synaptic activity.

Once we assessed that our MO system contains mature dopaminergic neurons with post-natal nigral identity, resembling those found in human adult SN, we investigated the possibility that this model may as well recapitulate the pathological features of an age-related neurodegenerative condition as PD.

To this end, we selected two subjects affected by PD and carrying the *GBA1* L444P mutation, which is the most common genetic risk factor for PD known worldwide. In one of these patients, this variant was present on both alleles and manifested with a neuronopathic type of GD associated with a complete deficiency of enzymatic activity. Notably, we included in the study human SN specimens, including that of one of the PD patients from whom we derived MOs, and used these as a positive control against which we verified results obtained in MOs.

MOs of *GBA1*-mutated patients were able to reproduce the reduction of the GCase enzymatic activity observed in patients (Gegg et al., 2012) and, most strikingly, displayed accumulation of GlcCer, which is still a debated finding in PD brains (Huebecker et al., 2019; Gegg et al., 2015; Boutin et al., 2016). To the best of our knowledge, previous studies of *GBA1*-related PD models from patients did not report substrate accumulation from enzymatic deficiency, with the exception of two reports describing increased levels of GlcCer in iPSC-derived neurons of *GBA1* patients compared with controls (Schöndorf et al., 2014; Burbulla et al., 2019). The remarkable accumulation of lipid substrates that we observed in *GBA1*-mutated MOs thus suggests that, by recapitulating features of adult brain and a more advanced maturation of neurons, this cell culture system allows faithful reproduction of lipid accumulation occurring in brains due to GCase deficiency.

Interestingly, GlcCer accumulation has also been reported in the SN of PD brains independently of *GBA* mutational status (Huebecker et al., 2019). However, in studies conducted on extra-nigral areas (*i.e.*, striatum, cerebellum, and temporal cortex) of brains of PD subjects, substrate accumulation was not detected (Gegg et al., 2015; Boutin et al., 2016). Whether this discrepancy is related to the fact that the brain regions not primarily affected in PD may be less prone to show GlcCer accumulation or really lack substrate accumulation still needs to be elucidated. Moreover, it is feasible that GlcCer accumulation may not be detectable in PD brains due to the loss of specific neuronal populations, which may have been depleted due to significant lipid dysfunction. In this regard, MOs can be analyzed before extensive neuronal loss occurs, thus allowing analyses of the cell types primarily involved in lipid dysregulation and toxic accumulation.

Once we demonstrated the ability of MOs to reproduce GCase deficiency and GlcCer accumulation, we provided evidence that they also recapitulate α -syn pathology and, as such,

may provide insights on the debated role of this protein in the pathogenesis of PD. α -syn is a 140 amino acid protein which localizes at the pre-synaptic terminals and is widely expressed throughout the central nervous system. It binds to acidic phospholipids through its amino-terminal repeats, when it multimerizes and becomes α -helical (Longhena et al., 2019).

The physiological function of α -syn is incompletely understood. A role in the synaptic function has been hypothesized: as a monomer, it probably participates in synaptic vesicle exocytosis by enhancing vesicle priming, fusion, and dilation of exocytotic fusion pores. In its multimeric membrane-bound state, α -syn may act as a molecular chaperone, assisting in the folding of synaptic fusion components, promote the docking and clustering of synaptic vesicles at the active zone, and regulate dopamine release by associating with the dopamine-transporter (Sharma and Burré, 2022).

α -syn has an amino-terminal region that consists of seven imperfect 11-amino acid repeats, through which it physiologically folds into α -helices in the presence of negatively charged lipids (Goedert et al., 2013). Point mutations in the *SNCA* gene associated to PD are located in the same repeat region and are thought to promote the transition of the natively unfolded protein to multimeric structures. In most cases of PD, the conversion of α -syn protein from soluble monomers to oligomers and insoluble aggregates is thought to be a key pathogenetic event. However, mechanisms that lead to α -syn aggregation and how its accumulation causes neurodegeneration are still unclear.

