Chapter 6

Summary and general discussion
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Parkinson’s disease (PD) is a complex multisystem disorder causing progressive loss of dopaminergic and non-dopaminergic neurons in the central nervous system (CNS) and peripheral nervous system (PNS). Understanding disease progression and the multifaceted molecular mechanisms underlying the neuropathology in Parkinson’s disease (PD) is essential to develop novel neuroprotective or disease-modifying therapeutic strategies for this devastating disease. The theoretical framework on disease progression in the preclinical and clinical phase of PD presented by Braak and colleagues in 2003 (1;2) led to many discussions and studies on the clinical relevance of α-synuclein-immunoreactive Lewy bodies (LBs) and Lewy neurites (LNs) and selective vulnerability in early stage PD (3;4). Braak and colleagues showed that the distribution of LBs and LNs in the CNS is not random but predictable, which was later confirmed by others (5-8). The pathology most likely starts in predelicted sites, i.e. olfactory bulb (OB), dorsal motor nucleus of vagal nerve (DMV) or in the PNS (9;10). Importantly, the classification by Braak et al. (1), emphasizes the vulnerability of non-dopaminergic nuclei and systems, most likely contributing to non-motor symptoms in early stages of the disease.

Little is yet known about the molecular mechanisms contributing to neuronal cell death, selective vulnerability and LB pathology in the premotor stages of PD. Elderly with incidental Lewy body disease (iLBD) may serve as a model to study these mechanisms and discover novel therapeutic targets.

The overall aim of this thesis was to gain insight into molecular mechanisms leading to neurodegeneration and α-synuclein pathology in the premotor and motor stages of PD. To this end, we studied dopaminergic cell loss and LB pathology in the substantia nigra (SN) in pathologically-confirmed iLBD and PD donors with Braak α-synuclein stages ranging from 0 to 6 (Chapter 2). Furthermore, the transcriptome of postmortem brain tissue of iLBD and PD donors was studied in an attempt to identify known and novel molecular pathways contributing to neurodegeneration and α-synuclein pathology in selective vulnerable regions in early stage PD (Chapters 3-5).

In chapter 1, we reviewed the literature regarding non-motor and motor symptoms during disease progression of PD and the main neuropathological features of PD and PD-related disorders. The progression of the α-synuclein pathology throughout the brain is thought to correlate with the clinical progression of the disease and provides a framework for studying PD progression (1;3;11). Linkage, genome sequencing and GWAS studies have provided evidence for mitochondrial defects, oxidative stress, protein clearance and synaptic dysfunction in PD based on the identification of mutations or duplications in genes, such as DJ-1, Parkin and α-synuclein, implicated in juvenile and early-onset PD (12-14). Transcriptome studies using human post-mortem material have aided in our understanding of pathogenetic mechanisms involved in PD, pinpointing mechanisms such as inflammation, synaptic dysfunction, aberrant protein clearance and altered protein synthesis to play a key role in end-stage PD (15-17). Based on literature, we expect a role for autophagy and protein synthesis and synaptic dysfunction to play a role in the early stages of PD.

In chapter 2, we studied neuronal loss in the SN in donors with Braak α-synuclein stages ranging from 0 to 6. Donors with severe tangle, amyloid-β or other concomitant pathology were excluded, so degeneration based on Alzheimer’s disease pathology or other neurological or psychiatric disorders is highly unlikely. We demonstrated that nigral neuronal loss already occurs in elderly in which the α-synuclein pathology is limited to the OB, DMV and locus coeruleus (LC) (Braak α-synuclein stage 1-2). This may indicate that the presence of α-synuclein aggregates in these regions is associated
with nigral neuronal loss, suggesting that loss of trophic support or retrograde degeneration could contribute to nigral neuronal loss. However, there may be also as yet unidentified factors other than α-synuclein that play a role in the degeneration of nigral or other neurons in PD. Our findings are in accordance with the recently published data by Milber and colleagues (18), who demonstrated that dopaminergic cell loss may precede local α-synuclein pathology in the SN in iLBD. We showed that the progression from Braak α-synuclein stage 3 to stage 4 is associated with a significant decline in neuronal cell density (up to 46%) followed by a less pronounced loss between Braak stages 4 and 5, and 5 and 6. This suggests that the disease progression is not linear but rather negatively exponential, as previously suggested by others (19;20). Moreover, a negative correlation was observed between neuronal density and local α-synuclein burden in the SN of PD patients, which is also in line with the findings in other studies (19;21;22). In our study, no correlation between disease duration and nigral neuronal density was found, which is in contrast to the findings by others (23;24). One explanation would be that the variability in disease duration in our cohort was substantial and the number of PD patients in our study was relatively low.

