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On the roles of genes in Parkinson's disease

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Cover *Mattias Karlen*

The *brain* and the *DNA sequence* represent the focus of the thesis on the genetic causes of Parkinson's disease. The seeds from the climbing shrub *Mucuna pruriens* contain high concentrations of levodopa, a precursor of the neurotransmitter dopamine which, was used to treat Parkinson's disease in ancient times.

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ABSTRACT

Parkinson's disease is a progressive neurodegenerative disorder which affects 1% of the population over the age of 60. In order to identify candidate genes with a potential role in Parkinson's disease pathology, we investigated genes which are involved in protein aggregation and the ubiquitin-proteasome system (*α*-synuclein and ubiquitin carboxy-terminal hydrolase L1, *UCH-L1*), oxidative stress (*DJ-1*), mitochondrial function (mitochondrial transcription factor A, *TFAM*), regulation of drug/toxin levels (multi-drug resistance 1, *MDR1* and alcohol and aldehyde dehydrogenases, *ADH* and *ALDHs*), as well as a gene with unknown function, but highly implicated in Parkinson's disease genetics (leucine-rich repeat kinase 2, *LRRK2*). (1) Using *in situ* hybridization, we characterized the cellular localization of candidate genes in both human and rodent tissues and found marked diversity in terms of areas and intensities of transcriptional activity. Some genes exhibited a widespread neuronal expression (*UCH-L1, DJ-1, SNCA*), one showed a particularly high expression in the dopamine target area striatum (*LRRK2*), some were expressed also in non-neuronal tissues (*LRRK2, DJ-1, MDR1*), and others exclusively so (*ADH1, ADH4*). (2) We also searched for genetic variability in a Swedish case-control sample consisting of 310 Parkinson patients and 315 controls, which resulted in identification of several potential risk factors (*LRRK2* G2019S; *MDR1* 1236C/T; *SNCA* rs2737029 (A/G) and rs356204 (A/G), as well as protective factors (*UCH-L1* S18Y) for disease. (3) Behavior, gene expression and/or brain neurotransmitter levels were studied in different transgenic and drug-induced rodent models (*Adh4-/-*; *α*-synuclein over expressing and *α*-synuclein-/-; *Darpp-32-/-* and *Darpp-32* T34A mutant and MitoPark mice and in 6-OHDA treated rats). A possible co-regulation between *Lrrk2* and *α*-synuclein gene activities was found. Moreover, *Adh4-/-* mice displayed alterations in substantia nigra dopamine levels, as well as in dopamine-related behavior. In conclusion, the findings in the present thesis suggest an important role for genetic risk factors in the pathogenesis of Parkinson's disease. Due to the great complexity of the disease, it seems likely that several molecular pathways and networks involving different genes and downstream effectors can affect the trophic support and/or survival of dopamine neurons, subsequently leading to Parkinson's disease.

LIST OF PUBLICATIONS

I. **Westerlund M**, Galter D, Carmine A, Olson L (2005) Tissue- and species-specific expression patterns of class I, III, and IV Adh and Aldh 1 mRNAs in rodent embryos. *Cell Tissue Res* 322(2):227-36

II. **Westerlund M**, Belin AC, Felder MR, Olson L, Galter D (2007) High and complementary expression patterns of alcohol and aldehyde dehydrogenases in the gastrointestinal tract: implications for Parkinson's disease. *FEBS J* 274(5):1212-23

III. Carmine Belin A, **Westerlund M**, Anvret A, Lindqvist E, Pernold K, Ögren S O, Duester G, Galter D. Modeling Parkinson's disease genetics: altered function of the dopamine system in Adh4 knockout mice. *Submitted manuscript*

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VI. **Westerlund M**, Carmine Belin A, Anvret A, Håkansson A, Nissbrandt H, Lind C, Sydow O, Olson L, Galter D. Cerebellar α-synuclein levels are decreased in Parkinson's disease and do not correlate with *SNCA* polymorphisms associated with disease in a Swedish material. *Submitted manuscript*

VII. **Westerlund M**, Carmine Belin A, Anvret A, Bickford P, Olson L, Galter D (2008) Developmental regulation of leucine-rich repeat kinase 1 and 2 expression in the brain and other rodent and human organs: Implications for Parkinson's disease. *Neuroscience* 152(2):429-36

VIII. Galter D, **Westerlund M**, Carmine A, Lindqvist E, Sydow O, Olson L (2006) LRRK2 expression linked to dopamine-innervated areas. *Ann Neurol* 59(4):714-9

IX. Carmine Belin A, **Westerlund M**, Sydow O, Lundströmer K, Håkansson A, Nissbrandt H, Olson L, Galter D (2006) Leucine-rich repeat kinase 2 (LRRK2) mutations in a Swedish Parkinson cohort and a healthy nonagenarian. *Mov Disord* 21(10):1731-4

X. **Westerlund M**, Ran C, Borgkvist A, Sterky FH, Lindqvist E, Lundströmer K, Pernold K, Brené S, Kallunki P, Fisone G, Greengard P, Larsson N-G, Olson L, Galter D. Leucine-rich repeat kinase 2 and other PARK genes in rodent models of Parkinson's disease. *Submitted manuscript*

XI. Galter D, **Westerlund M**, Belin AC, Olson L (2007) DJ-1 and UCH-L1 gene activity patterns in the brains of controls, Parkinson and schizophrenia patients and in rodents. *Physiol Behav* 92(1-2):46-53

XII. Carmine Belin A, **Westerlund M**, Bergman O, Nissbrandt H, Lind C, Sydow O, Galter D (2007) S18Y in ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) associated with decreased risk of Parkinson's disease in Sweden. *Parkinsonism Relat Disord* 13(5):295-8

XIII. Belin AC, Björk BF, **Westerlund M**, Galter D, Sydow O, Lind C, Pernold K, Rosvall L, Håkansson A, Winblad B, Nissbrandt H, Graff C, Olson L (2007) Association study of two genetic variants in mitochondrial transcription factor A (TFAM) in Alzheimer's and Parkinson's disease. *Neurosci Lett* 420(3):257-62

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AD	Alzheimer's disease
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
BBB	blood-brain barrier
CNS	central nervous system
COMT	catechol-O-methyltransferase
$\chi 2$	chi-square
DARPP-32	dopamine- and cAMP-regulated phosphoprotein of 32 kDa
DAT	dopamine transporter
DOPA	dihydroxyphenylalanine
DOPAC	dihydroxyphenylacetic acid
DOPAL	dihydroxyphenyl-acetaldehyde
GI	gastrointestinal
HPLC	high-performance liquid chromatography
HVA	homovanillic acid
i.p.	intra peritoneal
LB	Lewy body
LRRK	leucine-rich repeat kinase
MDR1	multi-drug resistance 1
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	mitochondrial DNA
6-OHDA	6-hydroxydopamine
PCR	polymerase chain reaction
RA	retinoic acid
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
SEM	standard error of the mean
SNP	single nucleotide polymorphism
TFAM	transcription factor A, mitochondrial
UCH-L1	ubiquitin carboxy-terminal hydrolase L1
UPS	ubiquitin proteasome system
WGA	whole-genome association

INTRODUCTION

Parkinson's disease

Parkinson's disease is a heterogeneous and genetically complex disorder of largely unknown etiology. Several hypotheses have been put forward for its causes, but it remains to be resolved how the different proposed mechanisms, individually or in concert, cause neurodegeneration. The disease acquired its present name from the British neurologist James Parkinson (1755-1824) who described the clinical symptoms of the progressive disease in An Essay on the Shaking Palsy (*paralysis agitans*, Latin) in 1817[1]. One of the earliest descriptions of Parkinson's disease originates from the ancient Indian medical system *Ayurveda* created more than 4500 years ago. According to the *Ayurveda* system, the disorder was referred to as *Kampavata* and it was treated with seeds from *Mucuna pruriens*[2]. The relieving substance of the plant was unknown at that time and it was not until the 1930's that the active component L-3,4-dihydroxyphenylalanine (L-Dopa) was isolated[3]. The finding had little impact at that time, since the involvement of dopamine in Parkinson's disease had not yet been discovered. In the early 1900's, the neurologist Frederic Lewy observed inclusion bodies in the brains of Parkinson patients, and the inclusions were hence given the name Lewy bodies (LBs). Today, these eosinophilic cytoplasmic inclusions constitute one of the pathological hallmarks of the disease. About the same time, Tretiakoff reported depigmentation, nerve cell loss and gliosis in substantia nigra[4] and emphasized the involvement of this brain area in Parkinson's disease. The key neurotransmitter involved in the disease is the catecholamine dopamine, which was identified in the brain by Arvid Carlsson and colleagues in 1958[5], and he later suggested that Parkinson's disease was related to brain dopamine[6]. In the following year, Ehringer and Hornykiewicz reported striatal dopamine deficiency in Parkinson's disease[7] and this important discovery was accompanied by a thorough mapping of the brain monoaminergic systems[8-13].

Etiology, pathology and treatment

The etiology of Parkinson's disease remains largely unknown, but appears to include genetic risk factors that alone or possibly in combination with environmental factors cause the disease. Key symptoms result from progressive degeneration of dopamine neurons in substantia nigra pars compacta, accompanied by neurodegeneration in other brain regions such as locus coeruleus and the dorsal motor nucleus of the vagus nerve. Loss of striatal dopamine innervation results in a clinical phenotype characterized by *bradykinesia*, slowness of movements, *tremor*, trembling in hands, arms, legs or head, *rigidity*, stiffness of trunk and limbs, and *postural instability* or

impaired balance. The slowly progressing symptoms typically start by affecting one side of the body, spreading to involve both sides and progressing into severe motor disabilities. Neuropathological investigations of Parkinson patients show degeneration of the neuromelanin-containing neurons of substantia nigra pars compacta in the midbrain leading to visible depigmentation of the area. The degeneration is most commonly accompanied by insoluble intracellular proteinaceous LBs and Lewy neurites in the brain stem and cortical areas[14].

