From the DEPARTMENT OF NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

NEURODEGENERATIVE DISEASES STUDIED IN HUMAN BRAIN AND RODENT MODELS

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Stockholm 2015

Cover: Two methods used in this thesis are the gait analysis of mice and immunohistochemical staining of brain tissue. Parts of the cover figure are adapted from (Mulherkar *et al.*, 2013).

Published by Karolinska Institutet.
Printed by EPrint AB 2015

ISBN 978-91-7549-851-5

Karolinska Institutet

Department of Neuroscience

Neurodegenerative diseases studied in human brain and rodent models

THESIS FOR DOCTORAL DEGREE (Ph.D.)

publicly defended in the Hillarp Lecture Hall, Retzius väg 8, Stockholm

Friday, March 13, 2015, 9 am

by

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ABSTRACT

Parkinson's and Alzheimer's disease are the two most common neurodegenerative diseases world-wide, but are still little understood. Papers in this thesis examine some of the possible pathogenic mechanisms with the help of mouse models and analysis of human post-mortem tissue.

Gene-based animal models have been developed to study pathological pathways during disease progression. Using mouse models with overexpression or ablation of disease-related genes we analyzed effects of pathogenic mutations on the function of the proteins. A prominent feature of patients carrying the G2019S mutation in leucine rich repeat kinase 2 (LRRK2) is a high load of Lewy Bodies, an intracellular protein accumulation highly enriched in α-synuclein. We investigated the interaction of LRRK2 and α-synuclein in transgenic mice and did not find an influence of LRRK2 expression on fibrillization of α-synuclein or dopamine neurons pathology, indicating separate pathways for the two genes causing PD.

Using another genetic model with a conditional knockout of the mitochondrial transcription factor A in dopamine neurons, the MitoPark mouse, we detected reduced dopamine release and pacemaker activity in the substantia nigra several weeks before these mice develop motor dysfunction. The mRNA levels of the hyperpolarization-activated cyclic nucleotide gated channels 1-4 (HCN1-4), responsible for the pacemaking activity, were not altered in MitoPark mice, indicating that post-translational modifications occur early in the presymptomatic stages of Parkinson's disease.

To evaluate behavioral and cellular changes related to L-DOPA therapy we used older MitoPark mice, which model late stages of Parkinson's disease. Chronic L-DOPA treatment normalized gait parameters but induced also progressing dyskinetic behavior in MitoPark mice. The treatment also caused a robust increase of TH mRNA expression in the striatum, as evidenced by RNA-Sequencing. The induction of TH in striatal neurons with an interneuronal phenotype was dependent on the degree of dopamine depletion and the L-DOPA dose.

In disease-affected brain areas of patients with Parkinson's or Alzheimer's disease we found a significant increase in small-sized cells expressing the lysosomal enzyme myeloperoxidase. This finding supports involvement of a neuroinflammatory component in these diseases and encourages the research for anti-inflammatory treatments. Another protein implicated in the pathogenesis of neurodegeneration is the serine peptidase HTRA2. We detected altered enzyme activity and expression of HTRA2 in frontal cortex samples from Alzheimer's disease patients. The association of a mutation in a HTRA2 allele with Alzheimer's disease in our case-control material further supported a role of mitochondrial dysfunction in the pathology.

Taken together, the studies presented in this thesis uncover changes in gene and protein expression in mouse and human samples, as well as behavioral changes in animal models of disease and will aid the development of better treatment options by increasing our knowledge of underlying pathological mechanisms.

LIST OF SCIENTIFIC PAPERS

I. Daher JP, Pletnikova O, Biskup S, Musso A, **Gellhaar S**, Galter D, Troncoso JC, Lee MK, Dawson TM, Dawson VL, Moore DJ. Neurodegenerative phenotypes in an A53T α-synuclein transgenic mouse model are independent of LRRK2. *Hum Mol Genet. 2012 Jun 1; 21(11):2420-31*

II. Good CH, Hoffman AF, Hoffer BJ, Chefer VI, Shippenberg TS, Bäckman CM, Larsson NG, Olson L, **Gellhaar S**, Galter D, Lupica CR. Impaired nigrostriatal function precedes behavioral deficits in a genetic mitochondrial model of Parkinson's disease. *FASEB J. 2011 Apr;25(4):1333-44*

III. **Gellhaar S**, Marcellino D, Abrams MB, Galter D. Chronic L-DOPA induces hyperactivity, normalization of gait and dyskinetic behavior in MitoPark mice. *Accepted in Genes Brain Behav.*

IV. **Gellhaar S**, Dillman A, Planert H, Silberberg G, Cookson M, Galter D. Significant effect of L-DOPA treatment on the transcriptome of a brain area relevant for Parkinson’s Disease: induction of TH expression in striatum. *Manuscript*

V. Westerlund M, Behbahani H, **Gellhaar S**, Forsell C, Belin AC, Anvret A, Zettergren A, Nissbrandt H, Lind C, Sydow O, Graff C, Olson L, Ankarcrona M, Galter D. Altered enzymatic activity and allele frequency of OMI/HTRA2 in Alzheimer's disease. *FASEB J. 2011 Apr;25(4):1345-52*

VI. **Gellhaar S**, Sunnemark D, Eriksson H, Olson L, Galter D. Numbers of myeloperoxidase immunoreactive cells are significantly increased in brain areas affected by neurodegeneration in Parkinson’s and Alzheimer’s disease. *Manuscript*

CONTENTS

Introduction 1
Parkinson's disease 1
Pathology 1
Causes 2
Treatment 4
Animal models of Parkinson's disease 5
Animal models of PD used in this thesis 6
Alzheimer's disease 10
Aims of the thesis 13
Material and Methods 15
Material 15
Human tissue samples 15
Rodent models of Parkinson's disease 15
Methodology 17
Behavior studies 18
Immunohistochemistry and Western blot 19
In situ hybridization 20
RNA extraction and qRT-PCR 20
RNA sequencing 21
Electrophysiology 22
Results 23
Mouse models of PD 23
Interaction of LRRK2 and α-synuclein, paper I 23
Neurobiological events in the premotor phase of PD in the MitoPark mouse, paper II 24
Establishment of MitoPark mice as a dyskinesia model, paper III 25
TH expressing cells in the striatum of MitoPark mice after L-DOPA treatment, paper IV 26
Neurodegenerative disease pathology in humans 27
Analysis of the expression and activity of HTRA2 in AD and PD, paper V 27
Myeloperoxidase-expressing cells in affected brain regions in AD and PD, paper VI 28
General discussion 29
Conclusions 31
Acknowledgements 33
References 36

LIST OF ABBREVIATIONS

AADC	L-amino acid decarboxylase
AD	Alzheimer's disease
APP	amyloid precursor protein
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
bp	base pair
cDNA	complementary DNA
COMT	catechol-O-methyltransferase
DA	dopamine
DAT	dopamine transporter
DNA	deoxyribonucleic acid
eGFP	enhanced green fluorescent protein
GFAP	glial fibrillary acidic protein
HCN	hyperpolarization-activated cyclic nucleotide gated
HTRA2	HTRA serine peptidase 2
IAP	inhibitor of apoptosis protein
I_h	hyperpolarization-activated current
IR-DIC	infra-red differential interference contrast
KO	knockout
LB	Lewy body
L-DOPA	L-3,4-dihydroxyphenylalanine
LID	L-DOPA induced dyskinesia
LRRK2	leucine-rich repeat kinase 2
MAOB	monoamine oxidase B
MPO	myeloperoxidase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	mitochondrial DNA
mRNA	messenger ribonucleic acid
PARK	genetic loci initially linked to autosomal forms of PD
PBS	phosphate buffered saline
PD	Parkinson's disease
qRT-PCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
RNA-Seq	RNA-Sequencing
ROS	reactive oxygen species
SNc	substantia nigra pars compacta

syn	α-synuclein
Tfam	mitochondrial transcription factor A
Tg	transgenic
TH	tyrosine hydroxylase
WT	wild-type
6-OHDA	6-hydroxydopamine

INTRODUCTION

Common features of many neurodegenerative diseases are aberrant or misfolded proteins, ubiquitin-proteasome system defects, oxidative stress, and mitochondrial perturbation, which all can result in cell death. Genetic mutations and certain environmental factors also increase the risk of developing a neurodegenerative condition. Familial forms are rare monogenic diseases and typically have an earlier onset than sporadic forms. In some cases, different neurodegenerative diseases are even linked to the same polymorphisms or mutations, thereby sharing similar pathological mechanisms. In the following, the two most common neurodegenerative diseases, Parkinson's disease (PD) and Alzheimer's disease (AD) will be described in more detail.

PARKINSON'S DISEASE

Parkinson's disease (PD) has a prevalence of about 1% at the age of 65 years increasing to 5% at the age of 85 years. Men are slightly more affected than women (Hirtz *et al.*, 2007).

PD was first described in 1817 by James Parkinson in "An Essay on the Shaking Palsy" (Parkinson, 2002), but had been known since 4500 BC in Ayurveda as Kampavata and was treated with ground seeds of Mucuna pruriens in ancient India (Manyam, 1990, Ovallath & Deepa, 2013). Kampa means tremor, which is the most prominent symptom occurring during rest and later gave rise to the name shaking palsy. Other than tremors, the cardinal symptoms are bradykinesia, postural instability, and rigidity. These motor symptoms are preceded by more unspecific non-motor symptoms such as constipation, olfactory dysfunction, sleep disturbances, or psychological symptoms such as depression (Klockgether, 2004). The disease starts slowly as neuronal dysfunction originating from the death of dopaminergic (DA) cells of the substantia nigra pars compacta (SNc) (Fearnley & Lees, 1991). More than half of the DA neurons have typically died when patients first contact a physician because of motor symptoms, which typically occur unilaterally in the beginning due to the unsymmetrical degeneration of DA SNc neurons (Fearnley & Lees, 1991). Dementia can be an early complication of PD but increases to 78% later on (Aarsland *et al.*, 2003). Biomarkers to detect the disease before motor-symptom onset have not been found yet, and are an important research topic.

Pathology

Although the loss of SNc neurons has been the focus of most studies, the first lesions might occur in neurons of the wall of the gastrointestinal tract, the dorsal motor neurons in medulla oblongata, neurons in the raphe nucleus and locus coeruleus (Braak *et al.*, 2003, Del Tredici *et al.*, 2002, Kupsky *et al.*, 1987). A hallmark of affected neurons are proteinaceous inclusions termed Lewy bodies (LBs) and Lewy neurites consisting of many proteins with α-synuclein as

a prominent component (Lewy, 1912, Spillantini *et al.*, 1997). LB pathology is believed to spread from the brainstem via the basal forebrain to cortex cerebri as the disease progresses (Braak *et al.*, 2003). The detection of LBs is however not essential for the diagnose of PD, as they are not found in all PD patients, and can be detected in the amygdala of patients with AD, and also neurologically healthy controls (Fearnley & Lees, 1991, Hamilton, 2000). Neurofibrillary tangles consisting of tau protein and Aβ plaques are detected in 15% of all PD brains and correlate with dementia (Compta *et al.*, 2011, Jellinger, 2007, Jellinger & Attems, 2008). The partly overlapping pathologies of neurofibrillary tangles, LBs, and Aβ plaques hint to similar pathways in neurodegeneration, although the cellular distribution varies between the diseases.