In chapter 3, we confirmed that previously described molecular mechanisms such as the immune response, protein clearance and axonal function are deregulated in the SN of PD donors compared to age-matched controls (16;17;25). Interestingly, pathways such as mammalian target of rapamycin (mTOR) and eukaryotic initiation factor 2 (EIF2) signaling were already deregulated in the SN of donors with Braak stages 1 and 2. In addition, the mTOR and EIF2 signaling pathways remain altered in Braak stages 3 and 4 and Braak stages 5 and 6, which illustrates the importance of these pathways during disease progression of PD. The alterations in mTOR and EIF2 signaling pathways have previously been linked to PD by post-mortem and peripheral mononuclear blood cells transcriptome studies (26). Influencing these pathways may hold the key to alter disease progression in PD and as alterations in EIF2 are observed in the blood cells, deregulated elements of the EIF2 pathway may serve as biomarkers for PD.

Neuropathological studies have shown that OB is one of the first brain regions affected with α-synuclein pathology in PD (27). However, neuronal loss in the anterior olfactory nucleus or loss of dopaminergic neurons in the OB is not detectable in PD patients (28;29). Transcriptome analysis of post-mortem OB tissue of control, iLBD and PD donors (chapter 4) revealed deregulation of genes and pathways involved in protein transport and disposal systems, and in glutamate signaling in early stage PD. Deregulated processes included protein folding, ubiquitination and proteasomal degradation, Golgi membrane traffic, protein deglycosylation, protein import in mitochondria, cytoskeleton dynamics, apoptosis, transcription and pre-mRNA splicing in end-stage PD.

In chapter 5, we have used the transcriptome of the OB, medulla oblongata (MO), LC and SN tissue of iLBD, PD donors and age-matched controls, to gain insight in region-specific and common molecular mechanisms underlying α-synuclein pathology and disease progression in early stage PD. Comparative analysis of the transcriptome profiles showed that pathways related to the immune response, protein synthesis and autophagy are deregulated in all above-mentioned regions in iLBD donors compared to controls, suggesting that these mechanisms may play a role in the pathogenesis of PD. A significant down regulation of pathways involved in endocytosis was observed, as well as an up regulation in endosomal markers and proteins that are involved in the sequestering and autophagic degeneration of proteins, including heat shock protein 70 (HSP70), phosphatidylethanolamine (PE) and lysosomal-membrane protein 2 (LAMP2) (30). In addition, we observed a significant down regulation of synaptic vesicle genes and genes involved in synaptic transmission in the brainstem and OB of iLBD compared to controls including solute carrier family 10, member 4 (SLC10A4) and synaptic vesicle glycoprotein 2 c (SV2C) (31;32) and an up regulation of genes related to plasticity (33). All regions display an up regulation of inflammatory pathways and genes in PD and iLBD donors.
compared to controls (34), including CTLA4 signaling in cytotoxic T lymphocytes and B cell receptor signaling. In addition, major histocompatibility complex, class II, DR alpha (HLA-DRA) was deregulated which indicates that the immune system may already triggered in the early stages of the disease (35). Based on our findings, it is not possible to determine whether the inflammation and immune response serves as protective mechanisms or, alternatively, contributes to the progression of the disease. Molecular mechanisms involved in the formation of α-synuclein aggregates in all regions in iLBD included the ubiquitin proteasome system (UPS) and mitochondrial dysfunction. In the SN, prior to the presence of α-synuclein aggregates, few transcriptional changes linked to mitochondrial dysfunction were found. Significant deregulation of mitochondria was observed in brainstem tissue samples with α-synuclein aggregates. In line with our observations, it has recently been shown that an impaired autophagy, mitophagy and dysfunctional UPS results in accumulation of dysfunctional mitochondria and α-synuclein aggregation in a mouse model of neuropathic Gaucher disease, a lysosomal disorder (36).

We identified several novel molecular pathways associated with α-synuclein aggregation in iLBD. These pathways include down regulated isoleucine and valine degradation and up regulated polyamine regulation. Valine and leucine are branched-chain amino acids (BCAA) and are thought to interact with mTOR and EIF4. The latter are elements of the autophagy process (37). As shown in chapter 3, mTOR activity is decreased throughout disease progression in the SN. Therefore regulation of valine and leucine might directly or indirectly regulate mTOR activity. Furthermore, an increase in BCAAs may promote chronic inflammation and neurodegeneration in a primary microglia cell culture (38). Further studies exploring mechanisms affected by altered levels of BCAAs are needed to elucidate the role of these pathways in PD pathology.