Although the pathology of Parkinson's disease is relatively well understood, there is limited understanding of the etiology, except for some genetic forms of the disease. Due to the diversity in pathology between Parkinson patients, it seems likely that several molecular pathways and networks involving different genes and downstream effectors, together affect the trophic support and/or survival of dopamine neurons (Fig. 1). The heterogeneity of the disease has attracted considerable scientific interest and has generated several hypotheses concerning possible causes, including protein misfolding, mitochondrial and ubiquitin-proteasome dysfunction, oxidative stress, inflammation, apoptosis and exposure to environmental toxins. However, it remains to be resolved exactly how the different genes already implicated in Parkinson's disease and others yet to be discovered, come together in the pathogenic events.

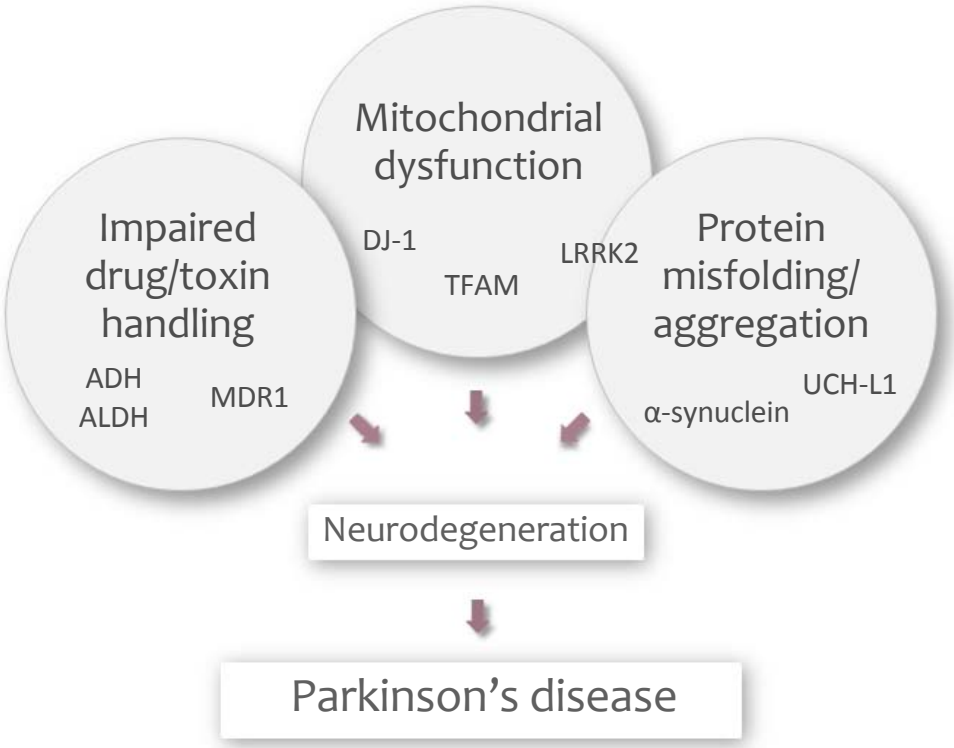


Fig. 1. Candidate genes for Parkinson's disease investigated in the present thesis and their suggested role in the disease pathogenesis. The output of the different pathways is suggested to affect the trophic support or survival of dopamine neurons, subsequently leading to neurodegeneration and Parkinson's disease.

The therapeutic strategies currently available for Parkinson's disease include drug therapy and surgical treatment[15]. The fist category includes drugs that serve to increase the levels of dopamine in the brain. Levodopa (L-dopa), the most commonly used drug, is a precursor of dopamine that passes the blood-brain barrier (BBB) and is converted to dopamine in the brain. L-dopa is efficient in relieving symptoms in the early phases of the disease, extending the period of time in which the patient can live a relatively normal life. However, the effect of the drug gradually declines as the disease progresses. Other types of drugs used alone or in combination with L-dopa, include enzyme inhibitors which decrease the breakdown of dopamine (e.g. monoamine oxidase-B, MAO-B and catechol-O-methyltransferase, COMT inhibitors) and dopamine receptor agonists which mimic the effect of dopamine. Another option is to deliver L-dopa by a pump directly into the small intestines[16]. However, in patients with advanced Parkinson's disease, complementary surgical treatment or deep brain stimulation (surgical implantation of an electrode into a selected brain area, usually the subthalamic nucleus) can be used. These treatments can have marked effects on disease symptoms, but do little to deter disease progression. Identification of new risk factors for Parkinson's disease can therefore be valuable in order to find ways of delaying, slowing or possibly preventing disease outbreak.

Dopamine metabolism

Degeneration of neurons in substantia nigra of Parkinson patients results in loss of dopamine innervation of striatum. Dopamine plays an important role in a wide variety of functions including movement, reward, motivation and cognition. Dopamine is formed from the amino acid tyrosine in a two step process in which tyrosine is first converted into 3,4-dihydroxyphenylalanine (DOPA) by the rate limiting enzyme tyrosine hydroxylase (TH), after which DOPA is converted to dopamine by DOPA-decarboxylase (or aromatic amino acid decarboxylase). Dopamine can in turn be converted into noradrenaline and adrenaline in other cell types than the dopamine neurons. Dopamine is packed into synaptic vesicles by the vesicular monoamine transporter (VMAT) and following release from the presynaptic nerve terminal, it diffuses out into the synaptic cleft and binds to dopamine receptors. Unbound dopamine is transported back into the presynaptic nerve terminal by the dopamine transporter (DAT), and repacked into synaptic vesicles. Dopamine can also be degraded to the inactive metabolites dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA) by the enzymes MAO and COMT. A bi-product formed in dopamine neurons is neuromelanin, which has given its color and name to the substantia nigra.

Genetic involvement in Parkinson's disease

The majority of Parkinson's disease cases are "sporadic" and the disease was long considered a non-genetic disorder. An early exception and probably one of the first documented examples of a genetic component in Parkinson's disease was the observations by Leroux in 1880[17], who suggest that heritable factors might increase disease susceptibility[18]. More than half a century later, Allen reported familial forms of parkinsonism inherited as a dominant trait in North Carolina, USA[19], and Henry Mjönes described autosomal dominantly inherited cases in Sweden[20]. A pioneering discovery was made in 1996 by Polymeropoulos and colleagues[21] who reported genetic linkage in an Italian family with an autosomal dominant form of the disease. The discovery was soon followed by identification of a mutation in the α-synuclein gene located at the PARK1 locus[22] and subsequently by identification of other chromosomal regions (PARK1-PARK13) with a suggested linkage to the disease. In six of these loci mutated genes have been identified causing autosomal dominant or recessive Parkinson's disease: α-synuclein (*SNCA*; PARK1), parkin (*PRKN*; PARK2), ubiquitin carboxy-terminal hydrolase L1 (*UCH-L1*; PARK5), PTEN-induced protein kinase 1 (*PINK1*; PARK6), *DJ-1* (PARK7), and leucine-rich repeat kinase 2 (*LRRK2*; PARK8)[23] (Fig. 2).

A genetic variant can be a mutation or a single nucleotide polymorphism (SNP), the latter of which is present at a frequency of >1% in the population. Other inter-individual genetic differences that may alter disease susceptibility include small RNA species and copy number variants. It has been estimated that approximately 10–15% of all Parkinson patients have a known genetic component involved in the disease, although this might become adjusted in the future as new genetic markers are identified[24]. A reason why the genetic influence on the disease has been underestimated is the relatively high age of onset, the reduced penetrance of some of the mutations and a complex genetic heredity. Other factors leading to an underestimation are that patients may not recall disease symptoms in affected relatives or that relatives may have passed away before developing any symptoms.

The impact of genetic factors in Parkinson's disease has gotten variable support from epidemiological studies[25-27]. The risk ratio has been reported to be increased in siblings and the offspring of affected patients, but the concordance between mono- and dizygotic twins has been found to be lower than expected. This opens for the possibility that gene-environmental interactions or epigenetic factors may play an important role in genetically predisposed mutation carriers. However, positron emission tomography (PET) data of monozygotic twins has shown higher concordance for decreased L-dopa binding, indicating marked heredity, albeit with other factors influencing the age of onset[28].