A further common pathological feature is chronic inflammation in affected brain regions in AD and PD. This process may be triggered by degeneration, in AD even preceding the stage of aggregated proteins, and contribute to disease progression. Results from mouse models suggest that neuroinflammation in AD occurs even before the detection of aggregated proteins (Heneka *et al.*, 2005). Activated astrocytes and especially microglia release proinflammatory cytokines, free radicals, chemokines, members of the complement cascade, as well as inflammatory enzymes. The activation of microglia may also impair the function of endothelial cells, damage the blood-brain barrier and allow the entrance of leukocytes (Brochard *et al.*, 2009, Togo *et al.*, 2002, Yenari *et al.*, 2006). Leukocytes contain the enzyme myeloperoxidase (MPO), which has been shown to be increased in brain tissue in PD and AD (Choi *et al.*, 2005, Green *et al.*, 2004). MPO is expressed in the myelocytic lineage until the stage of promyelocytes in the bone marrow. It is held in lysosomes of monocytes, as well as in the azurophilic granules of neutrophil granulocytes which play a role in the microbicidal defense (Borregaard & Cowland, 1997). During the respiratory burst it is released from the granules and produces cytotoxic hypochlorous acid (HOCl) (Harrison & Schultz, 1976). It has been reported to be expressed in microglia, astrocytes, neurons and plaques in hippocampus and cortex of AD patients and microglia in the midbrain of PD patients (Choi *et al.*, 2005, Green *et al.*, 2004, Maki *et al.*, 2009, Reynolds *et al.*, 1999).

Another supporting factor for the role of inflammation in neurodegenerative diseases is that non-steroidal anti-inflammatory drugs slightly reduce the risk of PD (Etminan *et al.*, 2003, Samii *et al.*, 2009).

Causes

Already in 1880, Leroux discovered a familial aggregation of parkinsonism previously believed to be sporadic (Leroux, 1880). A Swedish family with mendelian Parkinson was reported in 1949 and later the cause was shown to be duplications and triplications of α-synuclein (Mjönes, 1949, Puschmann *et al.*, 2009). Twin studies strengthened genetic causes of neurodegeneration and in 1997 the first gene causing familial PD, α-synuclein, was mapped

by linkage studies (Duvoisin & Johnson, 1992, Polymeropoulos *et al.*, 1997). It was termed PARK1 and in the following years further genes and loci were mapped. In the group of sporadic PD cases there might be many with a genetic cause that has not been identified yet due to methodological limitations or polygenetic inheritance.

Incomplete penetrance of mutations may hint to an interplay of the genetic variation with other genetic or environmental factors. Langston and coworkers discovered one environmental factor and described the fast development of parkinsonian symptoms in young drug addicts using contaminated "synthetic heroin" (Langston *et al.*, 1983). This contained 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which can cross the blood-brain barrier and is converted to 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B (MAOB) (Markey *et al.*, 1984). MPP+ is transported into cells harboring the DA transporter (DAT). Inside cells MPP+ accumulates in mitochondria and inhibits complex I of the electron transport, which leads to the reduced production of ATP, the production of reactive oxygen species and eventually to cell death (Ramsay & Singer, 1986, Sinha *et al.*, 1986). Other risk factors such as traumatic brain injuries have received much attention in the last years after the diagnosis of PD in individuals who have had head injuries, for example boxers, football, and ice hockey players (Goldman *et al.*, 2006). Another new area of research in PD is epigenetics. Without modifying the genome, epigenetic modifications may alter risk or susceptibility to environmental factors (reviewed in (Ammal Kaidery *et al.*, 2013)).

In addition to α-synuclein, detoxifying enzymes attracted attention in reaction to the discovery of PD inducing toxins. An important role of mitochondria emerged not only due to the impact of toxins on mitochondria but also by findings that genes involved in familial PD including parkin, PINK1 and DJ-1, are involved in the maintenance of a healthy pool of mitochondria. Several different features can be affected in mitochondrial homeostasis in PD. Mitochondrial motility may be impaired by mutations or alterations of expression in PINK1, Parkin, α-synuclein or leucine-rich repeat kinase 2 (LRRK2), as these proteins all interact with the microtubule-mediated trafficking of mitochondria in the cell (Gillardon, 2009, Lee *et al.*, 2006, Weihofen *et al.*, 2009, Yang *et al.*, 2005). Dynamic fusion and fission of mitochondria is also needed to maintain critical mitochondrial functions and altered expression of PINK1, for example, increases the amount of dysfunctional mitochondria in cell culture studies (Yang *et al.*, 2008).

Mitochondria supply energy to the cell in the form of adenosine triphosphate (ATP), which is generated by the respiratory chain in the inner mitochondrial membrane. The organelles contain their own DNA (mtDNA) and 13 of the 37 genes in mtDNA encode subunits of the respiratory chain and 24 encode the translational machinery in the mitochondrion. Mitochondria undergo regulated turnover, called mitophagy and mtDNA replication is therefore a continuous process in both dividing and non-dividing cells. Throughout life mutations and deletions in mtDNA accumulate and can cause functional impairment in SNc DA neurons and other cells (Bender

et al., 2006). Dysfunctional mitochondria may lead to increased reactive oxygen species (ROS) being formed, which in turn damages mtDNA by oxidation (Miquel, 1992).

Mutations in the mtDNA polymerase γ (POLG), the enzyme responsible for the synthesis and proofreading of mtDNA, have been found in idiopathic PD (Luoma *et al.*, 2007). The MitoPark mouse models the consequences of impaired mitochondrial function in DA neurons by genetic removal of the mitochondrial transcription factor A (Tfam) in these cells, leading to progressive degeneration of SNc DA neurons and resulting PD-like motor deficits (Ekstrand *et al.*, 2007).

Treatment

The seeds of Mucuna pruriens, which were used in ancient India to treat PD, contain L-3,4-dihydroxyphenylalanine (L-DOPA), which was isolated in 1937 (Damodaran & Ramaswamy, 1937, Manyam, 1990). The oral treatment with this DA precursor is still the gold standard treatment, as there is no cure or prevention for PD. L-DOPA can cross the blood-brain barrier where it is taken up and converted to DA by L-amino acid decarboxylase (AADC) to increase the striatal DA content (Lloyd *et al.*, 1975). It is administered together with a peripheral AADC inhibitor to limit adverse effects of circulating DA (Cotzias *et al.*, 1969). The effect of L-DOPA on motor performance lasts a few hours in the first several years of treatment. With prolonged administration, decreased efficiency of L-DOPA treatment and negative side effects, such as dyskinesia, pathological gambling and psychotic behavior is observed (Barbeau, 1969, Weintraub *et al.*, 2010).

Dyskinetic symptoms are highly debilitating side effects and eventually limit the use of L-DOPA, in a third of all patients after about five years after the onset of motor symptoms (Ahlskog & Muenter, 2001, Chapuis *et al.*, 2005). The side effects occur more often in early onset PD (Fabbrini *et al.*, 2007). The probability of developing dyskinesias increases with higher dose of L-DOPA as well as a longer treatment duration (Zappia *et al.*, 2005). L-DOPA induced dyskinesias (LID) are choreic movements, small amplitude, dance-like, or dystonic movements, a twisting of the limbs due to involuntary contraction of muscles, or a combination of both types (Fabbrini *et al.*, 2007). These side effects are coupled to peak plasma levels of L-DOPA after administration (Tolosa *et al.*, 1975). To avoid the pulsatile L-DOPA plasma concentrations, the administration of a L-DOPA gel via an intestinal pump has been developed. The treatment has been demonstrated to be effective in advanced PD, but carries the disadvantages abdominal surgery and technical problems including dislocation or blocking of the catheter and intestinal perforation or obstruction (Fernandez *et al.*, 2014, Fernandez *et al.*, 2013).

Drug treatments to increase presynaptic DA include inhibitors of MAOB or catechol-O-methyltransferase (COMT), enzymes which catalyze DA breakdown. In the past few years an initial therapy with DA agonists instead of L-DOPA has been examined in greater detail with various results (Group *et al.*, 2014, Parkinson Study, 2000). Deep brain stimulation of the

subthalamic nucleus or the globus pallidus pars interna has proven to be effective in advanced PD, when drug therapy no longer improves symptoms (Deep-Brain Stimulation for Parkinson's Disease Study, 2001).

Another treatment option, which is still in the experimental phase is the implantation of cells that are reprogrammed to functional DA neurons. The first engraftment of DA-cell rich fragments of human embryos into PD patients was performed in 1988 and was constantly improved to be able to use the patient's own cells for implantation in the future (Hartfield *et al.*, 2014, Lindvall *et al.*, 1988). This research also led to the discovery of new possible pathological pathways, such as a prion-like spreading of α-synuclein into before unaffected cells in culture and that genetic predisposition increases the susceptibility of reprogrammed cells to degenerate (Reyes *et al.*, 2014, Sanchez-Danes *et al.*, 2012).

ANIMAL MODELS OF PARKINSON'S DISEASE

The discovery that certain toxins caused parkinsonian symptoms led to the first animal models of PD. The most common model is generated by stereotactic injection of 6-hydroxydopamine (6-OHDA) into the rat brain to cause degeneration of DA neurons (Ungerstedt, 1968). A feature of this model is the possibility to generate a strictly unilateral loss of the uncrossed nigrostriatal DA system. The description of parkinsonian symptoms in drug users injecting contaminated heroin led to the development of new PD animal models based on systemic poisoning with MPTP to cause neurodegeneration (Langston *et al.*, 1983). Dependent on the administration regimen, acute, subacute or chronic, different lesion grades modeling different disease stages can be achieved. Due to the systemic injection, the DA lesion is bilateral and produces motor, as well as non-motor symptoms (Anderson *et al.*, 2007, Fifel *et al.*, 2014, Fredriksson & Archer, 1994, Ogawa *et al.*, 1985). Toxin-induced models display a fast DA depletion, whereas the slowly degenerating phenotype of PD is more difficult to model. In addition, the injection can lead to side effects not related to PD. More recently, the discovery of genetic mutations as a cause for PD have led to the generation of transgenic rodent models. These models are based on mutations found in familial PD, as well as on mutations found in candidate genes of sporadic PD. With the possibility to choose cell-specific promoters, the expression of a transgene can be directed to different brain areas. Transgenic animals are generally used to study pathological mechanisms of the mutation and most models actually fail to recapitulate motor- and non-motor features of PD. Mitochondrial dysfunction found in PD strengthens the implication of mitochondria-related genes in pathogenic mechanisms. Since no animal model has been developed yet that recapitulates all features of PD, the most relevant model needs to be chosen to address each experimental question.