Strengths and limitations of human post-mortem brain tissue for studying disease progression in PD

The use of human post-mortem brain tissue is essential for advancing studies of neurodegenerative disorders, particularly for understanding disease mechanisms involved in neuropathological processes in these complex disorders. Based on the studies in this thesis, some important comments and recommendations can be made for future transcriptome studies with human post-mortem material, which will be addressed in the following paragraphs.

Challenges in working with human post-mortem brain tissue

Human post-mortem brain tissue of well-characterized PD patients, iLBD and controls is rare. To obtain large sample sizes allowing detection of transcriptome alterations poses one of the greatest challenges in post-mortem research. Focusing on pathway alterations may overcome this challenge and allows to compare results between different methods and different cohorts (16). For the studies described in this report, we have used human brain tissue collected by department of Pathology (VUmc) and the Netherlands Brain Bank (NBB), a biobank which has been established by the end of 1985. All tissue was collected after short post-mortem delay (PMD) and extensive pathological assessment was done by experts (39). Another challenge in working with human post-mortem brain tissue is the matching of donor samples. The demographics of donors that enroll into a brain donation program might be biased towards several parameters, including sex, age and level of education. Although the etiology of sporadic PD is largely unknown, it is associated with age (40) and linked to environmental factors (41,42). Matching for these parameters between several research groups is important in scientific approaches to gain
insight in molecular mechanisms involved in early stages of PD and during disease progression. Sporadic PD is more common in men (41;42) and gender-related expression changes in human post-mortem material have been discussed in the literature (43). To explore the effect of gender in PD on transcripts in the brainstem and OB in PD and healthy donors, we conducted a pilot study. The results of this study showed that transcripts (n=475 probesets) that differed between male and female PD donors were linked to the X- and Y-chromosome, and were not related to the disease (unpublished data). Therefore, we included in the studies described in this thesis, brain tissue of both male and female donors.

Furthermore, we used stringent in- and exclusion criteria for the selection of all donors in our studies. Donors with concomitant pathology or other neurodegenerative or psychiatric disorders were excluded. PD patients were diagnosed by a neurologist and, in many cases, also examined by a psychiatrist or neuropsychologist during the course of their disease. Based on the post-mortem neuropathological assessment, we selected donor samples with Braak tangle NFT<III, amyloid score 0-B and no large infarcts or vascular pathology (44;45). Previously, it has been reported that tangle pathology is present in the brainstem in elderly donors with advanced AD pathological stages, particularly at Braak tangle stage V to VI (46). Consequently, in all groups included in our study, degeneration as a result of age-related tangle formation is highly unlikely.

Also, it has been postulated that certain drugs, such as morphine, given ante-mortem influence gene expression in the post-mortem brain (47). Therefore, donors receiving chronic administration of morphine in the last days or months before death were excluded. Specific agonal conditions, including coma and hypoxia, have also been reported to affect gene expression (48). However, matching for medication and ante-mortem processes remains a challenge using post-mortem material as donors participating in a brain donation program are usually affected by a disease prior to death.

**Human post-mortem brain tissue quality**

For microarray studies, the quality of the RNA is crucial; degradation of RNA is detrimental for the results. Therefore, the final step in selecting donor samples for transcriptome analysis is the quality of mRNA. Agonal factors have been reported to have a strong impact on gene expression profiles and quality of mRNA in the brain (48). More specifically, it has been suggested that coma, hypoxia and specific medication, would influence the transcriptome. These agonal factors have been shown to correlate with the pH of brain tissue (48;49). Based on previous reports, we only included brain tissue samples of donors with a CSF pH between 6.3 and 7 in the initial stages of the studies described in this thesis. Another factor that has been previously implicated in RNA quality is the PMD (49). Therefore, the inclusion of donors was restricted to cases with a PMD less than 12 hrs, irrespective of pH of CSF. In the last few years, a more accurate method for quantification of RNA integrity has been developed: the RNA integrity number (RIN).