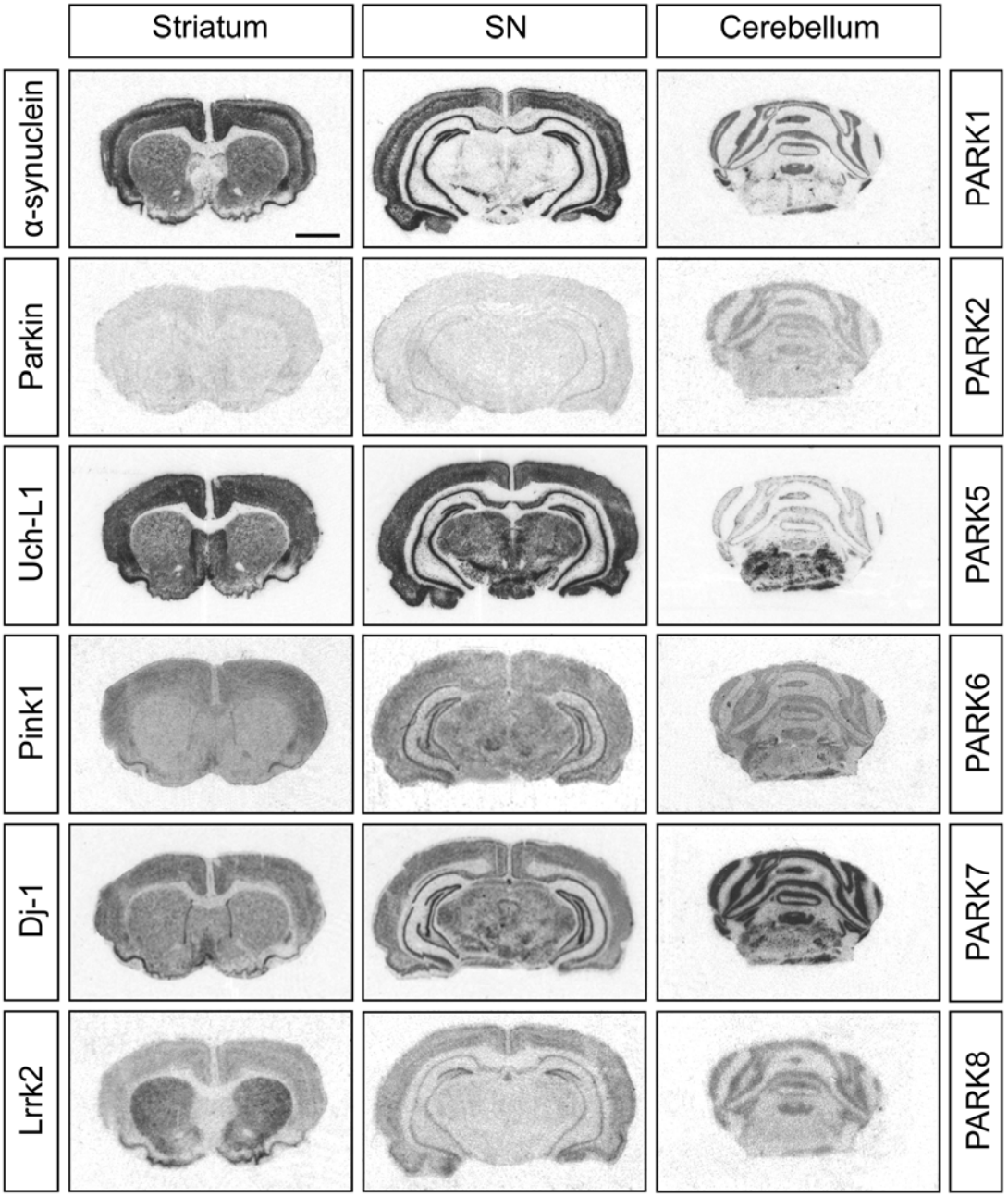


Fig. 2. mRNA expression patterns at three different rat brain levels (striatum, substantia nigra/hippocampus and cerebellum) of the six PARK genes *α*-synuclein (PARK1), Parkin (PARK2), Uch-L1 (PARK5), Pink1 (PARK6), Dj-1 (PARK7) and Lrrk2 (PARK8), linked to familial Parkinson's disease.

Identification of susceptibility genes in disease

Linkage and association studies are two approaches commonly used for the identification of genetic risk factors for disease. Linkage analysis enables hypothesis-free identification of chromosomal regions (loci) coupled to disease. The method is based on the segregation of a genetic marker, which has a known genomic location with the disease through several generations in a family. The analysis is most successful for loci with high penetrance, whereas analysis of mutations with low-penetrance and diseases with complex traits are more difficult.

Association studies

Association studies are based on the hypothesis that variants of a specific gene associate with an increased or decreased risk of disease, and are carried out using case-control materials. Candidate genes include genes located in regions already identified through linkage analysis, as well as genes for which an hypothesis about a role for maintenance of dopamine neurons can be put forward. A substantial number of association studies in Parkinson's disease have focused on genes involved in dopamine metabolism, protein aggregation, detoxification or mitochondrial function. Variable findings are frequently reported from association studies, possibly due to small sample sizes or variation in mutation frequencies between different populations. A strategy to increase reliability in association analyses is therefore to investigate large case-control materials from geographically distinct populations. Single association studies have limited power to detect true susceptibility genes, and hence have to be replicated in order to improve statistical significance. Other strategies include retrospective meta-analyses of multiple independent studies or collaborative multi-center studies with standardized methodologies and diagnostic criteria. In Parkinson's disease, collaborative analyses have led to the identification of the promoter polymorphism NACP-Rep1 in *SNCA* and the inversely associated missense mutation S18Y in *UCH-L1* as risk/protective susceptibility factors[29,30].

With an increasing number of SNPs available in public databases and the development of high-throughput, low-price techniques for genotyping, there is great interest in using whole-genome association (WGA) studies to unravel genetic susceptibility factors. The method is based on the scanning of a large number of genetic markers across the complete genome in order to find genetic variations associated with disease. In a large Parkinson's disease WGA study, conducted by Maraganore et al.[31] the authors analyzed 200,000 SNPs and identified eleven polymorphisms showing an association with disease. Given the large number of genes and mutations apparently involved in Parkinson's disease, it appears evident that multiple methodological approaches, including both linkage and association studies, are useful in the search for susceptibility factors. Moreover, studies of both

isolated and heterogeneous materials are important in order to identify pathogenic mutations in different populations.

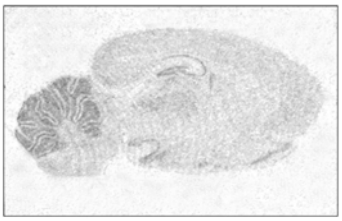
Candidate genes in Parkinson's disease

Despite identification of genetic loci linked to Parkinson's disease, the etiology of the vast majority of the cases remains to be clarified. We have selected candidate genes from several groups involved in different biological pathways which together or individually may contribute to disease pathogenesis (Fig. 1 and Table 1). Some genes investigated, have initially been implicated in Parkinson's disease through linkage analysis and have later been found to be involved also in sporadic disease, such as *SNCA*, *UCH-L1*, *DJ-1* and *LRRK2*. Others, such as the alcohol and aldehyde dehydrogenases (ADH and ALDH), multi-drug resistance 1 (*MDR1*) and mitochondrial transcription factor A (*TFAM*) have been identified as candidate genes based on their functions and possible relation to disease pathogenesis, and/or due to their previous association with disease in other populations. In the following sections, a general background of the genes investigated in the present thesis will be presented. The figures adjacent to each subheading represent sagittal sections through the rat brain hybridized to probes targeting the respective genes.

Table 1. Parkinson's disease susceptibility genes investigated in the present thesis.

Gene	Function	Chromosomal location	Paper
ADH1	Ethanol metabolism	4q21-q23	I-II
ADH4	Retinol metabolism	4q23-q24	I-III
ALDH1	Aldehyde metabolism	9q21.13	I-II
MDR1	Drug transport	7q21.1	IV-V
SNCA	Presynaptic signaling and membrane trafficking	4q23-q25 (PARK1)	VI, X
LRRK2	Cytoplasmic, associates with the mitochondrial outer membrane	12q12 (PARK8)	VII-X
UCH-L1	Hydrolyzation of C-terminal adducts of ubiquitin	4p14 (PARK5)	X-XII
DJ-1	Protection against oxidative stress and cell death	1p36.23 (PARK7)	X-XI
TFAM	Mitochondrial transcription	10q21	X, XIII

A group of genes that has caught our interest is the group of genes involved in regulation of endogenous and exogenous toxin levels, such as *ADH*, *ALDH* and *MDR1*. By regulating the uptake, metabolism and/or distribution of drugs or toxins, these genes might constitute an interface where environmental factors and genetics meet in the pathogenesis of Parkinson's disease. If genetic variability in these types of genes results in altered protein levels and function, this could alter the susceptibility to environmental factors and thereby predispose to or protect against disease.

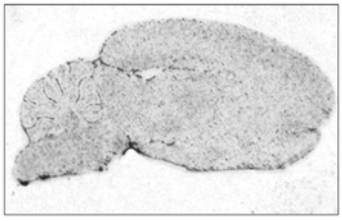


Alcohol and aldehyde dehydrogenases

Several reasons make ADH and ALDHs suitable susceptibility genes in Parkinson's disease: (1) ADHs and ALDHs are involved in the metabolism of alcohols and aldehydes present in food and air or endogenously produced during lipid peroxidation. They are present in tissues such as the epidermis and the epithelial lining of the gastrointestinal (GI) tract forming a physical and enzymatic defense to the environment[32-36]. (2) ADH and ALDHs are involved in retinoid metabolism, which is essential for cell growth and development[37]. (3) ALDH1 seems to be of particular importance for dopamine neurons in the midbrain since the gene is highly and selectively expressed in substantia nigra and this gene expression has also been found to be affected in Parkinson's disease[38,39]. Dopamine has the ability to condense with aldehydes to form tetrahydroisoquinolines (TIQs), a reaction which is used in the Falck-Hillarp fluorescence histochemical method to render catecholeamines fluorescent[40]. In neurons the reaction can be harmful if dopamine condenses with aldehydes intracellularly to form salsolinol[41], a substance related to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). (4) Last, genetic variability in two classes of *ADH*s have shown to associate with an increased risk of Parkinson's disease. A truncating STOP codon in *ADH1C*[42] and an *ADH4* promoter mutation which results in a 25-30% reduction of transcription activity both associate with disease[43,44].

The human *ADH*s are located in a cluster on the long arm of chromosome 4 and are subdivided into class I-VI or *ADH1-ADH6*[45]. Class I ADH, or ADH1, is abundantly expressed in liver and has the highest catalytic efficiency for oxidizing ethanol among all ADHs. ADH3 (heading figure), the ancestral form of ADH, functions as a glutathione dependent formaldehyde dehydrogenase, and ADH4 is highly efficient in retinoid metabolism. ALDH1 (or class I ALDH), located on human chromosome 9q21, acts on a broad spectrum of substrates including retinal aldehyde, biogenic amines and products of lipid peroxidation[46]. Another important function of ALDH1 is the

conversion of the dopamine metabolite 3,4-dihydroxyphenylactealdehyde (DOPAL), an endogenous aldehyde found to be toxic to dopamine neurons *in vitro* and *in vivo*[47,48] to DOPAC[49]. Intracellular formation of toxic aldehydes might explain why dopamine neurons in substantia nigra are particularly vulnerable.