Animal models of PD used in this thesis

The 6-OHDA model

The 6-OHDA rat model is the oldest and most often used model to study PD pathology. It was first described in 1959 and used for the modeling of PD in animals in 1968 (Senoh & Witkop, 1959, Ungerstedt, 1968). 6-OHDA is an analogue of catecholamines and taken up into the cell via DAT and the noradrenaline transporter (Luthman *et al.*, 1989). To exert its toxic effect only on DA cells it is often used together with a noradrenaline reuptake inhibitor. It is systemically toxic, cannot cross the blood-brain barrier and has to be injected into the brain by stereotaxic surgery. Following uptake into the cell, it accumulates and is oxidized, generating hydrogen peroxide, which leads to the production of reactive oxygen species and cell death (Cohen, 1984, Jeon *et al.*, 1995). Like MPTP, it is toxic to complex I of the electron transport chain (Cleeter *et al.*, 1992). Depending on the site of injection and concentration of 6-OHDA, different degrees of DA depletion are produced. Injection into the medial forebrain bundle causes extensive lesion, into the striatum a slow, progressive and partial lesion, and into the SNc a more specific lesion (Francardo *et al.*, 2011, Sarre *et al.*, 2004, Sauer & Oertel, 1994). A bilateral lesion, though more closely related to PD in humans, is not often used as it impairs the survival of the animal and requires intense care (Cenci *et al.*, 2002, Ungerstedt, 1971). The severity of a lesion can be assessed by injections of apomorphine or amphetamine and results in turning behavior when a certain degree of striatonigral neurons is destroyed (Hefti *et al.*, 1980). Other regions, for example the lower brain stem, are not affected by 6-OHDA and cells do not show a LB pathology. Motor symptoms, such as akinesia and tremor, are seldom reported in the unilateral model, but non-motor symptoms as cognitive deficits, depression-like behavior and cognitive impairment, have been detected (Branchi *et al.*, 2008, Lindner *et al.*, 1999, Tadaiesky *et al.*, 2008). The unilateral 6-OHDA model is suited for the preclinical testing for antiparkinsonian drugs (Ilijic *et al.*, 2011). The fast DA depletion does not give time to assess the slowly progressing motor impairment and the molecular changes happening before the onset of motor symptoms. In most studies, the unlesioned hemisphere is used as the control, but in these studies the authors do not take into account the compensatory effects elicited by the unlesioned hemisphere (Kozlowski *et al.*, 2004, Morin *et al.*, 2013, Terzioglu & Galter, 2008).

α-Synuclein transgenic mice

The first PARK gene, α-synuclein, was found and mapped in 1997 in an Italian kindred, as well as in Greek families with autosomal dominant PD (Polymeropoulos *et al.*, 1997). In addition to point mutations, also duplications and triplications of the wild-type α-synuclein gene have been shown to cause disease (Farrer *et al.*, 2004, Nishioka *et al.*, 2006). Patients with α-synuclein mutations have an autosomal dominant inheritance, an early onset and rapidly progressing disease. α-Synuclein is concentrated at presynaptic nerve terminals and

accumulates to form the main component of LBs (Jakes *et al.*, 1994, Spillantini *et al.*, 1997). The protein is unfolded in its native state but can bind to many ligands and proteins which leads to its folding (Deleersnijder *et al.*, 2011, Weinreb *et al.*, 1996). Several possible pathogenic mechanisms of A53T α-synuclein have been discovered as for example the inhibition of tyrosine hydroxylase (TH) activity in cell culture (Perez *et al.*, 2002). The A53T mutation is located in the N-terminal α-helix domain and is in cell culture toxic to catecholaminergic neurons and impairs protein degradation via ubiquitinylation (Petrucelli *et al.*, 2002, Stefanis *et al.*, 2001). It forms dimers and oligomerizes fast (Conway *et al.*, 2000, Krishnan *et al.*, 2003). The insoluble fibrils accumulate and form a part of LBs and Lewy neurites (Irizarry *et al.*, 1998, Wakabayashi *et al.*, 1997). α-Synuclein inclusions occur not only in SN DA neurons, but also in locus coeruleus, the dorsal vagal nucleus, and the enteric nervous system and oligomeric, soluble α-synuclein can be detected in human blood plasma and postmortem cerebrospinal fluid and is increased in plasma of patients with PD (El-Agnaf *et al.*, 2003, El-Agnaf *et al.*, 2006, Hughes *et al.*, 1992, Wakabayashi *et al.*, 1990).

One of the α-synuclein models is the A53T α-synuclein overexpressing transgenic mouse under the control of the mouse PrP promoter (Lee *et al.*, 2002). In this model, human mutated α-synuclein mRNA is expressed throughout the brain with a predominant expression in neurons, including SNc neurons, and with the protein present mostly in presynaptic terminals. Some, but not all, LB containing neurons undergo neurodegeneration. These mice have a late onset of motor symptoms, such as reduced spontaneous activity, and die prematurely due to the degeneration of neurons (Lee *et al.*, 2002, Martin *et al.*, 2006, Von Coelln *et al.*, 2006).

G2019S LRRK2 transgene and LRRK2 knockout

Funayama and coworkers found by linkage analysis a new locus being associated with PD in a Japanese family with autosomal dominant PD, which they termed PARK8 (Funayama *et al.*, 2002). Later, mutations in LRRK2 were found to be the cause (Paisan-Ruiz *et al.*, 2004, Zimprich *et al.*, 2004). Patients with LRRK2 mutations have autosomal dominant heredity with a late disease onset which does not differ from the idiopathic form (West *et al.*, 2005). LRRK2 is an protein with 6 domains and the most common mutation G2019S, accounting for about 3-6% of the familial PD cases and 1-2% of the sporadic cases, is related to an increase in kinase activity (Di Fonzo *et al.*, 2005, Gilks *et al.*, 2005, Kachergus *et al.*, 2005, Nichols *et al.*, 2005, West *et al.*, 2007). Most of the postmortem brains of G2019S LRRK2 patients contain LBs (Wider *et al.*, 2010).

In vitro studies have indicated that α-synuclein is a phosphorylation target of LRRK2 and that it might be implicated in the formation of LBs, as C-terminal epitopes of LRRK2 were detected in the halo of some LBs in the midbrain (Greggio *et al.*, 2006, Qing *et al.*, 2009). Furthermore, α-synuclein has been suggested to be a phosphorylation target of LRRK2 and a polymorphism in α-synuclein may modify the penetrance of LRRK2 mutation carriers in decreasing the age at onset (Botta-Orfila *et al.*, 2012, Qing *et al.*, 2009).

In paper I two LRRK2 mouse models were used for the analysis of interaction of LRRK2 with A53T α-synuclein.

A mouse model overexpressing human G2019S LRRK2 under the control of a CMV-enhanced human platelet-derived growth factor β-chain (CMVE-PDGFβ) was generated (Ramonet *et al.*, 2011). Aged G2019S LRRK2 mice display 18% reduction of SNc DA neurons, but unchanged density of TH positive nerve endings in striatum and unchanged levels of DA. The locomotor activity in aged G2019S LRRK2 mice is not impaired. Though no influence on behavior or histological level could be detected except for the SNc degeneration, electron microscopy revealed autophagic vacuoles in the cell body and axons, as well as abnormal condensed mitochondria (Ramonet *et al.*, 2011).

Mice with deletion of parts of exon 39 and all exon 40 of LRRK2, thereby disrupting LRRK2 function, were previously described (Andres-Mateos *et al.*, 2009). Brain development is not impaired, numbers of SNc neurons and TH immunoreactivity in the striatum are not changed in these mice. Likewise, levels of DA, serotonin and their metabolites are unchanged in different brain regions. In general, the LRRK2 knockout neither influences DA neuron degeneration, nor does it induce other features of PD under physiologic conditions in this model (Andres-Mateos *et al.*, 2009).

The MitoPark mouse model

The impairment of mitochondria was considered to be relevant to PD pathology before the first mutation in a gene causing PD was found and can happen at several different levels. Deletions in the mtDNA in SNc neurons of patients with PD were detected, though it was also reported that the number of deletions increased with age in neurologically healthy controls (Bender *et al.*, 2006). Neurons in the SNc are particularly affected by mtDNA deletions, as the frequency of mtDNA deletions was less in putamen and frontal cortex of PD patients (Bender *et al.*, 2008). Mitochondrial DNA encodes for 37 genes, of which 13 are respiratory-chain subunits. The subunits are part of complexes I, III, IV and V; only complex II is entirely encoded by nuclear DNA. The transcription requires several factors, which are encoded both by mitochondrial and nuclear DNA. One of these factors is the nucleus-encoded mitochondrial transcription factor A (Tfam), which binds to the mtDNA and promotes transcription (Dairaghi *et al.*, 1995). It also regulates mtDNA stability, replication and copy number and a homozygous systemic knockout is embryonically lethal (Clayton, 1991, Ekstrand *et al.*, 2004, Larsson *et al.*, 1998, Wang *et al.*, 2001). In these homozygous knockout embryos, the mtDNA is severely reduced, as is oxidative phosphorylation, leading to apoptotic cell death.

In MitoPark mice, Tfam is specifically knocked out in DA cells expressing DAT. The expression of DAT starts in the rat during embryonic development (Coyle, 1973). DAT mRNA in the mouse is expressed in SNc neurons, and much less expressed in parts of the VTA (Sanghera *et al.*, 1994).

MitoPark mice are generated by crossing two mouse strains: mice in which loxP-sites are inserted before exon 6 and after exon 7 of the Tfam gene, and mice expressing the cre-recombinase under the DAT promoter (Ekstrand *et al.*, 2007, Larsson *et al.*, 1998). Crossing DAT-cre mice with loxP-flanked Tfam mice leads to the disruption of Tfam in DA SNc neurons (Ekstrand *et al.*, 2007). As expected from previous studies, the mtDNA expression is severely reduced and the expression of COX subunit I mRNA is decreased already at the age of 6 weeks with a decrease of its enzyme activity found in 20-week-old mice. The DA cell loss in SNc is slowly progressive and corresponds with a decrease of DA levels in SN, VTA and cortex. Dysfunctional mitochondria resembling inclusion bodies observed in DA neurons in MitoPark mice do not stain for α-synuclein (Ekstrand *et al.*, 2007). In accordance with the decreasing DA levels in striatum, decreased vertical activity is detected at the age of 12 weeks and horizontal activity at the age of 24 weeks, modeling the early postural instability and bradykinesia in PD patients. The response to L-DOPA is DA depletion dependent in MitoPark mice (Ekstrand *et al.*, 2007, Galter *et al.*, 2010).