The RIN algorithm is based on a selection of features of the RNA electropherogram, such as the fraction of the area in the region of 18S and 28S compared to the total area under the curve and the height of the 28S peak in the electropherogram (50;51). In a previous study, a RIN value >7 was suggested to result in reliable gene expression measurements using microarray platforms based on the post-processing quality RNA measurements 3'/5'-actin and GAPDH ratios (15). Acceptable RNA degradation was a 3'/5'-GAPDH ratio lower than 10 and a 3'/5'-actin ratio lower than 3. In our dataset, we evaluated the validity of 1) pH of the CSF, 2) PMD and 3) RIN as indicators for RNA degradation based on the actin and GAPDH 3'/5'-ratios. In 12 brain tissue samples, the 3'/5'-ratio of the housekeeping genes actin and GAPDH were analysed and correlations with the RIN, PMD and/or the pH of CSF were determined. We found that there was no correlation between PMD and pH of CSF on the one hand, and actin and GAPDH ratio's on the other hand (PMD and actin: r=-0.14,
p=0.45; PMD and GAPDH: r=0.09, p=0.64; pH and actin: r=-0.12, p=0.52; pH and GAPDH: r=-0.02, p=0.92). However, RIN correlated with actin (r=-0.37, p=0.02) and GAPDH (r=-0.43, p=0.01). On the basis of these results, we concluded that RIN is an appropriate measurement for RNA degradation. Moreover, we observed that a RIN value of 5 is sufficient to obtain 3'/5'-actin ratio <3 and 3'/5'-GAPDH ratio <10. Based on these findings, we included tissue samples with RIN >5. The difference with RIN measurements in brain tissue provided by the NBB compared to the study of Bossers et al. (15) (6.2< RIN<9.5) might be explained by the different methods used for RNA isolation; Bossers et al. (15) used a column which eliminates mRNA species < 200 base pairs. The presence of short mRNA is taken into account in calculating the RIN number, resulting in a lower RIN number with a higher number of short RNA.

To determine whether the RIN varied between brain structures within a donor, we collected OB, MO, SN and amygdala (AM) from 14 cases, of which we had at least three of the four regions. We used repeated-measures ANOVA to investigate whether the RIN differed between the various brain regions of one individual. We found no significant differences between RIN of brain regions within one brain (F(3)=1.33, p=0.33; unpublished data). This indicates that if a low RIN is measured in one region, this predicts the RIN in other regions and, consequently, the donor can be excluded from transcriptome study completely.

To summarize, when selecting human post-mortem brain tissue samples for transcriptome studies, it is important to match the samples on age and ante-mortem factors, and include samples with acceptable RIN quality (RIN>5). A low RIN measured in one region of the brain indicates a low quality of RNA in multiple brain regions.

In the studies described in this thesis, we have shown that genome-wide transcriptome studies on human post-mortem material provide new insights into the pathogenesis of the disease based on the expression levels of mRNA transcripts. However, a recently developed technique called RNA sequencing (RNA-Seq), has proven to provide more information on the transcriptome level than microarray analysis, for example regarding RNA splicing alterations in AD (52-55). This new technology uses the advances of next generation sequencing and allows to look at alternative gene spliced transcripts, post-transcriptional changes, gene fusion, mutations and changes in gene expression. This technique could provide additional insights into the mRNA alterations and region-specific mRNA splicing events in early stage PD.

Towards understanding molecular mechanisms involved in α-synuclein aggregation in early stage PD

In this thesis, we have described deregulation of pathways previously associated with PD as well as novel pathways linked to disease progression in PD. Genes in these pathways that are a key element in the functioning of the pathway might represent new targets for the development of treatment strategies. However, our data are on the transcriptional level and additional experiments are necessary to confirm the role of these pathways and genes in disease progression in PD. In the remaining part of this chapter, we will discuss several experimental approaches that might aid in elucidating the role of the described molecular mechanisms contributing to the progression of PD.

Validation of targets and pathways

To work towards understanding the role of mTOR and EIF2 pathways in disease progression in PD and involvement of UPS, mitochondrial dysfunction and BCCAs in α-synuclein pathology, it is
important to validate our results by different techniques and in another population to assure that the results are representative for a large cohort of PD patients. To validate our findings in a technical manner, we are currently performing qPCR experiments in SN, MO and LC tissue to study the transcriptome alterations of specific genes with a high fold change between the groups (FC>+-1.50), and which are also associated with pathological processes of interest, such as synaptic dysfunction and autophagy. These genes include SLC10A4, SV2C, LAMP2 and HLA-DRBA (see chapter 5). For further biological validation, we will include several tissue samples of new donors in the qPCR in the transcriptome study (independent samples). To validate and study the cellular origin of the transcriptome changes of the identified elements, in situ hybridization and immunohistochemical techniques will be used.