Such fundamental and unsolved questions call for a model able to recapitulate α -syn pathology as seen in PD brains. In this view, transgenic models have failed for the most part, as they do not show the same modality of accumulation and formation of LBs, and even when overexpression is obtained and accumulation is observed, this does not correspond to the human pathological process (Goedert et al., 2013). Similarly, previous iPSC-derived models do

not fully recapitulate Lewy pathology. One study showed thioflavin T-stained fibrils in TH-positive neurons in MOs from PD patients with *LRRK2* mutations, but no clear evidence of α -syn fibrils was described (Kim et al., 2019). Another recent study reported the finding of LB-like inclusions in a genetically modified model consisting of midbrain-like organoids derived from human embryonic stem cells in which *GBA1* was knocked out and α -syn was overexpressed (Jo et al., 2021). However, no patient-derived model with a convincing PD-like pathology has been demonstrated thus far.

Here, we reported that MOs from subjects with *GBA1*-related PD developed insoluble α -syn aggregates in the shape of morphologically defined structures mirroring the typical LBs and LNs of PD neuropathology. The aggregated nature of such inclusions was corroborated by their resistance to proteinase K digestion. To our knowledge, this is the first demonstration of Lewy-like structures in a cell system derived from PD subjects, indicating that the genetic background of patients is a sufficient condition for the recapitulation of α -syn pathology *in vitro*. Moreover, the presence of insoluble α -syn and the localization of thioflavin-S/ α -syn double-positive inclusions in TH-positive neurons in *GBA1*-PD MOs reflects the classical fibrillary organization of α -syn in dopaminergic neurons observed in PD brains.

Additionally, we showed that the inoculation of α -syn extracts from PD MOs into wild-type MOs is sufficient to cause the appearance of thioflavin-S/ α -syn LB-like inclusions in injected tissues, thus supporting previous evidence on the properties of pathological α -syn to self-propagate and induce protein aggregation (Desplats et al., 2009; Volpicelli-Daley et al., 2011; Mougenot et al., 2012; Luk et al., 2012; Masuda-Suzukake et al., 2013). Importantly, the similar seeding activity for α -syn observed in *GBA1*-PD MOs and in brain homogenates of α -synucleinopathies (*i.e.*, PD, MSA-P) suggests that the α -syn aggregates in MOs as well as the tissue microenvironment of MOs share common features with the human brains.

Taken together, our data support the validity of the MO model as a suitable tool to study how the pathological accumulation of α -syn may impact on the resilience of dopaminergic neurons. In this regard, we explored the relationship between α -syn and synapsin III (Syn III), a pre-synaptic phosphoprotein which has been described as a component of LBs and is implicated in α -syn aggregation and in the associated nigrostriatal neuronal toxicity (Longhena et al., 2018; Faustini et al., 2018; Faustini et al., 2020). Interestingly, silencing of Syn III prevents the formation of α -syn inclusions in primary murine DA neurons and impedes α -syn aggregation and neurodegeneration in a mouse model of PD (Faustini et al., 2018).

In line with previous observations in *post-mortem* brains of sporadic PD patients, both the SN from the PD patient with a heterozygous *GBA1* mutation and MOs of *GBA1*-related PD exhibited increased Syn III levels compared with controls. Notably, the finding of a positive linear correlation between α -syn and Syn III protein levels further supports the existence of a critical interplay between the two proteins in PD pathology.

Finally, we investigated the potential of MOs as a patient-derived model for the screening of candidate compounds. Currently available therapeutic approaches to GCase deficiency include GCase activity restoration (*i.e.*, small molecule chaperones for GCase aimed at trafficking the mutant protein trapped in the endoplasmic reticulum to the lysosome), substrate reduction, and enzymatic replacement therapy (ERT). While ERT and substrate reduction therapy are approved treatments to address GCase insufficiency in GD, there is no specific therapy for *GBA1*-related PD.