Dopaminergic neurons express TH and immunostaining for this protein allows histological study of these cells. For the electrophysiological study these neurons can either be made visible by the intracellular expression of a fluorescent protein or by a large amplitude, slowly activated current (hyperpolarization-activated current, I_h) that does not inactivate after prolonged membrane hyperpolarization (Lacey *et al.*, 1989). The molecular basis for this I_h current is hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Ludwig *et al.*, 1998). In the heart, I_f, the correlate of the brain I_h, is described as a "pacemaker", which is activated by hyperpolarization and shows slow kinetics (Brown & Difrancesco, 1980, Difrancesco, 1993). I_h exerts stabilization on the resting membrane potential and influences the input resistance (Chen *et al.*, 2001, Magee, 1998). In the SNc, it distinguishes DA from non-DA cells, but the importance of HCN channels for the pacemaking activity has been shown to decrease three weeks after birth (Chan *et al.*, 2007, Lacey *et al.*, 1989). There are four known isoforms, HCN1 to 4, which display differential expression patterns in the rodent brain. HCN1 is mainly expressed in neocortex, hippocampus, cerebellar cortex and brain stem. HCN2 is ubiquitously expressed with the highest expression in thalamus and brain stem. HCN3 is expressed only at a low level in the brain and HCN4 is most highly expressed in thalamus, olfactory bulb and the medial habenula (Notomi & Shigemoto, 2004, Santoro *et al.*, 2000).

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common neurodegenerative disease affecting about 5% of the people at the age of 65 and up to 30% of people over the age of 85 (Galimberti & Scarpini, 2013). It is the most common form of dementia, accounting for more than half of all cases. Because of the increase in life expectancy, the number of people with dementia is believed to increase with a projection of up to 81 million in 2040, thereby increasing the costs for medical treatment and care (Ferri *et al.*, 2005).

The disease was described by the Bavarian psychiatrist Alois Alzheimer in 1906 with the neuropathological phenotype and the known hallmarks of the disease, plaques, tangles and memory deficits, though senile dementia was known since the ancient Greeks and Romans (Berchtold & Cotman, 1998). During disease progression episodic memory declines, aphasia develops and judgment, decision-making and orientation are impaired. In the brain of aged individuals plaques and tangles are formed before the clinical onset of symptoms (Davies *et al.*, 1988). The pathology starts in the transentorhinal cortex, continues to the entorhinal cortex, the hippocampus, amygdala and other cortical areas (Braak & Braak, 1991). The plaque and tangle loads are more severe in early-onset AD, and correlate in these patients with dementia severity (Prohovnik *et al.*, 2006, Roth, 1986). Tangles are composed of hyperphosphorylated tau protein which aggregates into insoluble fibrils (Grundke-Iqbal *et al.*, 1986, Nukina & Ihara, 1986). Abeta is physiologically produced by the cells and is the main plaque component (Haass *et al.*, 1992, Masters *et al.*, 1985). It can accumulate due to missense mutations in amyloid precursor protein (APP), the precursor from which Abeta results by proteolytic cleavage by β- and γ-secretase (Haass & Selkoe, 1993, Kang *et al.*, 1987, Vassar, 2001). Depending on the location of the mutation in the Abeta region, the production of the self-aggregating $Abeta_{42}$ peptide is enhanced, or the aggregation of Abeta is generally promoted (Ancolio *et al.*, 1999, Levy *et al.*, 1990). The duplication of the APP locus can also cause AD as a dosage effect (Rovelet-Lecrux *et al.*, 2006). The most common mutations causing AD are located in the highly homologous presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes. Patients with autosomal dominant inheritance of APP and PSEN mutations have an early onset of disease (Levy-Lahad *et al.*, 1995, Raux *et al.*, 2005, Rogaev *et al.*, 1995). Many genes have been proposed to affect the risk of AD, but the apolipoprotein E (APOE) ε4 allele is the most prevalent (Saunders *et al.*, 1993, Strittmatter *et al.*, 1993). Carrying one allele copy can lower the age of onset by about 9 years (Corder *et al.*, 1993).

Other hypotheses for the pathogenesis of AD have been proposed, one of them is disruption of mitochondrial function, which has been described in the brain of patients with AD (Blass, 1993). The serine peptidase HTRA2/Omi is localized to the mitochondrial inter-membrane space and appears to modulate quality control in mitochondria as mice with a deletion of HtrA2 have increased ROS levels, accumulation of misfolded proteins in mitochondria and die perinatally when HtrA2 is depleted systemically (Martins *et al.*, 2004, Moisoi *et al.*, 2009,

Suzuki *et al.*, 2001). A connection to AD was found when HtrA2 was identified as a PSEN1 interaction factor in a yeast two-hybrid screen (Gray *et al.*, 2000). Moreover, APP is cleaved by and Abeta interacts with HtrA2 (Park *et al.*, 2006, Park *et al.*, 2004). HtrA2 is expressed as a proenzyme, which is directed to the intermitochondrial membrane where it attaches and is proteolytically cleaved, resulting in the soluble, mature form (Hegde *et al.*, 2002, Kadomatsu *et al.*, 2007). In response to DNA damage or other apoptotic stimuli, mature HtrA2 is released from the membrane into the cytosol and contributes to apoptosis (Hegde *et al.*, 2002, Suzuki *et al.*, 2001, Yang *et al.*, 2003).

The drug treatment of AD has been mainly symptomatic, such as affecting neurotransmitter levels in their availability. The implantation of cells producing neurotrophic factors into the brain of AD patients is a new promising treatment option which is still in its experimental phase (Eriksdotter-Jonhagen *et al.*, 2012).

AIMS OF THE THESIS

Neurodegenerative diseases involve pathological processes, which profoundly change gene expression profiles as well as protein and cellular functions in the brain. In addition, long-term treatment of these diseases influences gene expression and function. The aim of the present thesis was to study cellular, molecular and behavior changes, which correlate with disease progression using both human brain tissue and animal models of disease.

The specific aims were:

- to localize gene expression in novel genetic mouse models of Parkinson's disease (papers 1, 2)

- to study effects of L-DOPA treatment at behavioral and cellular levels, as well as on the striatal transcriptome (papers 3, 4)

- to investigate gene activity and cellular localization in different brain areas affected by neurodegeneration in Parkinson's disease and Alzheimer's disease (papers 5, 6)

MATERIAL AND METHODS

MATERIAL

Human tissue samples

Post mortem human brain tissue used for our studies was kindly provided by The Harvard Brain Tissue Resource Center (Belmont, USA), The Netherlands Brain Bank, the Brain Bank at Karolinska Institutet (Stockholm, Sweden) and the Queen Square Brain Bank for Neurological Disorders (London, UK). Human spleen tissue was provided by the University of Maryland Brain and Tissue Bank (Baltimore, Maryland, USA). Tissues were kept at -80°C until further use. Sections of 14 µm were used for in situ hybridization in papers IV, V and VI, and for immunohistochemistry in papers IV, V and VI. In papers V and VI, proteins were quantified using western blot, and enzyme activity was assessed in paper V.

Rodent models of Parkinson's disease

In our studies we used different mouse models of PD. All mice, except for MitoPark mice in paper II, and mice used in paper I, were kept and bred in our animal facility. The mice were group housed under standardized conditions, 12 hour light/dark cycle, food and water ad libitum, room temperature at 21°C and 60% humidity. All experiments performed at our lab were approved by the Stockholm North Ethical Committee on Animal Research.

MitoPark mice

MitoPark mice used in papers III, IV and VI were kept and bred in our animal facility, and MitoPark mice used in paper II were kept and bred at the NIH, NIDA (Baltimore, Maryland, USA) under standardized conditions. Two mouse strains are needed for the breeding of MitoPark mice: $DAT^{-/cre}$ and $Tfam^{loxP/loxP}$. DAT-cre mice were created by inserting the cre gene into the DAT locus by multiple cloning steps. $Tfam^{loxP/loxP}$ mice were created earlier (Larsson *et al.*, 1998). Both lines are regularly backcrossed to a C57Bl6 background. MitoPark mice were created by Ekstrand and coworkers who crossed DAT-cre mice with mice with a loxP-flanked Tfam allele resulting in MitoPark mice (DAT/DAT-cre $Tfam^{loxP/loxP}$) (Ekstrand *et al.*, 2007). MitoPark mice were compared to their control littermates (DAT/DAT $Tfam^{-/loxP}$ or DAT/DAT $Tfam^{loxP/loxP}$). In paper II mice lacking the DAT-cre construct (DAT/DAT $Tfam^{-/-}$ or DAT/DAT $Tfam^{-/loxP}$) were used as control for voltammetric recordings.

TH-MitoPark mice

For the assessment of electrophysiological properties of the ectopically TH expressing neurons in the striatum of L-DOPA treated MitoPark mice in paper IV, MitoPark mice were crossed with Tg(Th-EGFP)DJ76Gsat/Mmnc mice obtained from the Mutant Mouse Regional Resource Centers, hereafter referred to as TH-MitoPark mice. These mice were generated by injecting a modified bacterial artificial chromosome (BAC) into the pronuclei of FVB/N fertilized oocytes. The BAC containing the TH gene is modified by replacing the mRNA protein coding sequences by sequences encoding the eGFP reporter gene (Gong *et al.*, 2003). Heterozygous mice were crossed with MitoPark mice to obtain TH-MitoPark mice (DAT/DAT-cre $Tfam^{loxP/loxP}$ $THeGFP^{+/-}$). These mice co-express TH and GFP in striatal neurons after L-DOPA treatment (Figure 1).

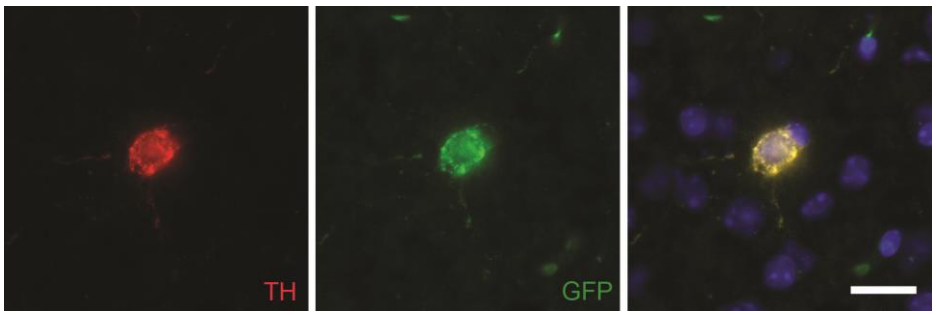

Figure 1: Co-expression of TH and GFP in the striatum of a L-DOPA treated TH-MitoPark mouse. Scale bar: 20 µm

α-Synuclein transgenic mice

A53T α-synuclein overexpressing mice were used in paper I to study the possible interaction of α-synuclein and LRRK2 in the pathogenesis of PD. These mice express α-synuclein under the control of the mouse PrP promoter, which is hindbrain selective (previously described by (Lee *et al.*, 2002)). They were backcrossed for at least ten generations and maintained on a C57BL76J background.

