Putting the second brain first
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Chapter 1

General introduction and outline of the thesis
Parkinson’s disease

Parkinson’s disease (PD) is, after Alzheimer’s disease, the second most common neurodegenerative disorder. First described as a “shaking palsy” by James Parkinson in 1817, the definition of PD has evolved to include various motor and non-motor symptoms. Rather than a resting tremor, bradykinesia is the cardinal motor feature and can be accompanied by a resting tremor, rigidity, gait disorders and/or postural instability. In addition, various non-motor symptoms are recognized as part of the clinical syndrome of PD. These include cognitive impairment, sleep disorders, such as REM sleep behavior disorder (RBD), and autonomic dysfunction, such as orthostatic hypotension and constipation. Whereas the neurological substrates for the motor symptoms mainly concern degeneration of dopaminergic nuclei in the substantia nigra, degeneration of cholinergic, noradrenergic, serotonergic and glutaminergic projections are related to the non-motor symptomatology and to some extent even the motor symptomatology, making PD a multi-neurotransmitter disorder. Regardless of the clinical commonalities necessary for the clinical diagnosis, PD is characterized by a large clinical heterogeneity. Not only does the constellation of motor and non-motor symptoms differ between PD subjects, the rate of progression can also vary greatly. Most PD treating clinics are probably familiar with examples of subjects whose complaints remain mostly limited to a resting tremor for over a decade versus subjects who require nursing home admission within a few years after diagnosis. The rate of progression is related to several clinical indicators.

Most notably, classifying PD based on predominant motor symptomatology in a tremor dominant (TD) and postural instability and gait disorders (PIGD) subtype seems to predict a relative benign and more malignant progression, respectively. Nonetheless, subtyping efforts have not yet led to personalized predictions of progression and subsequent counselling and treatment. The treatment of PD mainly consists of dopamine replacement therapies aimed at alleviating the motor symptom burden. The mainstay of the dopaminergic treatment is levodopa, a precursor of dopamine that can cross the blood-brain barrier after which it gets converted in dopamine. Levodopa is mostly prescribed in combination with a decarboxylase inhibitor (eg. carbidopa, benserazide) to prevent the conversion of levodopa to dopamine outside the brain. Other dopaminergic drugs include dopamine agonist, that directly bind the dopamine receptors in the brain, and MAO-B or COMT-inhibitors, aimed at reducing the breakdown of dopamine and increasing the bioavailability of levodopa, respectively.

As the disease progresses, the therapeutic window of the dopamine replacement therapy becomes more narrow, as more dopaminergic terminals are lost, leading to so-called ON-OFF fluctuations with respectively hyperkinesia and reduced therapeutic effects. Despite several dopaminergic drugs showing a more favorable profile with fewer dose-response fluctuations, patients might require more invasive advanced treatments, including deep brain stimulation, more continuous infusion of levodopa directly in the jejunum or infusion with the dopamine agonist apomorphine. Therapeutic strategies have also been developed for other symptoms, including cholinesterase inhibitors in selected patients with cognitive dysfunction, as well as anti-depressive and anti-psychotic therapies tailored to the PD population. Nonetheless, no treatment exists to date that affects the progression of the disease.

Alpha synuclein

The etiology of PD is believed to be multifactorial with environmental factors eliciting deleterious effects against a background of genetic vulnerability. Although, strictly speaking, monogenic forms of PD are not part of idiopathic PD, their discoveries provided insights in its etiology. The first identified monogenic form of PD concerned an autosomal inherited G209A substitution in the SNCA gene, discovered in familial PD cases in Italy and Greece in 1997. In the following years multiple genetic defects related to the SNCA gene were uncovered, including duplications and triplications. The SNCA gene codes for the protein alpha synuclein (aSyn), which makes up about 1% of total cytosolic protein content in neuronal tissue, where it resides in a native unfolded state. The physiological role of aSyn is still debated, but it seems related to neural development and synaptic transmission.