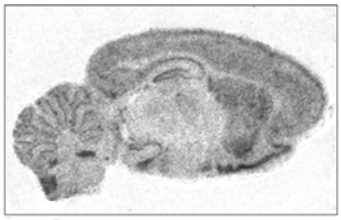
Multi-drug resistance 1

A family of proteins, which has been suggested to influence the susceptibility to Parkinson's disease by constituting a possible link between the genes and the environment, is the ATP-binding cassette (ABC) super family of transporter proteins. The large ABC super family in humans regulates bioavailability of xenobiotic compounds in the body. One of the family members most widely studied in Parkinson's disease is the 170 kDa protein P-gp (Permeability glycoprotein) encoded by the *MDR1* gene. The protein is composed of two homologous halves making up a cylinder shaped transporter in the cell membrane. It was first isolated from tumor cells where it plays a key role in multi-drug resistance[50]. MDR1 functions as an energy-dependent drug efflux pump removing toxic substances from inside the cell. It is expressed in various tissues in the body, including the gastrointestinal and nasal respiratory mucosa, where it prevents substances from being absorbed or inhaled[51,52], the BBB[53] and the placenta[54]. MDR1 has also been observed in tissues involved in detoxification and excretion, such as the liver hepatocytes and the proximal tubules of the kidneys where it accelerates secretion into bile and urine respectively[55,56]. The gene encoding *MDR1* has 28 exons and spans over 100kb on human chromosome 7q21.1[57]. The three polymorphic sites 1236C/T (exon 12), 2677G/T/A (exon 21), and 3435C/T (exon 26) have been found to associate with Parkinson's disease individually or as haplotypes[58,59]. Polymorphisms in the gene have also been associated with altered protein expression and function[60], possibly affecting the transporting capacity and therefore intracellular toxin levels.

α-Synuclein

Several lines of evidence point at *α*-synuclein (NACP, PD1), present at the PARK1 locus, as an important candidate gene in Parkinson's disease. The point mutation A53T in the coding region of *SNCA*, found in an Italian and three unrelated Greek families with autosomal dominant inheritance, was the first demonstration of a mutation linked to Parkinson's disease[22]. The finding was soon strengthened by the identification of *α*-synuclein as one of the major components of LBs[61], the proteinaceous intracellular inclusions found mainly in the brain stem of Parkinson patients. During the following years, other point mutations, A30P and E46K, were found in unrelated German and Spanish Parkinson's disease families[62,63], and *SNCA* gene multiplications in other families[64-68]. However, *SNCA* variants can only account for a small number of the familial Parkinson's disease cases. Identification of *SNCA* gene multiplications has clearly shown that gene dose can be critical in causing parkinsonism. Patients carrying a gene duplication, i.e. three copies of the gene, exhibit a 1.5 fold increase in *α*-synuclein levels whereas a triplication of the gene results in a 2-fold increase in protein levels[65]. In agreement with the gene-dose effect, triplication carriers exhibit an earlier age of disease onset compared to duplication carriers[66]. Evidence from several studies has also indicated an influence of *SNCA* promoter variability on susceptibility to sporadic disease. These findings were supported by a recent meta-analysis in which the NACP-Rep1 promoter polymorphism was significantly associated with Parkinson's disease[30]. Moreover, polymorphisms in intronic regions of *SNCA* have been identified as risk factors for sporadic forms of the disease[69,70].

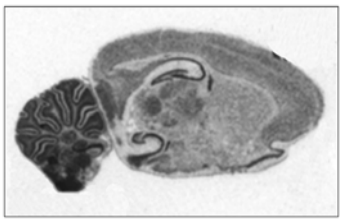
α-Synuclein is located in nerve terminals and is abundantly expressed in the brain. The protein was originally identified as a precursor protein of a non–β-amyloid component (NAC) of Alzheimer's disease amyloid plaques[71]. The function of *α*-synuclein is not fully understood, although a growing body of evidence suggests involvement in learning, synaptic vesicle mobilization, presynaptic function and maintenance of synaptic vesicle pools[72-74]. Mutated forms of the protein may be more likely than wild-type protein to aggregate and form proteinaceous inclusions, which is of high interest in the pathogenesis of the disease. LBs formed in the brain stem and cortical areas in Parkinson's disease have been identified in patients carrying mutations in *SNCA*, *UCH-L1*, and *LRRK2*, but they are usually absent in the brain from patients with *Parkin*-mutations. The mechanism whereby *α*-synuclein causes neurodegeneration is not known but may involve formation of protein aggregates composed of protofibrils or fibrils[75]. Interestingly, a protective role of LBs, by binding misfolded proteins has also been suggested.



Leucine-rich repeat kinase 2

Genetic variants in *LRRK2* now stand out as the largest known cause of Parkinson's disease accounting for up to 10% of autosomal dominant familial Parkinson cases. Parkinson patients with *LRRK2* mutations have typically late disease onset and a wide range of pathology which can differ between or within families, e.g. presence or absence of LBs. The physiological function of the protein remains largely unknown, but recent studies provide clues to possible functions, such as protein-protein interactions, maintenance of neurites and regulation of neuronal survival. LRRK2 is localized to membranous and vesicular intracellular structures such as mitochondria, vesicles, lysosomes and endosomes[76].

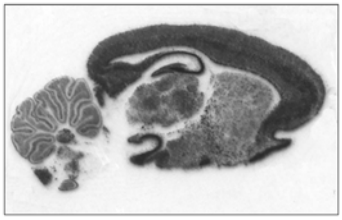
LRRK2 at the PARK8 locus on chromosome 12p11.2-q13[77] is linked to autosomal dominant Parkinson's disease[78,79] and it has also been found to be responsible for "sporadic" forms of the disease. The gene consists of 51 exons coding for a multi-domain protein consisting of ankyrin repeats, a leucine-rich repeat (LRR), a highly conserved Roc (Ras in complex proteins) and COR-domains (C-terminal of Roc), a tyrosine kinase catalytic domain of the mitogen-activated protein kinase kinase kinase (MAPKKK) class and WD40 repeats[78]. The first mutations in *LRRK2* were discovered in Basque Parkinson families, and the protein was therefore named dardarin from the Basque word *dardara* meaning tremor. Extensive research in the last years has identified several putatively pathogenic mutations in *LRRK2*, including R1441C/G/H, Y1699C, G2019S, I2020T and G2385R together with several other mutations which have not been shown to be pathogenic. The G2019S mutation, which is located in the kinase domain of the protein, is alone responsible for 5-6% of familial Parkinson's disease cases (and even up to 30-40% in Ashkenazi Jews and North African Arabs) and around 1-2% of apparently sporadic cases[80] and is thought to have evolved from common founders in Europe and Japan[81]. The G2385R polymorphism, which is common in Asian populations, but rare in other parts of the world, was discovered in a straight forward association study involving single SNPs. This variant is present in around 9% of Asian Parkinson's disease cases and 4% of controls, with a higher frequency in familial cases. Despite its high presence in Asia, previous large scale WGA studies have failed to identify G2385R as a risk factor for Parkinson's disease.



DJ-1

DJ-1 is a multifunctional protein with suggested involvement in oxidative stress response, protein folding, apoptosis, chaperone activity and transcriptional regulation. The 189 amino acid protein is highly conserved across species and is present both in brain and in peripheral tissues. Under normal conditions DJ-1 is found in the nucleus and cytoplasm[82,83] and it relocalizes to mitochondria under oxidizing conditions[84,85]. DJ-1 has been suggested to be a sensor of oxidative stress since it shifts its isoelectric point to a more acidic form following oxidative stress[86,87]. Moreover, wild-type DJ-1 has the ability to reduce the motor abnormalities and dopamine neuron death caused by 6-OHDA in rats[88].

The PARK7 locus on chromosome 1p36 was originally found to cause autosomal recessive early onset parkinsonism[89]. However, genetic variability in *DJ-1* is a rare event, accounting for ~1% of the Parkinson cases with early onset[90,91]. Clinically, patients carrying mutations in *DJ-1* have early disease onset, slow progression and good response to levodopa. The original finding of genetic variation in *DJ-1* was presented by Bonifati and colleagues, who showed a homozygous deletion of exons 1-5 and a substitution of the conserved amino acid leucine to a proline at position 166[92]. The L166P mutation has been found to destabilize the protein[93], alter folding properties[94] and reduce protein levels[91]. Subsequent screening of the *DJ-1* gene has lead to identification of several variable sites containing deletions, missense and nonsense mutations[90,95,96].



Ubiquitin carboxy-terminal hydrolase L1

UCH-L1 is a key component of the ubiquitin-proteasome system (UPS). The protein has a major role in ubiquitin-dependent proteolysis by recycling ubiquitin polymer chains and generating ubiquitin monomers[97]. The function of the UPS is to remove damaged proteins, and UCH-L1 dysfunction results in aggregation of proteins such as α-synuclein, which is considered as one of the possible pathogenic events of Parkinson's disease. UCH-L1 is highly and specifically expressed in neurons[98] and is one of the most abundant proteins in the brain, comprising up to 2% of the total soluble brain protein[97]. It has also been identified as one of the components of proteinaceous inclusion bodies in the remaining neurons of substantia nigra in

Parkinson's disease[99]. The protein is also known as PGP9.5 and has it has been widely used as a neuronal marker both in the central and peripheral nervous system.

The gene encoding *UCH-L1* is located on chromosome 4p14 in the fifth PARK locus which is linked to autosomal dominant Parkinson's disease. The first missense mutation, I93M, was identified in a German family with typical Parkinson's disease and disease onset around 50 years of age[100]. However, the linkage to chromosome 4p14 has been questioned since it has only been identified in a rare number of cases from a single family. A more common variant of the *UCH-L1* gene, S18Y in exon 3 [101] has been found to be associated with a reduced risk of Parkinson's disease or with a risk reduction in cases with early disease onset[102-108], although contradictory findings have also been reported[109-111].

Results from *in vitro* studies have suggested two opposing enzymatic activities of UCH-L1 towards α-synuclein, depending on its polymerization[112]. As a monomer, UCH-L1 hydrolyses poly-ubiquitin chains, which promotes ubiquitination and subsequently proteasomal degradation of α-synuclein. As a dimer, UCH-L1 exhibits ubiquityl ligase activity, ligating ubiquitin to α-synuclein via a K63 linkage which spares the proteins from proteasomal degradation. The S18Y mutation inhibits dimerization of UCH-L1 and thus favors α-synuclein degradation.

