Scope & Outline

JOANNA A. KORECKA
Scope

Parkinson’s disease (PD) is a progressive neurodegenerative disorder. According to the Parkinson’s Disease Foundation, 7 to 10 million people suffer from PD worldwide. Approximately 60,000 new cases are diagnosed each year in the US alone (www.pdf.org). In the Netherlands, more than 50,000 patients have been diagnosed with the disease (www.parkinsonfonds.nl). The combined direct and indirect costs of PD in the US, including treatment, social security payments and loss of income are about $25 billion dollars per year. Only 5% of the PD cases are familial, while the rest of the patients suffer from a sporadic form of the disease (Dauer and Przedborski, 2003). The principal risk factor for PD is age, with incidence rising significantly after age 50 (Driver et al., 2009). Despite extensive research efforts worldwide, the causes of sporadic PD remain unknown until this day. The current treatments, both pharmacological and surgical, diminish the symptoms of both familial and sporadic forms of the disease, and therefore temporarily improve the quality of life of the patients (reviewed in Olanow et al., 2009). As yet, no genuine regenerative treatments that stop the progression or cure the disease are available.

Neuropathological changes in sporadic PD are of a progressive nature and involve multiple neuronal systems. Lewy body formation and neurodegeneration start in the brain stem and olfactory bulb, ascending to the Substantia Nigra pars compacta (SN) nucleus and further progressing towards the neocortex, reaching sensory and prefrontal areas of the brain (Braak et al., 2003). Selective loss of dopaminergic (DAergic) neurons in the SN as well as extensive gliosis in that area are the best known neuropathological characteristics of PD, directly accounting for the motor symptoms. The familial forms of the disease are caused by mutations in genes such as SNCA, PARK2, DJ-1, PINK1, LRRK2 and PARK9 (reviewed in Hardy et al., 2006; Bonifati, 2007; Hardy, 2010). These genetic mutations helped investigators to identify a number of biological processes that potentially contribute to the development of familial PD. However, for sporadic PD the specific molecular alterations leading to the typical neuropathological changes in the SN still need to be elucidated. The etiology of the disease appears to be multifactorial, involving both biological and environmental components (reviewed in Dauer and Przedborski, 2003; Maguire-Zeiss et al., 2008; Olanow et al., 2009). Developing regenerative treatment requires more research into the basic cellular and molecular changes that occur in the brain of PD patients.

In the last years, in particular since the completion of the human genome project (Lander et al., 2001; Venter et al., 2001), a number of new “high-throughput” approaches have been used to further dissect the molecular changes in PD at a scale that was previously unthinkable. These approaches include large scale analysis of single nucleotide polymorphisms (SNP), genome-wide gene expression analysis using micro arrays, and more recently “deep” sequencing, and proteomics approaches (reviewed in Tsuji, 2010; Hardy, 2010; Lewis and Cookson,
2011; Greene, 2012). These techniques revealed molecular changes in multiple brain regions and in blood samples from PD patients.

The transition from studies of single ‘candidate’ genes to a broader unbiased analysis of multiple molecular changes in human postmortem tissue has resulted in the identification of new “molecular signatures” of PD. Genome-wide SNP analysis has identified several potential susceptibility genes [UCHL1 (LeRoy et al., 1998), SEMA5A (Maraganore et al., 2005), SNCA (Mizuta et al., 2006), MAPT (Simon-Sanchez et al., 2009)] for PD. Genome-wide gene expression profiling revealed the dysregulation of numerous new genes involved in processes that had previously been implicated in the disease, including genes involved in the ubiquitin/proteasome system, heat shock regulation, iron transport, chaperone activity, oxidative stress regulation, vesicular transport and neurotransmission, and most recently miRNA modulation of mitochondrial function. Importantly, microarray studies also revealed changes in the expression of genes implicated in pathways not previously linked to the disease, including extracellular matrix signaling, cell adhesion, the polyamine signaling pathway and axon guidance (reviewed in Lewis and Cookson, 2011; Greene, 2012).

