Genetic and clinical pharmacology studies in GBA1-associated Parkinson's disease

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CHAPTER 1

Introduction

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New insights in the pathobiology of Parkinson's disease and possibilities for pharmacotherapy

(Nieuwe inzichten in de pathobiologie van de ziekte van Parkinson en mogelijkheden voor farmacotherapie).

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Over 200 years ago, in 1817, James Parkinson first described the characteristics of the disease that now carries his name in *An Essay on the Shaking Palsy*. [1] In the two centuries following this, our knowledge concerning Parkinson's disease (PD) has increased substantially. The clinical spectrum has been much better characterized, consisting of both motor symptoms and many non-motor-symptoms, like cognitive-, fear-, mood-, sleep-, autonomic- and olfactory dysfunction. The classical concept that this affliction is pathologically distinguished by a progressive loss of the dopaminergic neurons in the substantia nigra pars compacta, has been replaced by a peripheral and central multi-system disorder, associated with cumulative aggregation of the protein alpha-synuclein (α-syn) in axons and synapses, and in cellular inclusion called Lewy Bodies.[2] The role of inflammation in cell loss is both reactive and causative, by both the innate and adaptive immune system.[3] Additionally, epidemiology and genetics contributed to insights how many factors can contribute to the development of this disease. In the Netherlands, the number of people with PD is estimated at 60. 000 [4] with a prevalence of approximately 1% in people above 60 years, up to 2. 5% in people above 80 years. [5] Currently, almost all pharmacotherapy aims to supplement the dopamine deficit, exclusively facilitating symptomatic relief (primarily of motor symptoms), while progression of the underlying disease-process continues. Development of a disease-modifying therapy is of great importance.

Etiology and Genetics

Better understanding of the underlying pathobiology is essential for the development of new drugs. In recent years, the importance of certain impairments in the quality assurance of cellular proteins (proteasome and lysosome) and mitochondria (mitophagy) as well as inflammatory mechanisms in PD pathobiology have become apparent. As a result, independent of primary disease mechanisms, intracellular aggregation of α-syn occurs in the great majority of patients.[6–8] The initiation of these processes and the exact role of α-syn and Lewy Bodies are still under discussion. Animal studies show that overexpression of α-syn alone does not cause neurodegeneration,

but only in combination with microbiome changes.[9] The presence of Lewy Bodies has been associated with neurodegeneration in the area in question, but there are also indications that it actually acts neuro-protectively by trapping dysfunctional α-syn.[10] It is crucial to understand the relationship between these different processes in order to better identify potential leads for the development of new drugs.

Regarding the role of genetic factors, in 5-10% of patients PD is explained by dominant or recessive inherited mutations (Table 1), implying that the vast majority of patients have sporadic disease, probably due to an interplay of genetic- and environmental factors. The first genetic causality was found in 1997 in the *SNCA* gene, encoding the protein α-syn. In the following years, hereditary factors involved in PD have been shown to play a role in important processes for the quality control of proteins and mitochondria, whereby dysfunctional mitochondria are cleaned up by the autophagy-lysosomal system.[8,11] For each of these processes, a relationship with α-syn has now been demonstrated. Several genome-wide association studies (GWAS) have shown up to 90 genetic factors (most robust for *SNCA*, *MAPT*, *LRRK2* and *GBA1* (Table 1)) that slightly increase the risk of the disease.[12] Depending on the type of mutation in the *SNCA* and Leucine-rich repeat kinase 2 (*LRRK2*) gene, this plays a role as a dominant inheritance or genetic risk factor in patients with the sporadic form of the disease. Better understanding of the function of these specific genes and elucidation of cellular processes provide insight into the pathobiology of PD, which in turn offers the potential for pharmacotherapeutic intervention. Below, the different genes are grouped according to their role in a particular cellular process and how different disease mechanisms are interrelated. Table 2 provides an update on investigational drugs targeting these mechanisms that were discussed in 2017.

Mitochondria and Quality Control

The mitochondrion is an organelle that allows for energy supply of the cell, and dynamically operates by moving, dividing, and merging constantly, to ensure quality. It interacts with other organelles such as the lysosome and endoplasmic reticulum (ER), where proteins are made. Mitochondria