Mitochondrial transcription factor A

Mitochondria are cellular organelles involved in oxidative phosphorylation, energy supply, calcium homeostasis, apoptosis and fatty acid oxidation. They possess a 16 kb double stranded circular genome which is separate from the nuclear genome and is composed of 37 intronless genes encoding 13 proteins, 2 ribosomal RNAs and 22 transfer RNAs. The proteins encoded constitute subunits I, III and IV of the mitochondrial respiratory chain. Transcription and replication mitochondrial DNA (mtDNA) involves the nuclear encoded protein TFAM[113] which exist in the cell in concentrations several times higher than the concentration of mtDNA[114]. TFAM is essential for mtDNA transcription and maintenance. Homozygous Tfam deletion causes early embryonic lethality[115]. However, over expression of human TFAM in mice does not increase the enzymatic activity of the respiratory chain complex, suggesting that mtDNA copy number is not correlated to the electron transport function[116].

Mitochondrial dysfunction has been implicated both in aging and neurodegenerative disorders such as Parkinson's and Alzheimer's disease (AD). A link between mitochondria and parkinsonism was supported by the finding that the

neurotoxic substance 1-methyl-4-phenylpyridinium (MPP+), an active metabolite of MPTP which caused severe Parkinson's disease in a small group of individuals, appeared to inhibit mitochondrial complex I selectively in dopamine neurons[117]. Moreover, four of the Parkinson's disease-linked genes identified to date, Parkin, PINK1, DJ-1 and LRRK2, have been implicated in mitochondrial function[76,85,118,119]. mtDNA is vulnerable since it has little capacity for self repair and is not protected by histones, resulting in an accumulation of mutations over time (for review see[120]). Adding to the high vulnerability is the fact that mtDNA, unlike nuclear DNA, is exposed to reactive oxygen species (ROS) in close proximity. Protection of mtDNA against damage by ROS formed in the respiratory chain is therefore important in order to sustain proper cellular functions.

AIM OF THE THESIS

The aim of the present thesis was to investigate susceptibility genes for Parkinson's disease using three major approaches:

- Investigation of gene and protein expression:
    - Characterization of cellular mRNA expression patterns in nervous and peripheral tissues
    - Quantification of mRNA levels in rodent brain during development, adulthood and aging
    - Quantification of mRNA and protein levels in normal and pathological human brain

- Identification of susceptibility genes in Swedish Parkinson patients:
    - Screening for novel mutations in candidate genes
    - Determination of mutation, polymorphism and haplotype frequencies

- Analysis of genetic risk in rodent models of Parkinson's disease:
    - Investigation of spontaneous and drug-induced behavior
    - Quantification of brain monoamine and monoamine metabolite levels
    - Quantification of brain gene expression levels

MATERIALS AND METHODS

mRNA and protein expression

Animal tissues

Animals were kept under standardized conditions and supplied with food and water *ad libitum* (paper I-IV, VII-VIII and X-XI). For preparation of fresh frozen tissues, animals were sacrificed by cervical dislocation and tissues including brain, individual peripheral organs or whole embryos were dissected, rapidly frozen on dry ice and stored at -80°C. Staging of embryos was based on appearance of a maternal vaginal plug (noon of the day of plugging = E0.5). Frozen tissues were sectioned at 14 μm, thawed onto coated super frost glass slides (Menzel-Gläser, Braunschweig, Germany) and stored at -20°C until use.

Human tissues

Human post mortem brain and peripheral tissues were provided by the Harvard Brain Tissue Resource Center (Belmont, MA, USA), the Brain and Tissue Bank for Developmental Disorders (University of Maryland, Baltimore, MD, USA), the Netherlands Brain Bank (Amsterdam) and the Queen Square Brain Bank for Neurological Disorders (University of London, UK) and stored at -80°C until use.

Oligonucleotide probes

The synthetic oligonucleotide probes used for *in situ* hybridization were approximately 48-51 base pairs long and had a GC content of >45%. The publicly available online software mfold (version 3.2) was used to calculate the folding energy[121,122]. Probe specificity was controlled by aligning the probe against all sequences available in a public sequence data base (www.ncbi.nlm.nih.gov/BLAST) and only sequences with high specificity were selected. Furthermore, several probes were designed for each gene and only probes that generated coherent results according to area of expression were used for analysis. Probe specificity was also assured by comparing the expression to published *in situ* hybridization, immunohistochemistry and Northern blot data when available. A random probe with similar length and GC content as the gene-specific probes was processed in parallel as a negative control and generated no specific hybridization signal above background level (Fig. 3).

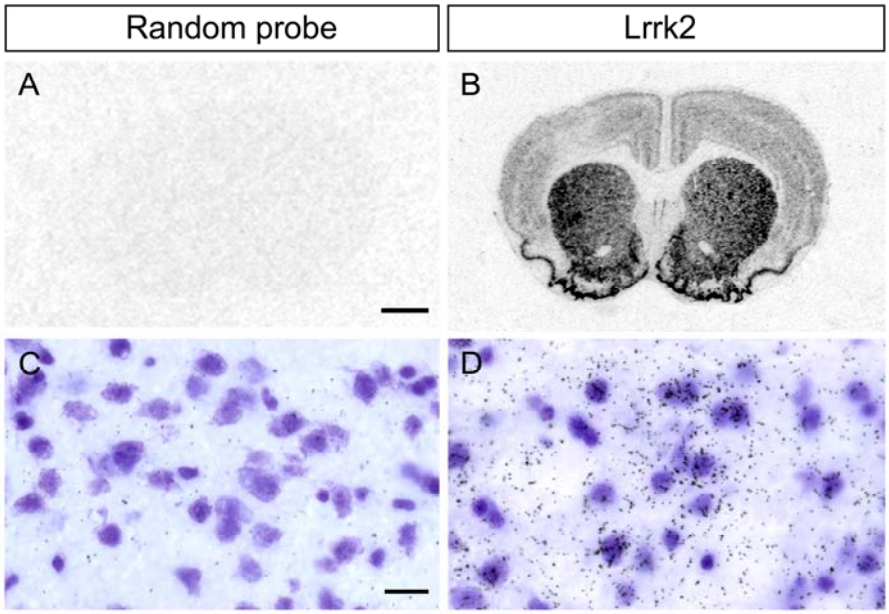


Fig. 3. Film and microscopic pictures of rat brain at the level of striatum. No specific hybridization signal is seen for the random probe on film (A) or at the microscopic level (C). Corresponding sections hybridized with a Lrrk2 probe revealed specific mRNA signal on film (B) and over medium spiny neurons in striatum (D). Scale bar (A-B)=2 mm; (C-D)=20 μm. (Modified from paper VII.)

In situ hybridization

In situ hybridization (paper I-II, IV, VII-VIII, X-XI) is a powerful method for localizing mRNA transcripts in the cell cytoplasm by complementary hybridizing a radioactively labeled oligonucleotide probe to a sequence of interest. The small size of the probes (~50 bases) allows for easy penetration into the cells or tissue. The method used in the present thesis is based on a protocol established by Dagerlind et al.[123]. Oligonucleotide probes were labeled with α-^{33}P-deoxyadenosine 5′-triphosphate (dATP) at the 3′-end (Perkin Elmer, Boston, MA, USA) using terminal deoxynucleotidyl transferase (TdT) (Amersham Biosciences, Buckinghamshire, England) and purified with ProbeQuant G50-Micro Columns (Amersham Biosciences). Cryosections were air-dried and hybridized overnight at 42°C with a cocktail containing 4xSSC, 50% formamide, 1x Denhardt's solution, 1% sarcosyl, 0.02 M Na_3PO_4, 10% w/v dextran sulfate, 0.2 M dithiothreitol, 0.5 μg/μl sheared salmon sperm DNA and the radio-labeled oligonucleotide. The following day, slides were washed in 60°C 1x SSC buffer for one hour and cooled to room temperature, followed by rapid dehydration in increasing concentrations of ethanol (70%, 95% and 99.5%) and finally air-dried. For visualization and quantification, slides were exposed over night to phosphoimaging plates. Alternatively, slides were exposed together with ^{14}C standards to autoradiographic films (Biomax, Eastman Kodak Co, Rochester,

NY, USA) for 5-21 days, depending on signal intensity. For microscopic evaluation of the signals, slides were dipped in NTB2 nuclear track emulsion (NTB2, Eastman Kodak) and exposed for 3-6 weeks, counter-stained with 0.5% cresyl violet and mounted.

Image analysis

To determine mRNA content, autoradiographic films or phosphoimaging plates were digitized and mRNA signal density was quantified using the software ImageJ (http://rsb.info.nih.gov/ij/). Optical density was converted to nCi/g as determined from a ^{14}C standard curve.

Statistical analysis

Comparison of brain mRNA or protein levels was performed using Student's t-test. Striatal mRNA signal intensities studied at different time points during development and aging were analyzed using a one-way ANOVA. The significance level in both analyses was set at $p<0.05$.

Western blot

Western blot was used to quantify the level of α-synuclein in human post mortem cerebellar samples. Fresh frozen tissue was sectioned at 40 μm on a cryostat and collected in Eppendorf tubes for homogenization in CelLytic buffer (Sigma-Aldrich, Stockholm, Sweden). Protein concentrations in the samples were determined using the Bradford assay (BioRad Laboratories AB, Sundbyberg, Sweden) and equal aliquots of protein were denatured at 70°C for 10 min under reducing conditions. Protein samples were run on a 4-12% Bis-Tris polyacrylamide gel in parallel with appropriate standard (SeeBleu plus2, Invitrogen, Lidingö, Sweden) and blotted onto a nitrocellulose membrane using standard protocols (Western Breeze kit, Invitrogen). Blots were blocked and incubated overnight at 4°C with primary antibody followed by incubation with alkaline phosphatase-conjugated secondary antibody. Blots were developed with a chromogenic substrate and band intensities were quantified using a BioRad imaging system (BioRad Laboratories AB). Levels of α-synuclein in each sample were expressed in relation to α-tubulin (α-synuclein/α-tubulin).