We have studied genome-wide alterations in gene expression in the SN of PD patients using Agilent microarrays (Bossers et al., 2009). As we were particularly interested in transcriptional alterations during the early stages of DAergic degeneration, we analyzed those parts of the PD SN that were relatively spared. This approach allows the identification of transcriptional changes in neurons that are potentially compromised by the disease but have not yet degenerated. We found 287 genes significantly differentially expressed in the PD SN. These expression changes may either be causal to the development and/or progression of sporadic PD, or may be the consequence of the disease process.

The primary challenge of genome-wide gene and protein expression studies is to translate the alternations in gene and protein expression into concrete biological mechanisms that underlie the degeneration of DAergic neurons in the SN of PD patients. Extracting this information from lists of target genes and proteins is essential to advance our understanding of the mechanisms underlying the disease.

The overall objective of the work described in this thesis was to face this challenge and to investigate the potential role of a relatively large set of genes in the degenerative process of DAergic neurons that occurs in PD. These genes were selected from the total group of 287 genes identified by microarray based on bioinformatics and literature study. Next, we used high-content cellular screens to examine the role of these target genes in DAergic neuron viability, neurite outgrowth and mitochondrial activity. This integrative approach allowed us to select a small number of targets for functional study in vivo. Overexpression of one of these targets, repulsive guidance molecule A (RGMA), in the SN of mice appeared to be sufficient to induce degeneration of DAergic neurons. Figure 1 pro-
vides an outline of the steps that together comprise the experimental strategy that was employed in this thesis.

Outline

Chapter 1 reviews recent cell replacement and gene therapy studies for PD and AD. Unlike the temporary symptomatic relief of current pharmacological (dopamine replacement) and neurosurgical treatments (deep brain stimulation) for PD, cell replacement and gene therapy are novel approaches that aim to either replace the damaged cells or prevent neuronal degeneration by stimulating regenerative and neuro-protective mechanisms in the affected tissue. Gene therapy for neurturin, a protective trophic factor for DAergic neurons, recently entered a phase II clinical trial. This demonstrates that this cutting edge therapeutic approach, based on adeno-associated viral vectors (AAV) to deliver the therapeutic gene, is well-tolerated and safe in human subjects (Marks, Jr. et al., 2010).

Chapter 2 describes the selection of 79 primary target genes from the total set of 287 dysregulated genes in the PD SN. These genes were selected, because based on bioinformatics and literature, they play a role in: 1) cell death- degeneration of neurons is a cardinal pathological feature in the SN of PD patients, 2) axon guidance, neurotrophic support and synaptic transmission - biological processes recently implicated in the development of PD, and 3) mitochondrial gene expression and cellular metabolism - interrelated processes clearly disturbed in PD. Cellular localization studies of 34 target genes using in situ hybridization and immunohistochemistry revealed that these genes are almost exclusively expressed in neurons in the SN, and not in reactive astrocytes or activated microglia.

The aim of chapter 3 was to establish an appropriate in vitro cell model to study target gene function. A good cell model to elucidate gene function in the context of PD should: 1) contain the main cellular and molecular properties that are characteristic of DA neurons, 2) be sensitive to environmental factors that are known to contribute to PD, and 3) be suitable for cellular screening, which allows examination of the function of many genes, proteins and specific compounds in a high-content manner. Using genome-wide transcriptional profiling combined with gene ontology and pathway analysis we characterized the molecular phenotype of SH-SY5Y cells after retinoic acid (RA) differentiation. Firstly, we found that RA induces the differentiation of SH-SY5Y cells and enhances their DAergic characteristics, while suppressing other transmitter phenotypes. Secondly, RA differentiated SH-SY5Y do indeed express 73% of our PD genes of interest. Finally, treatment of these differentiated cells with 0.01mM 1-methyl-4-phenylpyridinium (MPP+) reduces mitochondrial activity similar to DAergic neurons in vivo. Thus, RA differentiated SH-SY5Y cells can be used to model the effect of both environmental and genetic factors in the PD-associated neurode-
**Figure 1.** Schematic representation of the thesis outline. The project started with the identification of 287 genes differentially regulated in the human SN PD tissue compared to the matched controls (step 1). Based on gene ontology and Ingenuity Pathway Analysis, 4 biological functions have been identified to be altered in the PD SN tissue (step 2). From these functions, we have selected 79 primary genes of interest— the target genes (step 3), from which we have successfully localized 33 in human SN neurons (step 4). This allowed us to further develop appropriate PD-related in vitro and in vivo models (step 5), where the functional role of these genes could be tested. We performed high throughput siRNA knockdown screen of 62 genes and high content overexpression screen of 14 genes to explore the biological relevance of the investigated genes in PD related cellular function (step 6). Finally one gene was selected for in vivo validation, where its role in inducing cellular death of DAergic neurons in the mouse SN became clear (step 7). The chapter numbers describing the given steps are indicated in the bottom right corner of each box. Abbreviations: PD- Parkinson’s disease, SN- Substantia nigra pars compacta, DAergic- dopaminergic, RA- retinoic acid, MPP(+)-1-methyl-4-phenylpyridinium, AAV- adeno-associated virus, MPTP- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, LV- lenti virus, RGMA- repulsive guidance molecule A.
generative process. We therefore used these cells in functional gene screening assays.