The alterations in gene expression in the early stages of neurodegenerative diseases are thought to be intermediate compared to the expression levels of end-stage disease and controls (56;57), as previously described for AD (58). In our study we report that alterations in transcriptome in the early stages in PD are less pronounced compared to end-stage PD and the variation in gene expression in the iLBD group is larger compared to control and PD group. Therefore the identification of specific genes involved in pathogenesis of PD remains challenging. However, pathway analysis allows us to extrapolate the individual subtle findings linked to pathological alterations into a biological framework. In a meta-analysis of PD transcriptome studies, Zheng and al. (57) have combined data of 410 samples from patients with PD and healthy controls, which also included 16 samples of donors with possible iLBD. They have identified a down regulation of genes which are expressed in response to peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), and showed in a SH-SY5Y human neuroblastoma cell line that activation of PGC-1α rescues dopaminergic loss.

In order to study the functional consequences of deregulation of autophagy, reduced endocytosis and immune response for neuronal (dys)function and/or survival, in vitro and in vivo models can be used. High-throughput neuronal cellular assays based on morphology have been developed for functional evaluation of the genes and pathways implicated in PD (59). Influencing pathways identified in our study, such as mTOR and EIF2, might advance our understanding of the pathways involved in disease pathogenesis as well as identify potential therapeutic targets. Recently, Volpicelli et al (60) used primary neuronal cultures of mice to explore the role of prion-like propagation in neuronal cells. Interestingly, they observed propagation of the aggregates from cell-to-cell, and misfolded α-synuclein was observed to recruit endogenous α-synuclein to misfold, leading to LBs and LNs. In this model, it may be possible to evaluate the role of genes involved in α-synuclein aggregates, such as SLC10A4 (32) and SV2C (61). Also, genes implicated in endocytosis and autophagy could be evaluated, such as LAMP2, PE and clathrin. Individual knock-down of these genes by transfection of vectors using appropriate short hairpin RNAs might provide insight in the key modulators of aggregation and dysfunction in PD by studying the rate of α-synuclein aggregation and cellular death. In addition, levels of BCAAs and polyamines can be controlled and altered in these cellular assays to evaluate the role in α-synuclein aggregation.

Other options include in vivo modulation of the identified pathways. For example, mTOR can be modulated using Rapamycin in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model for PD. Previously, it has been shown that administration of Rapamycin reduces dopaminergic cell loss in the SN after chronic MPTP treatment, suggesting that mTOR modulation can rescue dopaminergic cells (62). Currently, Rapamycin is also used in human trails (63;64) and inhibits the activity of mTOR1. Recently, a mouse model has been developed in which non-transgenic mice have been inoculated with misfolded α-synuclein and these mice displayed Parkinson-like motor symptoms and α-synuclein pathology (65). Modulating mTOR through Rapamycin in this non-transgenic mouse model might provide useful insight into the role of autophagy in PD.
In addition, targeted genes from the transcriptome analysis can be approached in this model using viral-vector based gene silencing or over expression using this non-transgenic mouse model.

**Final remarks**

In this thesis, we have shown that neuronal cell death in the SN is already detectable in iLBD prior to local α-synuclein pathology, and related to the local burden of α-synuclein pathology in PD. We have shown that molecular pathways, such as BCAAs and polyamine regulation, may play a role in different stages during the progression of PD pathology. Other crucial alterations in gene expression levels in the SN of iLBD include reduced endocytosis, activated immune response and axonal dysfunction. Mitochondrial dysfunction and dysfunction of UPS were observed in tissue samples with α-synuclein pathology of iLBD but not without, suggesting that these processes are an early event in the pathological cascade of PD. In addition, we showed that immune response, synaptic dysfunction and endocytosis are common mechanisms involved in α-synuclein aggregation in all included regions.

Here, we have used the Braak α-synuclein staging scheme to gain insight in the molecular mechanisms during disease progression in PD. The nigral neuronal loss and deregulation of several PD-associated pathways in iLBD support the hypothesis that these donors represent the premotor phase of PD. The overlap between molecular pathways in brain tissue samples (25;66;67) and peripheral blood samples (26), including mTOR and EIF2 signaling, indicates that altered genes in these pathways hold promise to serve as diagnostic tools for PD.
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