Interestingly, SNCA mutations were all characterized by an autosomal dominant inheritance pattern and yielded a dose-effect response with triplications resulting in a more severe phenotype than duplications. The pathological significance of aSyn therefore seems to rely on toxic gain of function mechanisms, which most probably lie in its propensity to aggregate in amyloid-like fibrils through its ability to form β-sheets. Although it remains unclear which forms of aggregated aSyn exert cytotoxic effects leading to neurodegeneration, agglomeration of amyloid-like fibrils in so-called Lewy bodies and Lewy neurites is a pathological hallmark in most cases of (idiopathic) PD. As such, PD is similar to other neurodegenerative disorders like Alzheimer’s disease, amyotrophic lateral sclerosis and Huntington’s disease, which are all characterized by proteinopathies.

The Lewy body pathology in PD is self-propagating and spreads along neuroanatomical structures in a prion-like manner. Braak et al. suggest the spreading to follow a similar pattern in at least a subset of patients, as described in their six-stage model of disease spreading. Interestingly, the substantia nigra was not involved before stage three and aSyn infiltration of the brain would mainly occur through the peripheral pathways that terminate at the dorsal motor nucleus of the vagal nerve (DMVN). This not only suggests a prodromal period during which the pathology is already present without eliciting the characteristic motor symptoms, but also that PD might start outside of the brain, in particular along the vagal nerve in a proportion of patients.

Gastrointestinal origin

The idea of prodromal PD with a possible origin along the vagal nerve is also supported by the occurrence of non-motor symptoms several years before diagnosis, which can even predict a subject’s risk to develop PD in the coming years. RBD is the non-motor symptom associated with the highest conversion rate. Over 70% of subjects with polysomnography (PSG) proven RBD develop a synucleinopathy, most commonly PD, but also multiple system atrophy or Lewy body dementia. However, other constellations of non-motor symptoms can also predict phenoconversion and are incorporated in the research criteria to predict prodromal PD with over 80% certainty. Interestingly, constipation is one of the earliest prodromal symptoms,
occurring up to 20 years before diagnosis. In concordance, aSyn pathology has already been found in the enteric nervous system (ENS) of the gut wall during the prodromal stages of the disease. The early gastrointestinal symptomatology and pathology support the notion proposed by Braak et al. that PD might originate along the vagal nerve, in particular the gut. The pathology is then hypothesized to spread along the vagal nerve to enter the brain at the DMVN. In line with this hypothesis, truncal vagotomies are associated with a reduced risk of developing PD. Given the large clinical heterogeneity of PD, different etiologies might be relevant for different subtypes. Therefore, the proposed hypothesis of ascending pathology along the vagal nerve might only be relevant for a subgroup of patients. Functional imaging of neuronal innervation along the vagal nerve indeed indicates a dichotomy with a subgroup of RBD-positive PD patients having denervation of the gut and the heart at the time of diagnosis, whereas the innervation of these structures was relatively intact in RBD-negative subjects. Similarly, RBD-positive probable prodromal PD subjects were also characterized by denervation of the heart and the gut. From this, a dichotomous classification is proposed with RBD-positive subjects representing a so-called body-first subtype and RBD-negative subjects representing a so-called brain-first subtype. Both subtypes represent a different site of origin of aSyn aggregation and propagation, with the body-first subtype likely originating in the enteric nervous system. Based on the connectome of neurological structures, different spreading patterns, and therefore different clinical manifestations and rates of progression, can be predicted for both subtypes. Most importantly, gastrointestinal innervation is most likely cross-linked, whereas intrahemispheric connections greatly outnumber interhemispheric connections in the brain. Subsequently, the body-first subtype is likely characterized by a symmetric distribution of the pathology at the time of diagnosis, ascending through the cross-linked innervation of the vagal nerve to both hemispheres. On the contrary, the brain-first subtype likely has an asymmetric distribution of the pathology, mainly limited to one hemisphere. In line with this hypothesis, probable brain-first subjects indeed have a more asymmetric dopaminergic deficit as measured by FDOPA-PET than probable body-first subjects.