G2019S LRRK2 and LRRK2 knockout mice

In paper I, we studied the impact of a LRRK2 knockout (LRRK2 KO) and the overexpression of G2019S mutated human LRRK2 on the α-synuclein pathology in the hindbrain of a mutant A53T α-synuclein mouse model. The generation of LRRK2 KO mice, as well as transgenic mice overexpressing G2019S LRRK2, have been described previously (Andres-Mateos *et al.*, 2009, Ramonet *et al.*, 2011). Briefly, the CMV-enhanced human platelet-derived growth factor β-chain (CMVE-PDGFβ) is driving the expression of full-length human LRRK2 containing the G2019S mutation. This expression is neuron-specific and includes the DA neurons in the

SNc. In LRRK2 KO mice, part of exon 39 and the complete exon 40, which code for the kinase domain, are deleted and a stop codon is inserted thereby generating LRRK2-null mice. Mice are maintained on a B57BL76J background.

$Syn^{A53T}/LRRK2^{KO}$ and $Syn^{A53T}/LRRK2^{G2019S}$ mice

For the generation of $Syn^{A53T}/LRRK2^{KO}$ mice, first, double mutant mice (A53T-α-$Syn^{Tg}/LRRK2^{+/-}$) were generated by crossing hemizygous A53T-α-synuclein mice with LRRK2 KO mice. The progeny was crossed with $LRRK2^{+/-}$ mice to produce wild-type, $LRRK2^{KO}$, Syn^{A53T} and $Syn^{A53T}/LRRK2^{KO}$ mice. Double heterozygous $Syn^{A53T}/LRRK2^{G2019S}$ mice are generated by crossing heterozygous G2019S LRRK2 and A53T-α-Syn mice.

6-OHDA rat model

The injection of 6-OHDA into the striatum or medial forebrain bundle is widely used to achieve DA depletion in striatum of rodents (Ungerstedt & Arbuthnott, 1970). Unilateral stereotactic injections of 6-OHDA into the striatum of rats (4.4 mm posterior and 1.2 mm lateral to Bregma and 7.8 mm below the dura mater) were performed under isofluoran anesthesia. At 12, 24, 48 hours, 1 and 3 weeks after operations, rats were sacrificed by deeply sedating them with pentobarbital and transcardial perfusion with Lana's fixative (4% paraformaldehyde, 14% picric acid in PBS). Brains were immersed in 10% sucrose solution for cryoprotection.

METHODOLOGY

For the study of molecular, cellular and behavioral changes we used human tissue of patients with AD or PD and control tissue as well as animal models of PD and controls, some of which received a treatment. We examined the changes with suitable techniques depending on the research question. Figure 2 summarizes the material and methods used in this thesis.

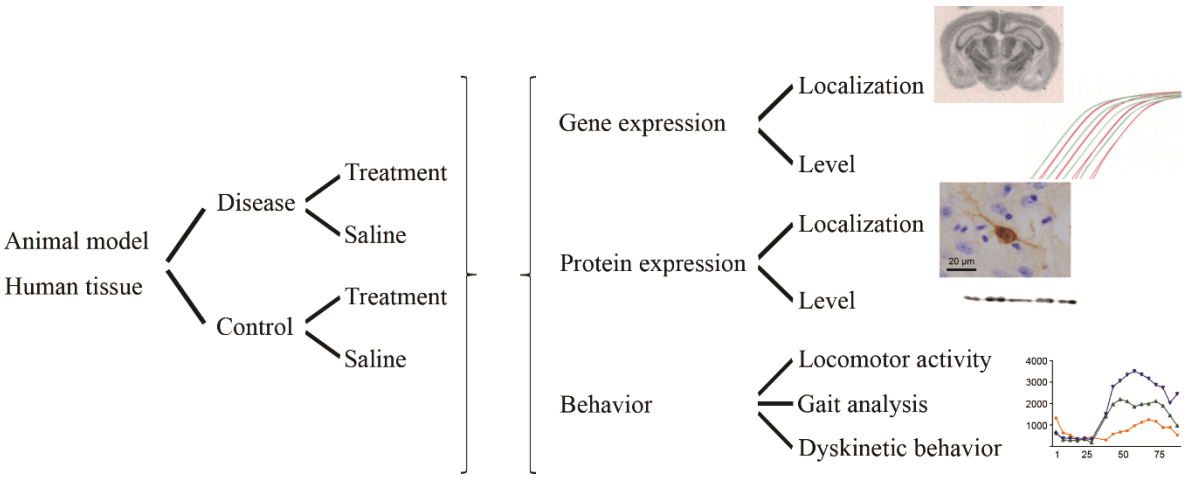

Figure 2: Overview over the material and methods used in this thesis

Behavior studies

The behavioral studies of MitoPark mice used in paper III were performed at the beginning of the dark period, the active phase of the circadian cycle of mice. Before each test, mice were habituated to the dimly-lit and noise controlled room for 60 minutes.

Open field activity

In paper III, we used an animal activity monitoring system (AccuScan VersaMax, AccuScan Instruments, Omnitech Electronics, Columbus, OH, USA) to detect drug-induced changes in open field activity. This system uses infrared-sensitive motion detection to record horizontal and vertical activity of a mouse in a Plexiglas activity chamber, measuring 42x42x30 cm (Figure 3). Mice were introduced to the chamber for 60 minutes before they received an injection and drug-induced activity was measured for 60 more minutes. The system collects activity data in five minute bins. Results were compared with two-way ANOVA using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

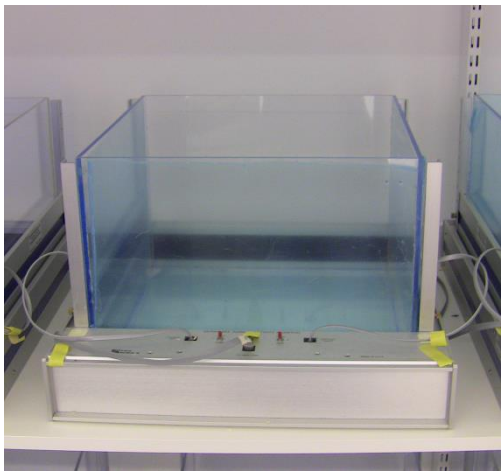

Figure 3: The AccuScan VersaMax system was used to assess locomotor activity of mice

Cylinder test

The cylinder test is widely used for quantification of motor performance in PD. Lundblad and coworkers used it to quantify abnormal involuntary movements in the unilateral DA depleted rat after L-DOPA treatment expressed as contralateral axial, limb and orolingual movements (Lundblad *et al.*, 2002). We adjusted these parameters in paper III due to the bilateral nature of the genetic DA depletion in the MitoPark mouse.

Glass cylinders were placed in front of a mirror. Thirty minutes after the L-DOPA or saline injection, mice were placed in the cylinder and videotaped for three minutes. With the help of the JWatcher software (Blumstein Dt, 2006) 120 seconds in the middle of the three minutes were evaluated and quantified using the following behaviors: duration of rearing, number of times mice reared, forepaw dyskinesia, forepaw dyskinesia with turning steps, three paw dyskinesia, third paw wall contact, duration of rotational behavior and time spent resting. We defined the dyskinesia score as the sum of duration mice spent with all of the three behaviors forepaw dyskinesia, forepaw dyskinesia with turning steps and three paw dyskinesia. Statistical analysis was performed using two-way ANOVA in paper III, with treatment and treatment duration as variables (GraphPad Software, San Diego, CA, USA).

Gait analysis

In MitoPark mice we measured gait parameters under voluntary and forced gait conditions before and after treatment. For the voluntary analysis we used the CatWalk System (Noldus Information Technology, Netherlands) which consists of a glass runway, a light bulb and a LCD camera (Figure 4). Paw placements are recorded by light, which escapes the plate when a paw is placed on the plate. Assessment of forced gait was performed on the TreadScan system (CleverSys Inc., Reston, VA, USA). Paw placements, which are detected by a LCD camera, are later analyzed by color differences from fur to paw. The following gait parameters were analyzed with the two systems if more than 11 consecutive steps were performed: base of support (BOS, average widths between either fore- or hind paws), stride length (distance between successive placements of the same paw) and swing speed (speed of the paw during the swing phase). Results were compared using Student's t-test (GraphPad Software, San Diego, CA, USA).

Figure 4: The Noldus® CatWalk ST system was used to perform voluntary gait analysis. Courtesy Noldus Information Technology BV

Immunohistochemistry and Western blot

Immunohistochemistry and Western blot are used for the detection of proteins. Immunohistochemistry allows cellular localization of proteins and Western blot the quantification of protein expression in tissues.

In papers III, IV and VI we performed immunohistochemistry on perfusion fixated rodent tissue, as well as post-fixated and fresh frozen human brain tissue.

Cryoections of 14 µm thickness were cut and mounted on slides (Superfrost Plus slides, VWR International AB, Sweden). The primary antibody was applied over night at 4°C and the respective secondary antibody for two hours at room temperature. For light microscopical visualization, the principle of avidin-biotin binding (ABC kit, Vectastain) followed by incubation with the chromogen substrate diaminobenzidine (Sigma Aldrich), as well as a nuclear counterstain with cresylviolet was used. For subcellular co-localization studies fluorescent labeled secondary antibodies with specific excitation and emission spectra were applied.

Western blots were used to quantify MPO expression in human brain tissue. Equal amounts of proteins were separated on a denatured gradient polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with non-fat milk and

incubated with the first antibody over night at 4°C. The specific antibody binding was visualized with a fluorophore coupled secondary antibody. The pixel density of the bands was measured with the freeware program Image J (Schneider *et al.*, 2012).

In situ hybridization

Oligonucleotide probes used for in situ hybridization (Good *et al.*, 2011, Westerlund *et al.*, 2008) were designed in ApE v1.13 developed by M. Wayne (http://biologylabs.utah.edu/jorgensen/wayned/ape/). The primers were designed to be 49 to 51 bases long, the melting temperature set to 72-76° and the GC content at 40-60% (Thermo Scientific, Ulm, Germany). Probes having a folding energy <-4 kcal/mol were selected and their sequence specificity was compared to the NCBI reference library (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In situ hybridization with radioactive labeled probes was used in papers I, II, III, IV, V and VI. With this method the mRNA expression in tissue can be visualized and quantified on film or microscopically analyzed if the slides are dipped in photographic emulsion. For in situ hybridization 14 µm sections of fresh frozen rodent or human tissue were used. Oligonucleotide probes were 3' labeled with ^{32}P-deoxyadenosine 5'-triphosphate using terminal deoxynucleotidyl transferase (Fermentas, VWR), cleaned and concentrated (ProbeQuant G-50 Micro Columns, GE Healthcare). The labeled probes were hybridized to the tissue over night at 42°C, washed in SSC and H_2O under highly stringent conditions and dehydrated using increasing concentrations of ethanol. The signal intensity was estimated using phosphoimaging plates (BAS reader 3000 system, Fujicolor Sverige AB) before exposure to autoradiographic film (BioMax MR Film, Eastman Kodak) or dipping in photographic emulsion (NTB2 solution, Carestream Health Inc., VWR) and developed after appropriate exposure time. For statistical analysis Student's t-test was performed in papers II, III and IV (GraphPad software version 5, GraphPad, San Diego, CA, USA).