Genotyping

Human DNA samples

The human DNA samples (paper V-VI, IX, XII-XIII) were obtained after informed consent and approval of the local ethics committee. DNA was prepared according to standard protocols. The sample set consisted of 310 Parkinson patients and 315 unrelated control individuals (Table 2). The samples were collected from the catchment area of Stockholm at the Karolinska University hospital and from the catchment area of Göteborg at the Sahlgrenska Hospital in Sweden. The patients were diagnosed according to the Brain Bank Clinical diagnostic criteria[124]. In the Stockholm and Göteborg materials 30% and 24%, respectively had a reported a family history of disease. Positive family history was defined as one or more first or second degree relative with disease, and was determined by interviewing the proband. However, this method might lead to an underestimation of the number of affected relatives, since relatives may be too young to have developed the disease, they may have passed away before any clinical symptoms were evident, they may not be in touch with the family any longer, or they may have been misdiagnosed. The control material was collected from the same geographical areas.

Table 2. DNA material used in the Parkinson's disease case-control association studies.

Status Origin	Female (n)	Male (n)	Total (n)	Mean age at sampling (years)	Mean age of onset (years)
Control					
Stockholm	71	73	144	40,8	-
Göteborg	105	66	171	69,0	-
Total			**315**		
Parkinson's disease					
Stockholm	73	121	194	68,5	60,9
Göteborg	49	67	116	67,5	59,2
Total			**310**		

Automated capillary sequencing

Automated capillary sequencing allows detection of novel genetic variants and the method was used to screen for mutations in exon 41 of the *LRRK2* gene in paper IX. Polymerase chain reaction (PCR) was carried out to amplify a fragment of genomic DNA using Taq polymerase and a primer pair specific for the region of interest. The amplified fragment was subsequently purified using a QIAquick PCR purification kit (Hilden, Germany) and the isolated fragment was sequenced with a single primer and

a DTCS kit, which makes use of randomly incorporated fluorescent labeled ddNTPs which terminate the elongation process. This will result in DNA fragments of different lengths, all terminating with a fluorescent ddNTP. The samples were mixed thoroughly with stop solution (1.5 M NaOAc + 50 mM EDTA), glycogen (20 mg/ml) and 95% ethanol (-20°C). To wash the DNA, samples were centrifuged followed by removal of the supernatant and adding of 70% ethanol (-20°C) in two runs. Prior to analysis, samples were resuspended in 40 µl of deionized formamide. Purified DNA fragments were separated using automated capillary gel electrophoresis (CEQ 2000 systems, Beckman Coulter Inc., Fullerton, CA, USA) according to the manufacturer's instructions.

Pyrosequencing

Pyrosequencing is a rapid method for high throughput genotyping of predefined variations in a DNA sequence of up to 20 bases. The detection of a SNP/mutation is based on the release of pyrophosphate during the incorporation of nucleotides into the newly synthesized DNA strand. Incorporation results in emission of light which is proportional to the number of incorporated nucleotides. Taq polymerase, a forward and a reverse primer (one labeled with biotin in the 5′ end) were used to amplify a 70-180 bp fragment of genomic DNA containing the variable site of interest. Biotinylated DNA sequences were immobilized onto streptavidine coated sepharose beads. Using a Vacuum Prep Tool (Biotage, Uppsala, Sweden) the DNA fragment was washed in 70% ethanol, denatured in 0.2 M NaOH and annealed to a sequencing primer (~15 bases) in the opposite direction as the biotinylated primer. Samples were analyzed on a PSQ 96MA system using a SNP Reagent Kit (Biotage AB).

Restriction fragment length polymorphism

The restriction fragment length polymorphism (RFLP) technique makes use of restriction sites in the location a genetic variation. Following PCR amplification the DNA fragment is subjected to a restriction endonuclease which cleaves the sequence depending on genotype. This results in DNA fragments of variable sizes which are separated on an agarose gel and visualized by UV translumination. We used the method in the case of the *LRRK2* R1441C/G/H mutation analyzed in paper IX, where the wild-type sequence was cut by the restriction endonuclease BstUI, leaving the mutated sequence uncut.

Statistical analysis

Differences in allele and genotype distributions were analyzed using a Chi-square ($\chi 2$) test and one or two-sided p-values. Distribution of genotypes in Parkinson patients and controls was tested for consistency with the Hardy-Weinberg equilibrium also using a Chi-square test.

Determination of haplotypes, a series of consecutive alleles present on a particular chromosome, was performed using the statistical software UNPHASED (version 3.0, ©Frank Dudbridge 2006) which performs analysis of multi locus haplotypes from genotype data in case-control sample sets. Calculations were carried out with haplotype reconstruction by an expectation-maximization algorithm based on observed genotypes for the individual SNPs. Statistical significance was defined as $p<0.05$.

Animal models

Adh4-/- mice

Adh4 knockout (*Adh4-/-*) mice were used to study disturbances in dopamine system related behavior (paper III). Generation of *Adh4-/-* mice has previously been described by Deltour et al.[125]. In these null mutant mice, the promoter and exon 1-6, corresponding to the amino acids 1-275 have been replaced by a neo cassette. The mice were backcrossed with C57BL/6BKL mice for ten generations before the experiments. To resemble the high age of onset in Parkinson's disease, all animals were aged under normal conditions to 12 months or more prior to experiments. Fresh frozen tissue from *Adh4-/-* mice was also used as a control of probe specificity in the *in situ* hybridization experiment performed in paper II.

Behavioral studies in Adh4-/- mice

Spontaneous and drug-induced locomotor behavior was studied in mice using a multi cage infrared-sensitive motion detection system[126] in which three parameters, motility, locomotion (horizontal activities) and rearing (vertical activity), were simultaneously recorded. The horizontal movement was detected by 48 photo sensors located in 4x4 cm squares covering the floor of the locomotor cage. *Motility* was defined as a movement covering one photocell (4 cm), whereas *locomotion* was defined as movement covering 8 photocells (32 cm). *Rearing* (animal is standing on its hind limbs) was recorded by six rearing detectors 4 cm apart, placed 10 cm above the cage floor. Standard transparent A3 macrolon cages with 40 ml of wooden shavings on the floor were placed on the motility meters. Activity measurements were performed

between 9 am and 3 pm and the wooden shavings and the cages were changed between each run.

Prior to experiments, animals were habituated to the low-noise, dimly-lit and ventilated experimental room for 30 minutes. Animals were placed in the experimental cages and the spontaneous locomotor activity was recorded for a period of 60 minutes (exploratory activity 0-20 minutes; habituation 20-60 minutes). *d*-amphetamine sulphate (*d*-amphetamine) (Apoteksbolaget, Stockholm, Sweden) was injected in a dose of 3.0 mg/kg intra peritoneally (i.p.), the animals were placed back into the cages and the drug-induced behavior was recorded for 60 minutes.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) and electrochemical detection was used in paper III to measure levels of monoamines and monoamine metabolites (dopamine, DOPAC, HVA, noradrenaline, serotonin and 5-HIAA in different brain regions of *Adh4* knockout, heterozygous and wild-type mice[127]. Following decapitation, mouse brains were rapidly removed, cooled in 4°C saline and dissected. Brain samples were frozen on dry ice and stored at -70°C until use. For analysis, samples were weighed and sonicated in 5 volumes or 30 µl of 0.1 M perchloric acid and centrifuged. Samples were separated on a reverse-phase column (Supelcosil™, LC-18, 75 mm x 4.6 mm, 3 µm particle diameter) and levels of monoamines and monoamine metabolites were determined by comparison to standards. The mobile phase consisted of a 0.05 M sodium phosphate/0.03 M citric acid buffer with 0.1 mM EDTA and variable amounts of methanol and sodium-l-octane sulphonic acid and had a flow rate of 0.4 ml/min. Detection was performed by a glassy-carbon electrode detector set at +0.7 V vs. an Ag/AgCl reference electrode. All monoamines and their metabolite levels were compared to wild-type animals (set to 100%).

6-OHDA lesioning

Stereotaxic injection of 6-OHDA in Sprague-Dawley rats under halothane anesthesia was performed 4.4 mm posterior and 1.2 mm lateral to Bregma and 7.8 mm below dura mater. Animals were sacrificed 12, 24 and 48 h, 7 days and 3 weeks after surgery and brains were rapidly dissected, frozen on dry ice and kept at -80°C until use. Completeness of lesioning was tested by amphetamine-induced rotational behavior 20 days after operation.

RESULTS AND DISCUSSION

Many of the candidate genes implicated in Parkinson's disease are widely expressed in the brain, without showing any particular restriction to the dopamine system. However, the Parkinson's disease associated genes *ADH1C* and *ADH4* do not even relate to any central or peripheral nervous tissues. Thus genes expressed solely in peripheral tissues may also be of relevance for the pathogenesis of Parkinson's disease. In a series of experiments we therefore mapped the peripheral mRNA expression of alcohol and aldehyde dehydrogenases in embryonic and adult rodent tissues and studied the effects of the *Adh4* gene on dopamine system-related behavior in *Adh4* knockout mice.

Alcohol and aldehyde dehydrogenases (Papers I-III)

I. Localization of Adh and Aldh in rodent embryos

We used *in situ* hybridization to characterize the spatial and temporal expression patterns of *Adh1* and *Adh4* mRNAs in mouse and rat embryos and compared it to corresponding adult tissues, in an attempt to promote further understanding and possible involvement of alcohol and aldehyde dehydrogenases in the pathogenesis of Parkinson's disease. Parkinson's disease is generally characterized by late onset (although early onset cases occur). Nevertheless, because the progressive degeneration of dopamine neurons is a slow process, and because a large majority of the dopamine neurons must probably be lost before symptoms appear, the degenerative events may begin early in life. It is therefore of great importance to study the activity patterns of genes not only during aging, but also during development, to clarify times and locations for possible early pathogenic effects.