Chapter 4 describes the results of high content screening to determine the functional effects of 62 target genes in SH-SY5Y cells in the absence or presence of MPP(+). siRNA-mediated knockdown of 62 genes and LV-overexpression of 14 genes demonstrated that 12 genes significantly affected various parameters including cellular viability, mitochondrial activity, and neurite outgrowth. Four genes were selected for further validation based on 1) being upregulated in PD SN as illustrated by the microarray study, and 2) induced dramatic changes in two or more of the measured cellular functional readouts. Overexpression of RGMA decreased neurite count, while its knockdown induced apoptosis. Overexpression of the transcription regulator of cellular survival prothymosin alpha (PTMA), neuron specific gene silencing transcription regulator carboxyl-terminal domain small phosphatase 1 (CTDSP1) and a cytoplasmic phosphoprotein involved in synaptic plasticity (WWC1), all decreased both cellular viability and neurite outgrowth.

In chapter 5 we describe the development of a mouse model that mimics the early stage of PD based on a chronic low dose MPTP treatment (15mg/kg) twice a week for 5 weeks. This treatment induces dopamine dependent behavioral deficit accompanied by a loss of tyrosine hydroxylase (TH) positive nigrostriatal axon terminals in the striatum, and gliosis, but no cellular death in the SN. This model bears a striking resemblance to the cellular changes that have led to the so-called ‘dying back’ hypothesis of PD, and provides an interesting window of opportunity to study the mechanisms that underlie early neurodegenerative events that may initiate the cellular death of DAergic neurons.

In chapter 6 targeted viral vector-mediated gene transfer is tested as means to study the function of target genes in murine DAergic neurons in vivo. The ability of different AAV vector serotypes to drive reporter gene GFP expression in DAergic neurons in the mouse SN was tested, and the performance of two different promoters (the classical, ubiquitously active CMV promoter and the neuron specific synapsin promoter) were evaluated. Transgene expression by AAV serotype 7 harboring the synapsin promoter resulted in the most efficient transduction of TH positive neurons in the mouse SN and induced the highest GFP expression in nigro-striatal axonal projections. This vector was therefore used in chapter 7 to study the role of RGMA in vivo in the mouse SN.

The cellular screens in chapter 4 resulted in the identification of a small set of genes that affected mitochondrial activity, neurite growth or viability of DAergic cells. In chapter 7 we show that AAV7-mediated overexpression of RGMA in the mouse SN results in behavioral abnormalities that are typical for loss of striatal DA input, a gradual degeneration of DAergic neurons, and micro- and astroglial activation in the SN. These data suggest that the upregulation of RGMA in human SN neurons in PD may be causally involved in PD-associated motor symptoms and the degeneration of SN DAergic neurons.
Chapter 8 provides a general discussion of the most striking results presented in this thesis. We speculate on the mechanism of RGMA signaling and its role in the degeneration of DAergic neuron, but also propose other possible genes identified in the *in vitro* screen to be equally important in the context of PD, such as PTMA, CTDSP1 and WWC1. We conclude this thesis with future prospects of our research and its contribution to the development of new therapeutic strategies for PD.