naturally decline in quality and may then be cleared by mitophagy. New mitochondria arise again through mitochondrial division. The right balance between well-functioning and less well-functioning mitochondria is essential for healthy cell function. Impaired mitophagy leads to an imbalance with mitochondrial dysfunction and neuronal degeneration as a result.[13] *Parkin, PINK1* and *DJ-1* are three genes with autosomal recessive inheritance which all are concerned with the quality control system of the mitochondrion.[8,11] All three genes are characterized by an early-onset presentation with good levodopa response.[11,14] *Parkin* and *PINK1* collaborate in clearing dysfunctional mitochondria. *DJ-1* may protect against mitochondrial damage from oxidative stress and is involved in the *Parkin-PINK1* system, regulating mitochondrial fusion and division.[13] A homozygous mutation in one of these genes disrupts this system to such an extent that the quality of the mitochondria begins to fail. For α-syn it has been shown as well to influence mitochondrial function, because it precipitates in mitochondria, causes oxidative stress and ultimately leads to mitochondrial dysfunction. Conversely, it is known that mitochondria play an important role in axonal transport, including that of α-syn, both anterograde (to the synapse) and retrograde (to the cell body). In case of mitochondrial damage, the equilibrium in transport shifts to retrograde transport, possibly resulting in the accumulation of α-syn in the cell body as a result.[15]

Maternal inheritance is seen in only a small portion within familial PD, which is consistent with mitochondrial inheritance. [16] Acquired mitochondrial damage may also play a role in PD. In the early 1980s, some young drug addicts presented with an acute syndrome almost indistinguishable from PD, after self-injection of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). [6] MPTP inhibits complex 1 in the mitochondrion and limits mitochondrial transport.[15] Post-mortem studies of PD patients have also shown a reduced complex 1 activity in the substantia nigra.[16] A further indication of mitochondrial involvement is based on animal testing, in which parkinsonism is induced after administration of complex 1 inhibiting herbicides and pesticides. The herbicide paraquat, which is now prohibited in the EU, is possibly associated with an increased risk of PD. Due to methodological challenges, this relationship has not yet been proven beyond doubt.[8,17]

Several forms of gene therapy targeting mitochondrial mechanisms, including *parkin* and *PINK1*, are currently in the preclinical stage of development. Clinical trials of gene therapy in PD have so far shown too little effect to get past phase 2 trials.[14] Nilotinib inhibits the non-receptor tyrosine kinase Abelson (c-Abl). In PD, c-Abl is over- active and phosphorylates α-syn and *parkin.* This phosphorylation inhibits the function of *parkin* and the autophagy of α-syn. Nilotinib is used in the treatment of certain forms of leukemia, but lower dosages may improve autophagy of α-syn.[18,19] A 6-month[20] and another 12-month[21] randomized placebo-controlled trial showed no clinical effect (but it's questionable whether this could be expected in this time frame, based on the mechanism). A phase 3 trial will likely follow.

Given the role of oxidative stress in the development of mitochondrial damage, several agents with an antioxidative effect have been investigated, such as coenzyme Q10 and MitoQ. None of these drugs were developed beyond phase 3 trials, due to the lack of a demonstrable effect on the course of the disease.[14] A recent phase 3 trial for inosine, a uric acid precursor that also has antioxidant functions, was terminated early due to lack of effect.[22,23]

Autophagy-lysosomal system

The autophagy-lysosomal system is a system that clears up waste products and dysfunctional organelles through different routes.[11,24] Small waste substances can be directly absorbed by the lysosome, larger substances need help with this and are accompanied by a chaperone. Organelles are first encapsulated (autophagy) and then fuse with the lysosome to be degraded. Several proteins are involved in transport to the lysosome, including *LRRK2* and *VPS35*, which in the case of a mutation can lead to autosomal dominant inheritance of PD.[25,26] Mutations in *LRRK2* explain about 10% of familial PD and mutations in *VPS35* only 0. 1-1% of familial PD. [11] Response to levodopa seems comparable to idiopathic PD for both genes.[26,27] So these are rare mutations, but both emphasize that a disorder in this autophagy-lysosomal system can contribute to the development of PD.

LRRK2 is a complex and large protein, with various enzymatic and interaction domains, among others involved in different transport processes.[7,11] A mutation in *LRRK2* blocks one of the transport systems to the lysosome, namely the chaperone-mediated autophagy system, which also facilitates transport and degradation of alpha-synuclein.[25] Blockade of this transport system leads to α-syn accumulation and -aggregation outside of the lysosome.[25] There is growing evidence of the other functions of *LRRK2*; *LRRK2* also appears to be involved in mitochondrial fusion and transport, and cytoskeletal dynamics, which provide transport within the cell in general.[28]

Research into drugs that target *LRRK2* has been under development for some time, mostly focused on inhibition of the *LRRK2* kinase domain of the enzyme. Because *LRRK2* is a complex molecule with several active domains, it has a greater risk of off-target effects, seen in lung and kidney preclinically, also if the kinase domain is inhibited selectively. There are several agents that penetrate the blood-brain barrier, which are undergoing further preclinical testing.[28] Two investigational *LRRK2* kinase inhibitors (DNL151 and DNL201) finished a first-in-patient study, of which the results are being awaited (ClinTrials: NCT04056689 and NCT03710707).