RNA extraction and qRT-PCR

Total RNA was extracted from frozen tissue using a tissue homogenizer and trizol. Complementary deoxyribonucleic acid (cDNA) was synthesized (SuperScript III First-Strand Synthesis System, Life Technologies) and the abundance was measured (Sybr Green, Applied Biosystems) on a QuantStudio 6 qRT-PCR system (Applied Biosystems). Primer pairs with products of 120 to 150 bp were chosen when they had a ~100% efficiency and gave a single product on the dissociation curve. Quantitative RT-PCR was used in paper IV for the validation of RNA-Sequencing (RNA-Seq) data. We used Ppid as housekeeping gene. Statistical analysis was performed using one-way ANOVA (GraphPad Software, San Diego, CA, USA).

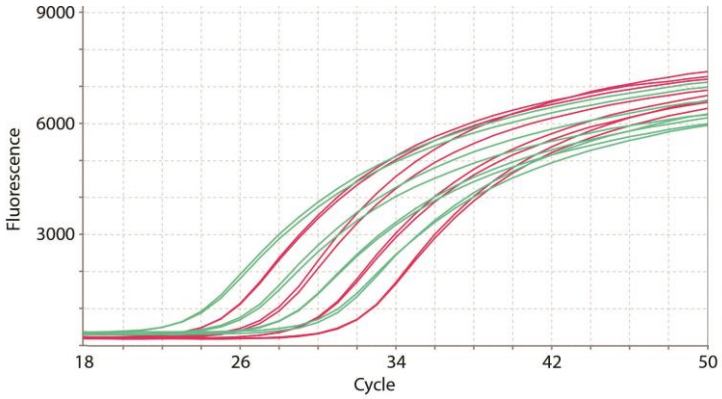

Figure 5: Representative standard amplification curves in a qRT-PCR run.

RNA sequencing

RNA-Seq is a high throughput method for the comparison of the transcriptome between study groups. It can also detect and quantify different splice variants and RNA editing of changes in expression levels. We used this method in paper IV to compare changes in expression levels in control and MitoPark mice treated chronically with either saline or L-DOPA. To perform this technique, the striatum was lysed, total RNA extracted with Trizol and a tissue douncer, messenger RNA pulled down with oligo(dT) priming and transcribed into cDNA (see figure 6). Adaptors are ligated to the cDNA to be able to attach them to the matrix on the flow cell where they are multiplied into clusters of the same sequence using PCR. Six libraries are pooled and hybridized to a lane with 100-bp paired end sequences. For the analysis of the sequence, fluorescently labeled nucleotides and a polymerase are added. The nucleotides are blocked at the 3' group so that only one nucleotide can be incorporated in one run. The incorporated nucleotides are imaged and give a signal for each cluster of cDNA sequences bound to the cell, and then another round is started with the addition of nucleotides and the polymerase, stepwise building the new strand of DNA. The order of nucleotides at each cluster is compiled from the fluorescent signal of the spot at each step, and all sequences are identified by annotation to the mouse reference genome. Several programs were used to align the sequences, for annotation and normalization. RNA-Seq has advantages over microarray in gene expression quantification. It does not rely on probe design and therefore novel genes can be detected. The dynamic range is bigger than that of microarrays and results are more closely related to absolute protein expression levels than microarray results (Fu *et al.*, 2009, Ning *et al.*, 2012, T Hoen *et al.*, 2008).

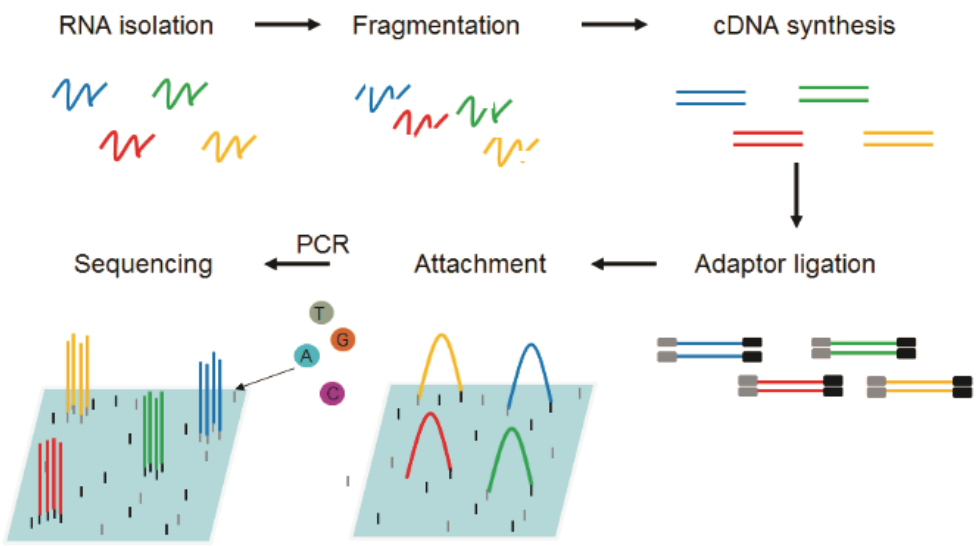

Figure 6: Schematic presentation of the RNA-Seq steps. Adapted from (Van Der Brug *et al.*, 2010) and (Mardis, 2008).

Electrophysiology

Electrophysiological measurements were used in paper IV to characterize TH expressing cells in striatum of L-DOPA treated TH-MitoPark mice. TH expressing cells were visualized by their eGFP expression using IR-DIC microscopy. To perform the measurements, brains were sliced in ice-cold extracellular solution, kept at 35°C for 30 minutes, and moved to room temperature before recordings. Recordings were performed at a temperature of 35°C. GFP fluorescent neurons were patched and identified by their passive and active membrane responses to step and ramp current injections. The current injections were chosen stepwise and started with an initial near-threshold step pulse to capture key active and passive properties to identify the cell type (Wang *et al.*, 2004).

RESULTS

MOUSE MODELS OF PD

Interaction of LRRK2 and α-synuclein, paper I

Polymeropoulos and coworkers discovered and mapped the first gene found in familial PD, α-synuclein (Polymeropoulos *et al.*, 1997). The disease causing mutation was an exchange of alanine on position 53 to threonine leading to fibrillization and accumulation of the protein in LBs in DA neurons of the SNc, a pathological characteristic of PD (Spillantini *et al.*, 1997). A few years later LRRK2 was mapped to chromosome 12 and six disease-causing mutations were discovered in different domains of the protein (Funayama *et al.*, 2002, Paisan-Ruiz *et al.*, 2004, Zimprich *et al.*, 2004). The most common mutation is the exchange of glutamine on position 2019 to serine in the kinase domain, leading to an increase in kinase activity, which causes toxicity and increased neurodegeneration (Smith *et al.*, 2006, West *et al.*, 2007). In the brains of PD patients harboring the G2019S LRRK2 mutation LBs are a common pathological finding, which led to the hypothesis of a possible interaction of these two proteins during the development of PD.

In paper I we studied the possible interaction between LRRK2 and α-synuclein by using transgenic mouse lines. The already well established mouse model with A53T α-synuclein under the control of the hindbrain-selective prion protein promoter was crossed with mice with a knockout of endogenous LRRK2 or overexpression of human G2019S mutant LRRK2 (Lee *et al.*, 2002, Von Coelln *et al.*, 2006). To characterize the expression patterns of the transgenic proteins we performed in situ hybridization of brain tissue sections from the different transgenic mouse lines. Both the A53T-α-synuclein and the G2019S-LRRK2 transgenes were broadly expressed in brain areas affected by neurodegeneration and were co-expressed throughout the brain at 9 and 12 months of age. The impaired acoustic startle response of A53T-α-synuclein mice was not changed by LRRK2 deletion or overexpression, neither was the premature lethality of these mice. At 13 to 14 months of age, the abnormal accumulation of phosphorylated human α-synuclein in the brainstem was modestly reduced in the absence of LRRK2 and slightly increased by the presence of human G2019S-LRRK2 overexpression, analyzed with a specific antibody against the pathological form of human α-synuclein. Glial fibrillary acidic protein (GFAP), a marker for astrocytes, was increased in the reticular formation and in the SNc in end-stage A53T-α-synuclein mice, whereas a marker for microglia, Iba1, was not affected by LRRK2-G2019S or LRRK-KO. Also the neuron numbers in the SNc stained for TH and Nissl were not changed by the modulation of LRRK2 expression. The results reported in this study do not support the hypothesis that LRRK2 expression influences A53T-α-synuclein pathology in the hindbrain.

Neurobiological events in the premotor phase of PD in the MitoPark mouse, paper II

The onset of motor symptoms in MitoPark mice begins at around 12 weeks of age, when the number of DA neurons in the SNc of the mice is approximately 40 percent reduced. In paper II we studied the neurochemical and electrophysiological properties in MitoPark mice aged 6 to 8 weeks, before the onset of motor impairment. Whole-cell patch-clamp recordings were performed on SNc neurons to assess the hyperpolarization-activated cation current I_h, which is an electrophysiological marker of SNc DA neurons mediated by hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels (Lacey *et al.*, 1989). HCN channels mediate I_h current in response to membrane hyperpolarization.

DA neurons of MitoPark mice had a smaller I_h amplitude and a reduction in the maximum hyperpolarization-activated cation conductance compared to control mice. In a next step we examined by in situ hybridization if the reduced I_h resulted from a reduced expression of HCN channels and if the voltage dependence of the remaining pool of functional HCN channels was changed. The mRNA expression levels of the four known isoforms of the HCN gene were compared in MitoPark mice at the ages of 6, 10 and 21 weeks with two oligonucleotide probes per isoform. HCN2 and 3 were strongly expressed in the SNc and VTA of MitoPark and control mice and the expression did not differ between the ages or genotypes analyzed (Figure 7). HCN1 and 4 were only weakly expressed in this area.

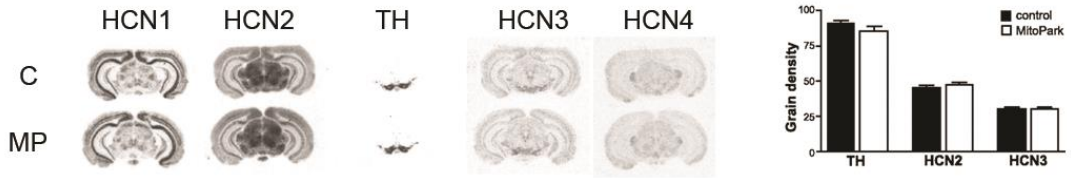

Figure 7: HCN1-4 mRNA expression on the level of the SN of a 10-week-old MitoPark mouse. Figure adapted from paper I, Good et al. FASEB J. 2011 Apr;25(4):1333-44

Current clamp recordings detected three different groups of SNc DA neurons, with one group of neurons having similar properties as the neurons in control mice. The other groups had divergent electrophysiological properties, which may represent increasing states of cellular disturbance in young MitoPark mice before the onset of motor impairment. These differences in MitoPark mice led to the assumption that there might be an altered striatal DA release. HCN channels are sensitive to levels of cyclic adenosine monophosphate (cAMP). An increase of Ca^{2+} in DA SNc cells in MitoPark mice, due to the mitochondrial failure, may lead to reduced cAMP levels, thereby decreasing HCN channel gating (Chan *et al.*, 2007). In vivo microdialysis in the dorsal striatum of control and MitoPark mice did not show a significant difference in the extraction fraction of DA or the basal extracellular concentration. However, the perfusion stimulated overflow of DA was significantly reduced in MitoPark mice. The DA

uptake mediated by DAT was unchanged in striatum of MitoPark mice but the vesicular DA release was reduced at the age of 6 to 8 weeks. Taken together, early physiological changes due to the mitochondrial failure in DA neurons are detectable long before overt behavioral deficits. Similar modifications are likely to happen in PD patients many years before the disease is diagnosed, and MitoPark mice are likely a promising model to investigate the initial neuronal changes during the early, pre-clinical stage of PD.