In agreement with its known role in alcohol metabolism, the *Adh1* gene was found to be transcriptionally active in embryonic rodent liver. It was also found to be active in the developing epidermis, as well as in the conjunctival, olfactory (Fig. 4) and intestinal epithelium in line with its suggested role in protection against toxic levels of alcohols. These *Adh1* expressing tissues form physical barriers and serve as a first line of defense against possibly harmful compounds in the environment. Similarly, presence of *Adh1* in the liver constitutes a second line of defense. We also reported presence of *Adh4* mRNA in the developing mouse olfactory epithelium (Fig. 4) and adrenal gland, suggesting these tissues as endogenous sources of retinoic acid (RA)

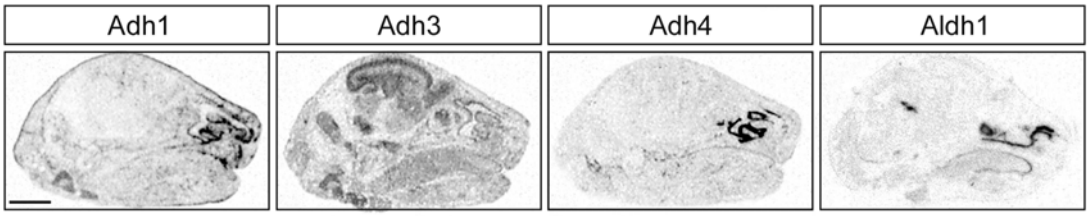


Fig. 4. mRNA expression of alcohol dehydrogenase (*Adh*) *1*, *4* and aldehyde dehydrogenase 1 (*Aldh1*) in the olfactory epithelium of E19.5 mouse embryos in comparison to the ubiquitously expressed *Adh3*. *Adh1* and *Adh4* are both absent from the brain, whereas *Aldh1* is present in substantia nigra. Scale bar = 2 mm. (Modified from paper I.)

during embryonic development. Adh4 is essential for RA synthesis and the expression levels of RA are generally high in the embryonic brain[128]. Moreover, the temporal expression of *Adh4* during embryonic development parallels that of RA[37].

Adh3 has a broad expression and a spectrum of substrates that differs from that of *Adh1* and *Adh4*. We observed a low, ubiquitous expression of *Adh3* mRNA from the earliest time points investigated in both mice and rats, which was completely different from the high and restricted expression of *Adh1* and *Adh4*. Due to its widespread expression, *Adh3* served as a morphological marker to facilitate orientation in film autoradiograms of embryos. In line with a previous study in adult tissues[129], our results indicate that *Adh3* is the only investigated class of *ADH*s expressed in the mouse and rat brain during late embryonic development. This suggests that the essential RA synthesis in the embryo is carried out by Adh4 in tissues other than the brain (e.g. in the olfactory epithelium or the adrenal gland) and subsequently transported to the target tissue via the circulation or simply by diffusion, since the RA is highly lipophilic and can travel across membranes for short distances (as is the case of an embryo).

II. Localization of Adh and Aldh in rodent gastrointestinal tract

In paper II we mapped the cellular localization of alcohol- and aldehyde dehydrogenases (*Adh1*, *Adh3*, *Adh4* and *Aldh1*) in the rodent GI tract to determine the possible roles of these enzymes in protection against toxins entering the body via the intestines. The importance of pathological changes in the GI tract of patients with Parkinson's disease has recently been emphasized by Braak et al.[130] who reported α-synuclein-positive inclusions in nerve plexa of the GI tract.

To characterize and discriminate between the three classes of alcohol dehydrogenases in the rodent GI tract, we used several class- and species-specific oligonucleotide probes together with control tissues with known expression profiles. Our results demonstrate that *Adh1*, *Adh3*, *Adh4* and *Aldh1* together provide a continuous lining of transcriptional activity along the entire GI tract, with

characteristic regional differences, pointing to the important role of alcohol and aldehyde detoxification in the tract. *Adh1* and *Adh4* are expressed mainly in non-overlapping populations of cells in the epithelial lining with *Adh4* being the dominating gene in the upper tract (i.e. tongue, esophagus and stomach) and close to the external environment where the cell turnover is rapid and the RA synthesis used for cell growth is high[36]. *Adh1* on the other hand, showed the highest expression in the lower tract (duodenum, jejunum, ileum colon and rectum, and was found deeper in the mucosa where it presumably participates in the metabolism of alcohols. *Adh3* exhibited a characteristic expression pattern clearly different from that observed for *Adh1* and *Adh4*. The *Adh3* gene was active in almost all tissues at low levels resembling the observations in embryonic tissues (paper I). The ubiquitous expression of *Adh3* is in line with the suggested house-keeping function of the gene. A general observation based on papers I and II was that the mRNA expression was less widespread in rats than mice. The striking lack of *Adh1* in embryonic rat adrenal gland (paper I) and adult small intestine (paper II), tissues with high expression in mice suggests a greater need of ethanol/retinoid metabolism in mice or that these functions are covered by other enzymes in rats.

Based on the observed tissue distribution in embryonic and adult rodents, the ADH and ALDHs may constitute defense enzymes, protecting againts alcohols (ADH1), aldehydes (ALDH1) and formaldehydes (ADH3) as well as being involved in the developmentally important retinoid metabolism (ADH4).

III. Modeling genetic risk of Parkinson's disease in Adh4 knockout mice

To study the role of alcohol dehydrogenases in Parkinson's disease, we performed behavioral studies on mice lacking one or two *Adh4* alleles. Mice in which the promoter region and exon 1-6 have been replaced by a neo cassette are null mutants[125] and do not show any obvious phenotypic differences compared to wild-type litter mates. To model the late onset of Parkinson's disease, animals were aged to 12 months or older before being analyzed. We studied the effect of the *Adh4* gene on dopamine system-related activity in wild-type (*Adh4*+/+), heterozygous (*Adh4* +/-) and homozygous (*Adh4* -/-) knockout mice using an infrared-sensitive motion detection system. During the initial hour of spontaneous activity recordings we did not observe any significant differences in activity between the three groups, except for increased rearing in the *Adh4*+/- compared to *Adh4*+/+ mice. Following injection of *d*-amphetamine, which increases the amount of dopamine in the synaptic cleft by blocking the reuptake and reversing DAT activity, the *Adh4*+/- and *Adh4*-/- animals exhibited increased locomotion and motility over time (Fig. 5). The higher activity observed in the *Adh4* knockout animals suggests an over-activity of the dopamine system, possibly related to increased sensitivity or an up-regulation of dopamine receptors in striatum. Interestingly, the effect was also shown to be gene-dose

dependent with the homozygous knockout mice being more sensitive than the heterozygous mice.

To verify whether the altered behavior of *Adh4* knockout mice was related to changes at the neurotransmitter level, we measured dopamine and dopamine metabolites in substantia nigra, striatum and the frontal cortex using HPLC. We found significantly increased levels of dopamine and the metabolite DOPAC in substantia nigra in *Adh4*-/- mice, possibly due to over-activity in the dopamine neurons, while corresponding levels in striatum and frontal cortex were unaffected. Taken together, the results from the behavioral studies and biochemical analyses, suggest involvement of the *Adh4* gene in dopamine-related neurotransmission, strengthening previous association of *ADH4* polymorphisms with Parkinson's disease[43].

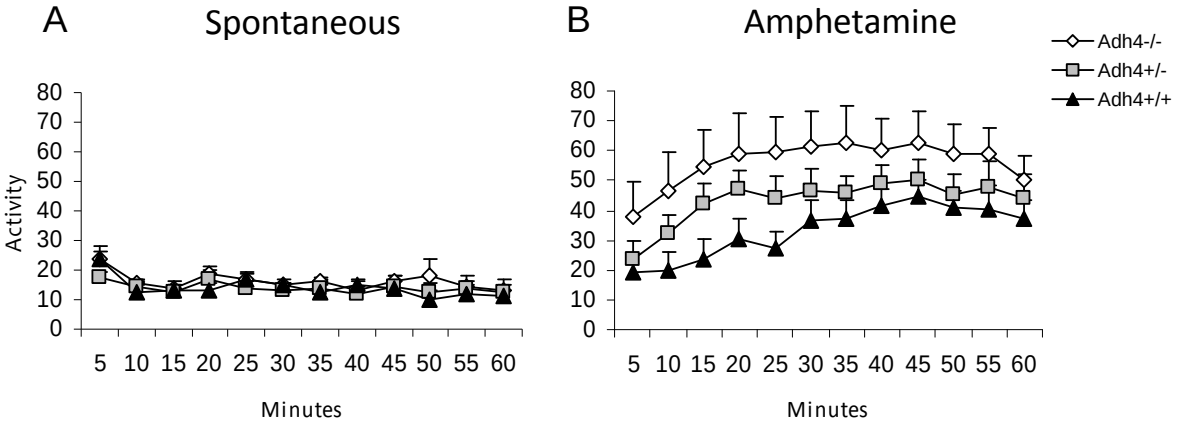


Fig. 5. (**A**) Spontaneous and (**B**) *d*-amphetamine-induced locomotion (movement of >32 cm) in *Adh4*+/+, *Adh4*+/- and *Adh4*-/- mice. Following administration of *d*-amphetamine (3.0 mg/ kg i.p.), *Adh4*+/- and *Adh4*-/- mice show a gene-dose dependent increase in activity over time compared to *Adh4*+/+ mice. (Modified from paper III.)

Multi-drug resistance 1/P-gp (Papers IV-V)

MDR1 plays an important role in regulating drug resistance and intracellular toxin levels and, based on these functions, its possible role has been studied in several disorders, including Parkinson's disease. Mutations in the gene may alter expression and transporting capacity of the protein, resulting in elevated intracellular levels of drugs. We investigated *MDR1* polymorphisms as possible susceptibility factors in Swedish Parkinson patients and we also characterized patterns of transcriptional activity of this gene in human and rodent tissues.