Reduced GCase activity in the brain of PD patients is associated with increased levels of α -syn (Murphy et al., 2014). In this respect, tackling GCase impaired metabolism to act on downstream effects of α -syn accumulation is a rapidly expanding avenue of research in drug development for PD (Sardi et al., 2013). Molecules engaging with the GCase pathway have

entered clinical trials for PD, such as the open-label clinical trial testing ambroxol in PD patients (ClinicalTrials.gov Identifier: NCT02941822) (Mullin et al., 2020) and the phase II trial on the administration of the GlcCer synthase inhibitor Venglustat in *GBA1*-PD patients (ClinicalTrials.gov Identifier: NCT02906020, recently terminated).

In our study, the use of ambroxol proved to restore GCase enzymatic activity in the *GBA1* heterozygous patient, but not in the GD/PD subject, implying that GCase residual activity, however reduced, is required for ambroxol to be effective. In MOs of both *GBA1*-mutated patients, ambroxol treatment increased levels of GCase protein, consistent with the reported ability of ambroxol to enhance the expression of GCase via the transcription factor EB (Magalhaes et al., 2018; Fois et al., 2015; Mullin et al., 2020). Interestingly, total α -syn and Syn III levels were reduced upon treatment with ambroxol in both *GBA1* patients. This may suggest that GCase and other enzymes upregulated by the non-specific action of ambroxol may have a direct role in protein disposal (Xilouri et al., 2013; Yap et al., 2011). Alternatively, it has been proposed that *GBA1* mutations may interrupt physiologic post-translational folding of GCase, preventing its transportation to the lysosome, and that GCase sequestration in the endoplasmic reticulum may induce an unfolded protein response and α -syn aggregation (Bendikov-Bar et al., 2013; Luan et al., 2013; Maor et al., 2013; Babajani et al., 2012). In this respect, by mobilizing the sequestered mutant GCase from the endoplasmic reticulum and facilitating its transportation to the lysosome, ambroxol may ultimately modulate α -syn levels.

On the other hand, GZ667161, a potent GlcCer synthase inhibitor, has been shown to decrease the levels of GlcCer and the number of insoluble α -syn aggregates in a GD mouse model and in mice expressing the A53T mutant α -syn (Sardi et al., 2017). Treatment of MOs with GZ667161 was successful in completely inhibiting the activity of the GlcCer synthase and preventing substrate accumulation. Interestingly, GCase protein amount was also reduced,

possibly as a consequence of reduced transcription and/or protein synthesis due to lower levels of GlcCer. Levels of total α -syn were not affected by exposure to GZ667161. This may be due to the timing of treatment, which was administered at a late time point (*i.e.*, 100 DIV), when α -syn pathology is already established.

Most importantly, both ambroxol and GZ667161 also had an effect on pathological forms of α -syn by reducing the area of proteinase K-resistant aggregates and therefore showing potential as candidate compounds for α -synucleinopathies.

By evaluating the efficacy of two compounds in patient-derived MOs, we showed that our model is amenable for testing of personalized therapies *in vitro*. Screening of drugs in MOs derived from iPSCs of patients with PD and other neurodegenerative diseases opens new possibilities of testing that support the concept of large-scale, human cell-based safety and toxicity screens, and aim at the development of patient-specific therapies. The non-invasive nature and virtually unlimited supply of this patient-derived approach would allow performing surrogate clinical trials *in vitro* – “clinical trial in a dish” – which are set to become the new testing ground, at the population level, of novel drugs on patients’ cells before moving into actual clinical trials. In this respect, assuming that MOs carrying a patient-specific genetic background are able to reproduce the neuropathological features of donor subjects, testing of molecules in MOs may predict their efficacy in brain tissues and represent a valuable pre-clinical platform for targeted drug development.

In conclusion, we presented an innovative MO-based culture system derived from patients with *GBA1*-related PD that represents the first model capable of reproducing the human α -synucleinopathy and the accumulation of GlcCer as observed in *post-mortem* brains. Demonstration of α -syn pathology rescue with molecules acting on GCase metabolism encourages the employment of MOs as a patient-specific tool for *GBA1*-related disorders and

other α -synucleinopathies and is another promising step towards a broader implementation of personalized medicine.

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