Establishment of MitoPark mice as a dyskinesia model, paper III

The aim of this study was the establishment of MitoPark mice as a genetic bilateral dyskinesia model. We used MitoPark mice at three different stages of DA depletion to study bilateral changes in motor behavior after chronic treatment with a moderate, clinically relevant dose of L-DOPA. Long-term treatment produced an age-dependent increase in locomotor activity and a normalization of gait parameters compared to control levels. Stride length as well as swing speed were increased in chronically treated compared to saline treated MitoPark mice after long-term treatment as studied using both, voluntary and forced gait analysis systems. Two days of treatment did not induce dyskinetic behavior, measured as the sum of the durations of forepaw dyskinesia, forepaw dyskinesia with turning steps and three paw dyskinesia.

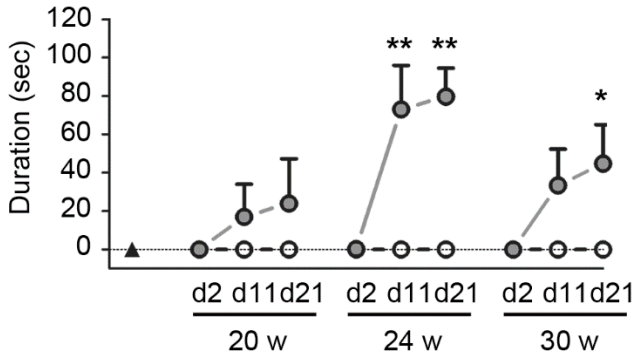

Figure 8: Dyskinesia score. Sum of the durations of forepaw dyskinesia, forepaw dyskinesia with turning steps and three paw dyskinesia of chronically saline (open circle) and L-DOPA (grey circle) treated MitoPark mice. The dyskinesia score of control mice is displayed as black triangle. Figure adapted from Gellhaar et al. Genes Brain Behavior 2015.

Eleven days of L-DOPA treatment induced an age-dependent increase in time of these bilateral behaviors in MitoPark mice. Saline treated MitoPark mice did not exhibit dyskinetic behavior but were rather immobile. No increase in activity was detected when control mice were chronically treated with L-DOPA. The expression of striatal FosB and prodynorphine was only increased in L-DOPA treated MitoPark mice.

Taken together, we showed that MitoPark mice have DA-depletion and treatment dependent changes in behavioral and biochemical measurements. The side effects of the treatment resemble those seen after the long-term L-DOPA treatment in PD patients, suggesting that the MitoPark mouse may serve a useful genetic and bilateral model for the study of drugs against LID.

TH expressing cells in the striatum of MitoPark mice after L-DOPA treatment, paper IV

In paper IV we studied the effect of chronic L-DOPA treatment on the transcriptome, protein expression and on electrophysiological properties of the striatum in a rodent PD model, the MitoPark mouse. The gene with the highest differential expression, as measured by RNA-Seq in the striatum after chronic L-DOPA treatment in MitoPark mice, was TH. Notably, L-DOPA did not induce expression of TH in striatum of control mice. We also found TH protein in cells of L-DOPA treated MitoPark mice, but did not detect any TH protein expressing cells in chronically saline or L-DOPA treated control mice, and just few in saline treated MitoPark mice. The induction of TH was DA depletion and L-DOPA dose dependent, as assessed by one and three days of treatment. Discontinued L-DOPA treatment reversed the expression of TH. An example of dose dependency for TH induction is shown in figure 9 in 20-week-old MitoPark mice.

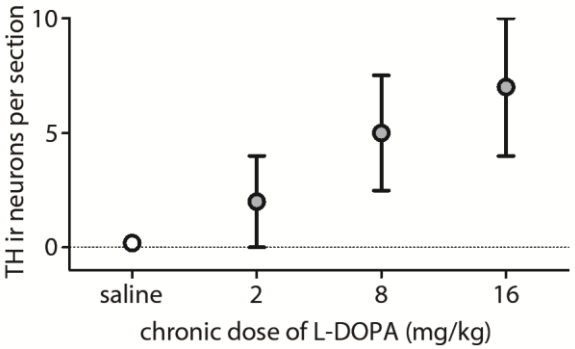

Figure 9: Increasing doses of L-DOPA induce an increasing number of TH ir cells in MitoPark mice (grey circles). 20-week-old animals were daily treated with 2, 8, or 16 mg/kg of L-DOPA for three weeks.

Markers of interneurons, such as calretinin, neuronal nitric oxide synthase (nNOS), glutamate decarboxylase, 67 kDa (GAD67) and parvalbumin colocalized with TH expression in striatum of chronically L-DOPA treated MitoPark mice. Many dopamine- and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32) immunoreactive (ir) medium spiny neurons were detected, but this expression pattern was never colocalized with TH expression. A co-expression with aromatic L-amino acid decarboxylase (AADC) indicates that the TH positive cells could produce DA.

By crossing MitoPark mice with mice expressing eGFP under the TH promoter, we generated TH-MitoPark mice. After L-DOPA treatment, eGFP expressing neurons in striatum were studied by electrophysiology which confirmed an interneuronal nature of these cells. In a next step we analyzed the relevance of these findings in human postmortem striatal tissue and detected an increase of TH mRNA expression in putamen and caudate nucleus of PD patients compared to neurologically healthy controls. This study demonstrates the induction of a subpopulation of TH ir neurons in the DA depleted striatum following L-DOPA treatment and MitoPark mice may be useful for further studies of the molecular effect of these TH ir neurons in the course of L-DOPA treatment.

NEURODEGENERATIVE DISEASE PATHOLOGY IN HUMANS

Analysis of the expression and activity of HTRA2 in AD and PD, paper V

The HtrA serine peptidase 2 (HTRA2) is localized in the mitochondrial intermembrane space and can mediate cell death following apoptotic stimuli by exposition of an inhibitor of apoptosis protein (IAP) binding motif or via its serine protease activity (Suzuki *et al.*, 2001). An N-terminal signal targets the precursor to the mitochondrial intermembrane space where it is cleaved to the proteolytically active form (Gray *et al.*, 2000). In 2005, the autosomal dominant mutations G399S and the A141S mutant allele in the HTRA2 gene were found in German PD patients (Strauss *et al.*, 2005). The resulting defect in protease activity led to an altered mitochondrial morphology and made cultured cells more susceptible to stress-induced cell death (Strauss *et al.*, 2005). Implication in AD was evidenced by the interaction of HTRA2 with Abeta, inducing neuronal apoptosis (Park *et al.*, 2004).

In paper V we studied the protein expression and enzymatic activity of HTRA2 as well as the mRNA expression in AD patient material. The HTRA2 variants A141S and G399S were analyzed in a Swedish case-control material for AD and PD. Protein quantification by Western blot revealed a significant decrease of the active form of HTRA2 in frontal cortex samples from AD cases, but unchanged levels of the unprocessed form. Unexpectedly, proteolytic activity of HTRA2 was significantly increased when compared to control tissue. Messenger RNA expression in frontal cortex samples indicated a slight non-significant reduction in AD tissue measured with two probes targeting different exons. The HTRA2 mRNA expression in the dentate gyrus was unchanged compared to control tissue. As expected, GFAP mRNA levels were significantly increased and UCH-L1 mRNA levels were significantly decreased in frontal cortex and hippocampus samples of AD compared to control tissue. The A141S variant was weakly associated with respect to genotype and allele frequencies between AD and controls, but not for PD and controls. The G399S mutation was absent in the Swedish AD case-control material and in the PD material no differences in genotype or allele frequencies were detected between PD and control samples. The unchanged levels of HTRA2 mRNA and of the unprocessed protein in the frontal cortex of AD patients suggest that pathological changes occur on posttranslational levels, as strengthened by the significant decrease of the active form in AD tissue. This could possibly be due to increased degradation, although the increase of enzymatic activity does not support such a hypothesis. Also, the disease associated mutations have been reported to decrease protease activity (Strauss *et al.*, 2005). This is not in line with our results, as one of the AD specimens contained the A141S variant, but the expression levels and protease activity did not differ from the other AD brain samples. Previous studies measured

the enzyme activity in vitro, which indicates that other factors, for example other genetic variants, influence the enzyme activity. For example, Plun-Favreau and coworkers reported that PINK1, the PARK6 gene, interacts and phosphorylates HTRA2 and that mutations in PINK1 lead to decreased phospho-HTRA2 levels (Plun-Favreau *et al.*, 2007).

Myeloperoxidase-expressing cells in affected brain regions in AD and PD, paper VI

Oxidative stress has been implicated in the pathogenesis of several neurodegenerative diseases. Phagocytes, macrophages and microglia constitute a major source of oxidants. They generate these with enzymes, such as MPO. This enzyme produces HOCl, and the levels of MPO have previously been reported to be increased in AD and PD brain samples (Choi *et al.*, 2005, Green *et al.*, 2004).

We analyzed the same human tissue samples with Western blot, immunohistochemistry and in situ hybridization for the expression of MPO. Western blot detected not only the bands for MPO, as assessed by human spleen lysate, but further bands with higher and lower molecular weight. Quantification of the band with high molecular weight showed an increased amount of MPO protein in PD striatum samples compared to control samples. Immunohistochemistry revealed MPO ir cells, which were small with a round or elongated shape (Figure 10).

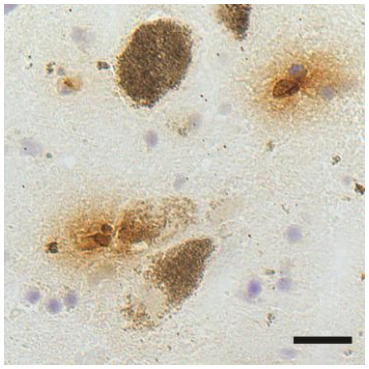

Figure 10: MPO ir cells (small brown and with a brown halo) located in close proximity to DA neurons (large cell filled with greyish neuromelanin granules) in the SNc of a PD patient. Scale bar: 25 µm.