Gut microbiota

Provided a possible gastrointestinal origin in a subset of PD subjects and the proposed influence of environmental factors, gut microbiota warrant interrogation on their role in PD. Within our gut, we host an environment of microbial communities, including bacteria, but also other micro-organisms, such as fungi and viruses. The entire environment is commonly referred to as the gut microbiome, with the term gut microbiota referring solely to the organisms within the environment, although the two terms are often used synonymously. The gut microbiome plays an essential role in health and disease. Its significance is often illustrated by the statistic that the number of gut microbiota is ten times, or another multitude higher than the number of cells in our body. Statistics that are actually quite controversial, not in the least due to the uncertain number of cells in the human body, with a more data-driven estimate suggesting a 1:1 ratio. A more certain comparison that is also more biologically relevant, would be the number of protein-coding genes. Compared to the human genome of around 20,000 genes, the human gut microbiome contains more than a 100-fold of that, with an estimated 3.3- to 9.9-million genes. Representing a plethora of biological functions, gut microbiota and host can co-exist in several ecological relationships. Often simply referred to as being either commensal or pathogenic, the range of ecological relationships actually entails the entire spectrum from mutualism to parasitism. Beneficial effects include the production of short chain fatty acids (SCFAs), which are bacterial metabolites produced through the digestion of dietary fibers and can have anti-inflammatory properties. On the other hand, detrimental health-effects can arise from a state of dysbiosis in which otherwise low-prevalent commensal microbes become pathogenic as they become more abundant.

In recent years, high-throughput DNA sequencing methods have become affordable for clinical researchers, allowing the study of gut microbiota composition without the need for cumbersome culture-based methods that could only capture a fraction of all microbes. The gut microbiome can vary based on the anatomical location and even its association with either the luminal or mucosal microenvironment. However, most gut microbiome studies only sequenced genetic material extracted from stool samples and therefore only provide information on the fecal microbiome. Several sequencing techniques can be used, but most gut microbiome studies are based on sequencing of the 16S ribosomal RNA (rRNA) gene or shotgun metagenomic sequencing. The 16S rRNA gene is prevalent in bacteria and several archaea and contains nine variable regions (V1-V9) that allow for taxonomic classification due to their evolutionary conservation. Shotgun metagenomic sequencing, on the other hand, provides an untargeted snapshot of all genes present in a sample. Shotgun metagenomics therefore has the advantages of providing a readout of the functional potential of the genetic content and includes all available microbes, whereas 16S sequencing is limited to taxonomic classification of bacteria and to some extent archaea. Also, shotgun metagenomic sequencing often provides taxonomic data up to species level, whereas 16S sequencing allows classification with lower resolution. However, this difference is ameliorated as 16S reference databases expand and multiple or even all 16S variable regions can be sequenced. Both methods rely solely on DNA sequencing and can therefore not determine whether the DNA fragment represents a living organism. For a true functional readout of the gut microbiome, rather than a readout of the functional potential, techniques including metabolomics or metatranscriptomics can be used, respectively providing information on present metabolites and transcribed RNA.

Microbiota-gut-brain axis

Gut microbiota play an important role in the bidirectional signaling between the brain and the gut, giving rise to the concept of a microbiota-gut-brain axis. With the availability of high-throughput sequencing, several gut microbiome studies have been performed within the context of neurological and neurodevelopmental disorders. Gut microbiome changes have been related to for instance autism spectrum disorder, Alzheimer’s disease and multiple...
sclerosis. Moreover, insights derived mostly from preclinical work show gut microbiota to influence behavior, brain development, inflammation and stress responses. Gut microbiota also modulate the PD-like pathology in preclinical models. For instance, the bacterial amyloid protein Curli was shown to cross-seed αSyn and thereby initiating αSyn aggregation in C. Elegans and rodents. An influential study by Sampson et al. showed the necessity of gut microbiota to initiate αSyn pathology and a PD-like phenotype in a rodent model of PD based on αSyn overexpression. Whereas specific pathogen free (SPF) mice with αSyn overexpression would develop αSyn pathology and a PD-like phenotype, the deleterious effects of αSyn overexpression seem nearly non-existent in germ-free mice that have no gut microbiome. Interestingly, when transplanting the germ-free mice with human gut microbiota from PD subjects and matched healthy donors, the mice again developed a PD-like phenotype. The phenotype was more severe in the mice transplanted with PD microbiota. This suggests that PD associated changes in gut microbiome composition can modulate its pathology.