Vacuolar protein sorting-associated protein 35 (*VPS35*) is part of the *retromer complex*, which recycles proteins from the lysosome to the Golgi system for reuse.[26] This is necessary for adequate functioning of lysosomal membrane proteins and enzymes. In the case of a mutation in *VPS35*, there is a reduced transport of the lysosomal membrane protein Lamp2a, which leads to decreased endocytosis of a *parkin* substrate, inducing apoptosis.[29] Impaired transport of enzymes interferes with lysosome function, which has preclinically been associated with α-syn accumulation.[7] These mechanisms, and potential pharmacotherapeutic targets, are specific for the subgroup of patients with such a mutation. However, recent investigations also suggest that *LRRK2* has a role in the pathogenesis of idiopathic PD.[0] It remains to be determined whether any therapy will be effective for all patients or only those with a specific mutation.

GBA1 associated PD

An enzyme in the lysosome, which is associated with sporadic PD is glucocerebrosidase (GCase), encoded by the *GBA1* gene. A damaging variant in the *GBA1* gene is currently the most common genetic risk factor for developing PD (GBA-PD);[31] a heterozygous mutation in this gene is found in 4-12% of people with sporadic Parkinson's, up to 15% in the Netherlands (**chapter 2**), and up to 20% in Ashkenazi Jewish.[32–34] Sequencing of this gene is methodologically challenging, due to presence of a pseudogene (risk of false-positive results) and due to susceptibility for an allelic imbalance when amplifying the gene (risk of false-negatives). The pseudogene is a highly homologous piece of DNA, which does not get transcribed, next to the functional gene. This pseudogene can contain mutations, which can falsely be attributed to the functional gene. This can be overcome by using a primer set unique to the functional gene. The allelic imbalance means that the two alleles are not amplified equally, which can result in a mutation not being detected adequately. This was in our case resolved by using a different polymerase enzyme (**chapter 3**). GBA-PD is associated with an average of five years earlier age of onset and faster progression of complaints, both motor and cognitive. Response to regular PD medication is similar to idiopathic Parkinson's.[31] It is considered a risk factor because most people with a *GBA1* mutation will not develop PD.[31] The type of mutation determines how much greater the probability is; this is increased by an estimated overall 2- to 7-fold (odds ratios [ORs]).[32–36] Since the absolute risk is still small and there are no therapeutic consequences, experience in genetic couseling for *GBA1* variants in PD is still limited. Despite the on average worse disease course, counseling requires sufficient nuance due to the high inter-individual variability in phenotype of GBA-PD (**chapter 4**). Certain intronic variants may also influence age at onset in a subgroup of patients (**chapter 5**). What is special about this gene is that it was not found by GWAS studies, but by clinical observation. A homozygous mutation in the *GBA1* gene may cause the rare lysosomal storage disease of Gaucher. It can present with hepatosplenomegaly, skeletal and blood disorders and in severe cases neurological abnormalities and early death.[37] In the early 1990s, enzyme replacement

therapy was developed as a breakthrough, which is a very effective treatment of the peripheral stacking of the GCase substrate glucosylceramide (GluCer). In the years that followed, it turned out that, despite this replacement therapy, patients with Gaucher often developed parkinsonism. This was explained by the fact that the therapy does not cross the blood-brain barrier, so that GluCer continues to accumulate in the brain. Based on these findings, *GBA1* seems a promising target for a potential first disease-modifying treatment in PD.

GBA1

Sufficiently decreased GCase activity leads to accumulation of GluCer in the lysosome, resulting in dysfunction of the autophagy lysosomal system, primarily seen in Gaucher's disease.[24] **Chapter 6** describes how certain glycosphingolipids (like GluCer) may be used as biomarkers in clinical trials and that GluCer is elevated in plasma only in GBA-PD compared to healthy volunteers. In preclinical models, mutations in the *GBA1* gene lead to accumulation of α-syn, but vice versa, induced α-syn overexpression also leads to decreased GCase activity. It is a self-amplifying process, aggravated by age-related degradation of the relevant enzymes.[11,31] Mutations in the *GBA1* gene can also cause the wrong folding of GCase, which can cause trapping of the misfolded enzyme in the endoplasmic reticulum (ER), which needs to be cleared via a dedicated control system. *Parkin* ubiquitinates misfolded protein, so that they are cleaned up by the lysosome or proteasome. Excessive use of this system creates 'ER stress', as a result of which other proteins, such as α-syn, can no longer be properly broken down.[38] Cell studies show GCase depletion also amplifies cell-to-cell transmission of α-syn.[39] Alpha-synuclein exocytosis takes place mainly when this is accumulated in a cell, as a back-up processing mechanism complementary to the lysosome, so that adjacent microglial can clean this up, which in a healthy situation have an increased capacity to phagocytosis and lysosomal degradation.[7]