These cells were also detected in the wall of larger blood vessels as well as in or around amyloid plaques in brain tissue from AD patients. The size and shape suggested these to be blood-derived cells, such as monocytes or macrophages, and not astrocytes or neurons. Binding to pyramidal neurons in the hippocampal CA1 region was most likely unspecific binding to modified proteins. In two rodent models of PD, the 6-OHDA rat and the MitoPark mouse, MPO ir was not detected in cells of the brain. Furthermore, MPO mRNA was not detected in human or rodent brain tissue, neither were hypochlorous acid modified proteins.

We conclude that MPO protein expression is significantly increased in hippocampus and frontal cortex of AD patients and in striatum and SNc of PD patients, brain areas specifically affected by the two forms of neurodegeneration, respectively. These results suggest that inhibitors of MPO might be useful in the treatment of AD and PD.

GENERAL DISCUSSION

The pathology of neurodegenerative diseases can be studied in human postmortem tissue and in animal models, which increase our understanding about the causes and possible treatment options of the disease. In studies presented in this thesis, we examined different animal models of PD and where possible, tried to validate these findings in human postmortem tissue.

The earliest animal models of PD were toxin induced models, which reproduce key motor symptoms and a fast degeneration of DA neurons. Even though different degrees of DA depletion can be achieved by different injection regimens, the slow development of DA cell death occurring in PD is difficult to reproduce. With genetic models such as the MitoPark mouse we are able to model the effects of progressive degeneration from pre-symptomatic to late stage PD in a time-frame of about eight months. That gives the possibility to study treatments and molecular changes at different stages of disease. We detected for example in young MitoPark mice an altered function of the hyperpolarization-activated cation current, which was not caused by altered mRNA expression of the four HCN channels. This reduced pacemaker activity of DA neurons in presymptomatic PD stages had not been demonstrated before and gives valuable information about pathological processes in human brain, long before DA neurons degenerate. At an older age MitoPark mice can be used to study treatment effects on behavioral and molecular level in order to improve the therapy of late stages of PD. Daily treatment with L-DOPA induced progressive LID in MitoPark mice and the induction of TH expressing cells in the striatum, changes that can also be detected in PD patients. We established the conditions to use MitoPark mice as a new, toxin-free LID model, in which novel anti-dyskinetic drugs can be tested to reduce the side effects of L-DOPA treatment.

Although rodent models show many of the PD symptoms, some features are not reproduced. The immunological components in neurodegenerative diseases are very difficult to study in models because they are seldom present. A good example is the increase of MPO ir blood-derived cells detected in disease affected regions in AD and PD cases which we failed to find in several mouse and rat models of disease.

The discovery of genetic causes of PD led to the development of transgenic animal models for the study of cellular changes caused by a mutation. The results found in cell culture do often not match those found in animal models and complicate the interpretation of the "real effect" of the mutation. Cell culture experiments showed that knockdown of LRRK2 induced an increased number of small LBs in cells with α-synuclein inclusion formation (Guerreiro *et al.*, 2013). Our results in transgenic mouse models failed to reproduce this finding. The introduction or ablation of LRRK2 together with mutated human α-synuclein in a PD mouse model did not result in altered aggregation of LBs, although both genes were expressed in the same brain area.

To date, animal models have been helpful in modeling PD and AD pathology, although all models have their limitations. Toxin-induced degeneration models the symptoms but does not reveal the cause of PD. Overexpression and knockout of disease-implicated genes and cell-specific ablation of e.g. the transcription factor Tfam in mouse models have increased our understanding of pathological mechanisms. However, we need to keep in mind that the expression of human mutated genes in animal models might induce a mismatch between the human and animal protein functions and pathways. In addition, all findings in animal models need to be validated and confirmed in human cases. One important limitation of analyzing human brain material is that we can study only a given time point, while we can study many pathways with various sophisticated methods in animal models. Yet, we need to keep in mind that only the comparison of results from animal models with human findings will lead to the development of better animal models that more closely resemble the human disease.

The use of animal models of diseases complements the analysis of post-mortem tissue from patients with neurodegenerative diseases in the effort to understand the underlying pathological mechanisms. Promising recent advancement has been made in the discovery of predictive biomarkers for early stages of disease, in order to find treatments to halt degeneration before irreversible damage occurs in the brain. Nevertheless, finding the cause (or causes) of neurodegeneration will remain a major research challenge.

CONCLUSIONS

We used a variety of mouse lines to model specific pathological or genetic findings reported in Parkinson's or Alzheimer's disease. Where possible, we corroborated our results in follow up analyses of human brain samples.

- In transgenic mouse models with overexpression or knockout of the PARK genes α-synuclein and LRRK2 we mapped the expression patterns of the transgenes. Although they were expressed in the same brain areas including the brain stem, we detected no influence on the number of DA neurons and α-synuclein pathology was largely independent of the presence of LRRK2, indicating separate pathological pathways.
- In young MitoPark mice, before the onset of motor symptoms, electrophysiological studies demonstrated that the pacemaker function and the neurotransmitter release was impaired. We studied the expression of HCN1-4 channels necessary for the pacemaker activity and did not detect any difference in mRNA expression levels in SNc compared to control mice, indicating post-translational adaptations. The study revealed striking dysfunctions of the nigrostriatal circuits in presymptomatic animals and provides a good animal model for early phases of PD.
- In middle aged and old MitoPark mice we demonstrated that chronic L-DOPA treatment normalized gait parameters, but also induced hyperactivity and dyskinetic movements in a DA-depletion dependent way. We established the requirements to use MitoPark mice as dyskinesia model and demonstrated the induction of dyskinesia markers in the striatum. This animal model of late phases of PD will probably be very useful to detect novel therapeutic targets to counteract L-DOPA induced adverse effects.
- In a further study on the effects of chronic L-DOPA treatment on striatal neurons in MitoPark mice we established that the induction of TH mRNA is one of the most robust effects on the transcriptome. We showed that TH was induced in neurons with histological and electrophysiological characteristics of striatal interneurons, and that the induction was dependent on the degree of DA depletion and the dose of L-DOPA. The TH induction in the mouse model was validated in human samples where we reported increased mRNA levels in putamen and caudate nucleus of PD cases.
- In human DNA and brain samples from AD or PD cases and matched controls we analyzed alterations in allele frequencies and gene activity of the mitochondrial serine peptidase HTRA2. In frontal cortex and hippocampus of AD cases we detected unaltered HTRA2 mRNA levels, whereas the level of the processed protein was significantly decreased and the enzymatic activity increased in frontal cortex samples from AD cases. In addition we discovered a weak association of one allele with AD, but not with PD, further supporting an involvement of this gene in neurodegeneration.
- In several brain areas affected by neurodegeneration in AD and PD we detected an increased number of MPO ir cells with the size and shape of blood-derived monocytes / macrophages. In our PD animal models these cells were not detected. Our results support a role for an inflammatory process in AD and PD and the study of anti-inflammatory treatments.

ACKNOWLEDGEMENTS

I would like to thank the many people who have supported and helped me throughout the years on this exciting journey towards a PhD.

My supervisors:

Dagmar, thank you for being a truly great supervisor, I really appreciate your frankness and our long discussions; for sharing your seemingly endless knowledge with me, for letting me grow as a researcher and for having trust in me.

Andrea, for always finding the time to answer my questions and for giving me the opportunity to delve into methods for studying genetic alterations.

Lars, for bringing great people together and creating a dynamic atmosphere in the lab, for your scientific advice and sharing your great knowledge.

Collaborators

Allissa Dillman, Mark R. Cookson, Daniel Marcellino, Dan Sunnemark, Håkan Eriksson, Henrike Planert, Gilad Silberberg, Maria Ankarcrona, Homira Behbahani, Darren Moore and Carl Lupica for exciting collaborations of which I hope there will be more in the future.

Eva Lindqvist, Karin Lundströmer, Karin Pernold, Margareta Widing and Katrin Wellfelt, for keeping the lab running, patient teaching, all support with animal work, and your technical expertise.

Bengt Winblad and Gunilla Johansson at Swedish Brain Power.

All members of the Department of Neuroscience:

Sandra Ceccatelli and Staffan Cullheim, the present and past head of the department.

Study directors Abdel el Manira and Gilberto Fisone.

Ida Engqvist, Filip Lindholm and Michael Wulff for help with computer issues I've had.

Elzbieta Holmberg, Anna Hammervik, Anders Johansson, Boje Persson, Christina Ingvarsson, Eliana Sobarzo, Eva Tegelberg, Frida Holmqvist, Karin Lagerman, Therese Brogårde, Thomas Johansson, Sofia Aminoff and Åsa Garmager, the administrative and technical staff at the department.

Niklas Lilja, Helena Ericsson, Anna Lindeberg, Ingela Berg, Linda Thors, Martina Andersson and Tua Finnman at the animal department, and specifically Anna Haglund for keeping track of all our mice.

Björn Meister, Helén Asp, Ingrid Blomgren, Lennart Brodin, Liz Strandelin and Peter Wallén for support with the teaching of students.

Past and present colleagues in the lab and at the department:

Alexandra Karlén, Anna Mattsson, Linus Olson, Anna Josephson, Gabriella Smedfors, Gustaf Wigerblad, Jacob Kjell, Jaime Ross, Josefin Koczy, Mimi Olofsson Westerlund, Lisette Graae, Simone Codeluppi, Teresa Fernandez Zafra, Tobias Karlsson, Guiseppe Coppotelli, Mehrafarin Ramezani, Caroline Ran, Anna Anvret, Ezri Juárez and Sophia Savage for scientific discussions and making it even more fun to go to work.

The Brené, Larsson and Paddock group, Adam Sierakowiak, Arianna Paganelli, Elin Åberg, Fredrik Sterky, Seungmin Lee, Silvia Paddock, Robert Karlsson and Stefan Brené.

Our master students Rianne Zwiers, Kris Maas, Theresa Schredelseker and summer students Julien Klimmt, Nathalie Grün and Kalyani Mudigonda for contributions to our projects and challenging me in various ways.

Richard, Michalina, Susanna, Sam, Tim, Teresa and Dorota for fun discussions and company.

Norija Zivkovic for all the early morning chats.

Friends

Karina for convincing me to perform research in neuroscience and Saskia for suggesting Sweden when I said I wanted to go just a little bit north from southern Germany.

Kerstin, Johanna, Christine und Nicole für durchtanzte Stockholmsnächte und gute Gespräche bei der einen oder anderen Flasche Wein.

Steffi, Jasmin, Frauke und Sven, für wundervolle Freundschaften, wenn auch hunderte Kilometer zwischen uns liegen.

Family

Christina, Jan och Benny för att ni med öppna armar välkomnade mig och alla mina tyska traditioner och egenheter.

Tante Renate, Onkel Wolfgang und Onkel Erich für Unterstützung über all die Jahre.

Mama, Papa und Martin für eure Liebe und Glauben an mich. Ohne euch wäre ich nicht da, wo ich heute bin.

Jonny, för alla äventyr

This work has been supported by Swedish Research Council, Swedish Brain Power, Karolinska Distinguished Professor Award, ERC Advanced Investigator grant 322744 and Swedish Parkinson Foundation.

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