**State-of-the-art**

Due to the possible gastrointestinal origin of PD, the link between gut microbiota, brain disorders and PD pathology, several case-control studies have investigated the gut microbiota composition in PD subjects. At the beginning of this thesis, the number of PD microbiome studies was steadily increasing. However, as can be expected in a rapidly developing field, PD microbiome studies differed greatly in terms of the methodologies used. Even though decentralized efforts to characterize the gut microbiome composition in PD come with the advantages of a rapidly increasing body of evidence, familiarity with different methodologies and diverse study populations, the outcomes and quality of the studies are not readily comparable. Moreover, almost all PD participants included in previous gut microbiome studies were already treated with dopaminergic medication, making it impossible to adequately disentangle PD associated changes from a possible confounding influence of PD treatment.

In addition, the brain-first and body-first subtypes, and their theoretical framework, described in the αSyn Origin and Connectome (SOC) model, were published during the creation of this thesis. Besides the general relevance of subtyping to explain the clinical and etiological heterogeneity of PD, this classification can shed light on the relation between gut microbiota changes and a PD subtype with a possible gastrointestinal origin of the disease.

**Aim and outline of the thesis**

The current thesis aims to advance our understanding of gut microbiome changes in PD, and explores the possibility of a gastrointestinal origin in a subgroup of PD, through:

1. Reviewing the existing body of literature to create an overview of established gut microbiome changes in previous gut microbiome studies in PD.
2. Reviewing the existing body of literature to create an overview of the different methodologies used in previous gut microbiome studies in PD.
3. Comparing the gut microbiome composition of treatment-naïve, de novo PD subjects to healthy controls.
4. Establishing the effect of dopaminergic medication on gut microbiome composition in PD.
5. Assessing several hypotheses proposed by the SOC-model concerning the relation between asymmetry of the pathology in PD and the clinical picture.

In chapter 2 we have performed a systematic review of the existing gut microbiome studies in PD. An overview of the outcomes is provided, as well as an overview of the used methodologies, which are reviewed for their strengths and weaknesses. Concrete recommendations are provided to increase the utility and comparability of both existing data and future gut microbiome studies in PD.

In chapter 3 we present the protocol of the DUTch PARkinson Cohort study of de novo PD subjects who are untreated at baseline (DUPARC). Data from the DUPARC cohort is used in the following chapters to assess the gut microbiome before and after initiation of dopaminergic treatment and to assess clinical and imaging markers in relation to the SOC-model.

In chapter 4 we have performed the first large gut microbiome study in treatment-naïve de novo PD subjects. Fecal samples were analyzed from a Finnish cohort consisting of several studies with homogenized procedures for stool sample processing, and from the DUPARC cohort.

In chapter 5 we show the influence of dopaminergic medication on gut microbiome composition in PD through a follow-up study of DUPARC participants one year after baseline. Participants who have initiated dopaminergic treatment within one year after the baseline measurement were included in the analysis to create a paired analysis of samples from the same participants before and after treatment initiation.

In chapter 6 several hypotheses concerning the SOC-model are assessed in relation to the asymmetry of the pathology in PD. No PSG-proven diagnosis of RBD could be used to differentiate between probable brain-first and body-first PD. Instead, we have dichotomized the DUPARC cohort in asymmetric and symmetric PD, by including participants in respectively the highest and lowest tertile of asymmetry of the striatal dopaminergic deficit, as measured...
by FDOPA-PET at the time of diagnosis. According to the SOC-model, this would lead to an enrichment with probable brain-first and probable body-first PD subjects, respectively. The SOC-model hypothesizes that symmetric PD subjects will have more autonomic dysfunction, as well as more pronounced and more symmetric brain atrophy, whereas asymmetric PD will show a more benign phenotype with brain atrophy mainly limited to the hemisphere with the largest dopaminergic deficit.

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