Since the *GBA1* mechanism in PD came to light, several companies are trying to develop a drug that targets this. Ambroxol, a small molecule chaperone, has been shown in animal studies to cross the blood-brain

barrier, activate GCase and lower α-syn.[40] In an open-label trial in GBA-PD, ambroxol was safe and well-tolerated and penetrated CSF.[41] Results of an RCT are being awaited (ClinTrials: NCT02914366). A second agent, venglustat (GZ/SAR402671), is a synthesis inhibitor of the substrate of GCase (GluCer), which in animal studies also leads to a reduction in α-syn and shows an improvement in memory experiments.[42] First-in-patient studies of venglustat showed favorable safety and tolerability, with CSF penetration and reduction of plasma and CSF GluCer,[43] but a 52-week efficacy trial unfortunately did not meet primary endpoints and further development for GBA-PD is stopped.[44,45] In **chapter 7 and 8**, results are discussed of LTI-291, a GCase activator.

Immune response

Activated microglial cells in the substantia nigra have been reported nearly a century ago and cytokine profiles have shown that the innate immune system is involved.[3] Recent studies now also show involvement of the adaptive immune system. Dopaminergic neurons present antigens via MHC-I in response of microglial cell cytokines, activated by α-syn, upon which cytotoxic T cells clear them. In addition, a specific MHC-II subtype is associated with PD and occurs in 30% of patients, in contrast to only 15% of healthy controls. This MHC-II subtype appears to be more sensitive to present α-syn and thus induce an immune response.[3] In a study with 67 PD patients, 40% of the patients had an immune response against an epitope of α-syn. *Parkin* and *PINK1* may regulate antigen presentation of mitochondrial peptides, representing the interface between the lysosomal and mitochondrial mechanisms in the pathogenesis of PD.[3,46]

Immunotherapy is in development, separated into active therapy, by means of vaccinations that induce a humoral and cellular reaction against α-syn aggregates, and passive therapy, by means of antibodies directly targeting α-syn aggregates. Preclinical research shows a decrease in α-syn aggregates and the several first-in-patient studies were safe and efficacy results are being awaited.[47–50] (ClinTrials: NCT04075318) Active and passive immunotherapy is also being investigated for amyloid beta and tau in

Alzheimer's disease and other related neurodegenerative diseases with different phase 1, 2 and 3 clinical trials. Despite promising preclinical results, patient studies in Alzheimer's disease unfortunately did not demonstrate clinical improvement.[50]

Other strategies

Some other developments have taken place based on epidemiological research. Various substances may be protective against developing PD, like the use of dihydropyridine calcium antagonists, beta agonists, nicotine and caffeine. For isradipine, a dihydropyridine calcium channel blocker, a 36-month RCT did not show a clinical neuroprotective effect.[51] An 18-month trial on transdermal nicotine showed a worsening effect compared to placebo.[52] A phase 3 trial on caffeine versus placebo showed no effect on motor symptoms after 18 months of treatment, but a slight worsening of cognition and dyskinesias in the caffeine group.[53] Beta agonists are currently being investigated. (Dutch public trial registry Trialregister: NL8002)

The effect of influencing the microbiome on the course in preclinical models of PD is developing rapidly. The role of the microbiome as a pharmacotherapeutic target in humans will become clearer in the coming years.[9,54]

An elaborate overview of investigational drugs targeting PD in various stages of development was recently published.[55]

Conclusion

As the complexity of mechanisms involved in PD is increasingly unraveled, a growing number of possibilities is uncovered to develop targeted pharmacotherapy. Thus, hope arises for a better perspective for patients with PD. Since the vast majority of patients have a non-familial form of PD, and the pathogenesis here is multifactorial, the question is whether pharmacotherapy will not consist of a multi-target approach, possibly adapted to the dominant mechanisms of an individual. This thesis focusses on the further unravelling of one of these mechanisms: the *GBA1* gene, encoding the lysosomal enzyme GCase. Several questions are addressed: How prevalent

are mutations in this gene in the Netherlands and does it affect disease onset (**chapter 2** and **5**)? What methodological challenges accompany the sequencing of this gene (**chapter 3** and **4**)? What biomarkers may be used in clinical trials targeting GCase (**chapter 6**)? And what are the effects of the novel GCase activator LTI-291, when first administered to healthy volunteers (**chapter 7**) and to GBA-PD patients (**chapter 8**)?

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