IV. Localization of MDR1 in human and rodent tissues

In paper IV we studied *MDR1* mRNA expression patterns in human and Sprague-Dawley rat tissues using radioactive oligoprobe-based *in situ* hybridization. In line with a suggested role in protection against and removal of intracellular toxins, *MDR1* mRNA was expressed in both human and rodent capillary endothelial cells in the brain, as well as in pia mater. *Mdr1* was also localized to blood capillaries in rat dorsal root and sympathetic ganglia (Fig. 6), possibly preventing toxins from accumulating in the nervous system. These observations strengthen the role of MDR1 in maintaining the BBB and other barriers.

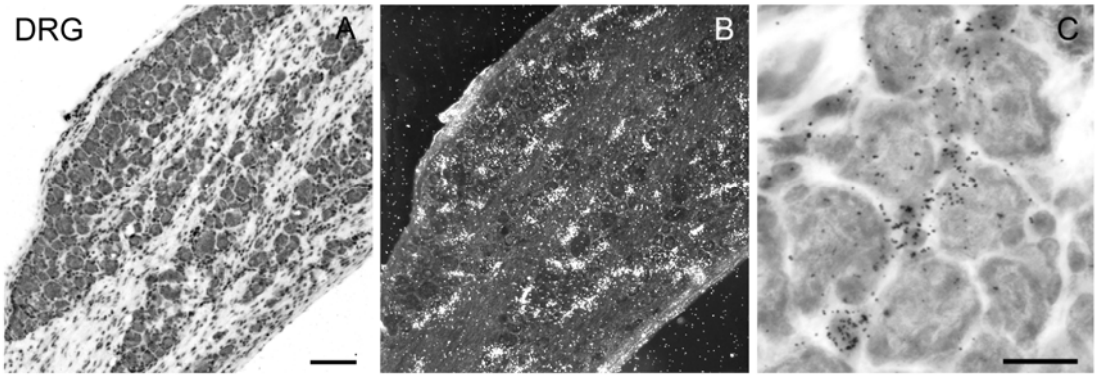


Fig. 6. Expression of *Mdr1* mRNA in endothelial cells of blood vessels of dorsal root ganglia (DRG) revealed by *in situ* hybridization. Panels **A** and **B** represent bright and dark field photomicrographs at low magnification and panels **C** is a bright field view at higher magnification. Scale bar (**A**-**B**) = 100 μm, (**C**) = 20 μm. (Modified from paper IV.)

In rodent peripheral tissues, *Mdr1* transcription was localized to epithelial cells of the small and large intestines, presumably to prevent toxic substances from being absorbed from the food. *Mdr1* mRNA was also found in the endothelial cells and hepatocytes of the liver and in the proximal tubules of the kidneys where *Mdr1* may accelerate the secretion of drugs into bile and urine, respectively. Previous studies have also located MDR1 to the placenta[54] and we reported robust *Mdr1* expression also in ovarian follicles and the Leydig cells of the testis. In these two situations, Mdr1 may serve a different role, such as participation in the secretion of steroids to the blood stream.

V. MDR1 polymorphisms in Parkinson's disease

To investigate a possible role of *MDR1* as a susceptibility gene in Swedish Parkinson patients we assessed the prevalence of three polymorphisms in our case-control sample set using pyrosequencing. The selection of SNPs was based on a previously reported association with disease[58]. In agreement with the previous findings we found association of the silent polymorphism 1236C/T (exon 12) with disease. However, no association was observed for SNP 2677G/T/A (exon 21) or 3435C/T (exon 26) in the Swedish population. We also reported significant association of the 1236C-2677G haplotype with Parkinson's disease and a trend towards association of the 1236C-2677G-3435C haplotype. The associated SNP 1236C/T is located in exon 12, and is not inducing an amino acid shift (synonymous SNP). The mechanism by which synonymous gene variants predispose to disease is unknown, but a possible effect could be altered mRNA stability or protein conformation. In a recent publication Kimchi-Sarfaty et al. suggested an effect of another silent polymorphism in *MDR1* (3435C/T) on co-translational folding and insertion of the protein into the cell membrane[131].

Several studies on *MDR1* polymorphisms have generated variable results in terms of susceptibility for Parkinson's disease, probably due to the heterogeneity between the populations studied. The overall genotype and allele frequencies of the three *MDR1* polymorphisms in our Swedish population were in agreement with other European populations[132,133], but not with Asian populations[58,59,104]. In addition to true differences between distinct human populations, variable results might be due to the relatively small sample sizes in some of the previous studies. It should also be noted that a disease-associated genetic variant may not itself be causative but can serve as a pointer to a chromosomal region in which the disease-causing variant resides.

In summary, the data presented in paper IV-V highlight the significance of investigating cellular mRNA expression patterns in order to better understand the normal physiological function of a gene product and to reveal its possible involvement in disease. Localization studies constitute a powerful tool for identifying tissues and cells in which disease-causing gene mutations can be expected to exert effects. Genetic variability in *MDR1* has been shown to have effects on protein expression and function and may also play a role in disease susceptibility and/or individual responses to drug treatments[134]. The findings also highlight the significance of investigating not only non-synonymous, but also synonymous genetic variations, and that it is of great importance to analyze SNPs in combination with haplotypes to identify susceptibility factors in disease.

α-Synuclein/SNCA (Paper VI)

VI. Polymorphisms and protein levels of α-synuclein in Parkinson's disease

SNCA gene variability and altered *α*-synuclein mRNA and protein levels have both been implicated in the pathogenesis of Parkinson's disease. In order to investigate the role of *SNCA* polymorphisms in our Swedish Parkinson's disease case-control material and whether the genotype had an effect on *α*-synuclein protein levels, we performed an association study of three SNPs previously associated with disease in a German material, and measured *α*-synuclein protein levels in postmortem brains from Parkinson patients and controls. Using pyrosequencing, we confirmed significant association of the two non-coding SNPs, rs356204 (G/A) and rs2737029 (G/A) with disease in our sample set. Genetic variations in *SNCA* have previously only been identified in familial cases of Parkinson's disease. However, two recent studies in a Japanese and a German sample[69,70], and our present study strengthen the involvement of *SNCA* polymorphisms as susceptibility factors also in sporadic Parkinson's disease. The two disease-associated SNPs are both located in a non-coding region of the gene (intron 4) showing that not only missense mutations, but non-coding polymorphisms can be associated with an altered risk of disease. This might be explained by linkage disequilibrium between a non-coding variant and a disease-causing mutation or by the influence of the non-coding SNP on transcriptional activity or mRNA stability. The ways in which polymorphisms in the *SNCA* gene may influence the risk of Parkinson's disease is not clear, but one hypothesis implicates impaired vesicular storage of dopamine, resulting in elevated dopamine levels in the cytoplasm and elevated dopamine metabolism, which in turn may lead to oxidative stress.

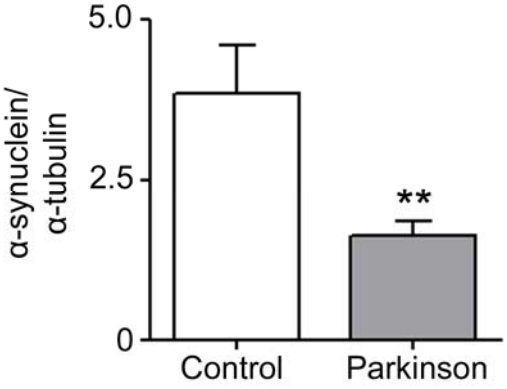


Fig 7. Western blot quantification of *α*-synuclein levels in human post mortem cerebellum of Parkinson's disease (n=16) and control (n=14) subjects normalized to *α*-tubulin. Levels are expressed as means ± SEM; groups were compared using Student's t-test. ** $p<0.01$. (Modified from paper VI.)

In addition to the genetic analysis we also investigated α-synuclein protein levels in post mortem human cerebellum and whether the levels were dependent of *SNCA* rs2737029, rs356204 or rs356219 genotypes in Parkinson's disease and control samples. Interestingly, we observed significantly decreased levels of α-synuclein protein (normalized to α-tubulin) in brain tissue from Parkinson patients, independent of the investigated SNP genotypes, age, tissue pH or post mortem interval (Fig. 7). This finding is supportive of and complements a recent study[135] in which the authors reported decreased *SNCA* mRNA levels in cerebellum, but not in any other brain regions investigated (substantia nigra, cingulate gyrus or medulla oblongata). However, other studies have pointed at reduced levels of *SNCA* mRNA also in midbrain/substantia nigra[136-138], although contradictory findings have been reported[139]. The reduced levels of soluble α-synuclein may be a result of aggregation of the protein in small granular aggregates, which have been shown to occur in cortex and striatum[140]. At the protein level, lower α-synuclein levels have also been observed in plasma and cerebrospinal fluid of Parkinson patients[141,142]. Despite the extensive research on α-synuclein in recent years, the function of the protein is still obscure and it remains to be solved how altered α-synuclein levels may relate to the neuropathology of Parkinson's disease.

Leucine-rich repeat kinase 1 and 2 (Papers VII-X)

To further aid to the understanding of LRRK2 function and how mutations in this gene may lead to disease, we characterized gene activity patterns in human and rodent tissues using *in situ* hybridization and compared the expression levels at different time points during development and aging. To determine the influence of *LRRK2* mutations in the Swedish population, we also performed a mutation analysis of three common *LRRK2* mutations in our Parkinson's disease case-control material.

VII. Temporal and spatial characterization of LRRK1 and LRRK2

In paper VII, we characterized the cellular localization of *LRRK2* mRNA in human and rodent brain and peripheral tissues at different time points during development and aging and we also compared the expression to that of the paralogous gene *LRRK1*, a related member of the ROCO protein family. To be able to follow the changes in *Lrrk1* and *Lrrk2* gene activities during development, we carefully mapped and quantified the expression levels in embryonic (E13.5-18.5) (Fig. 8), postnatal (P1-P29), adult and aging (4, 16 and 24 months) rat brain.

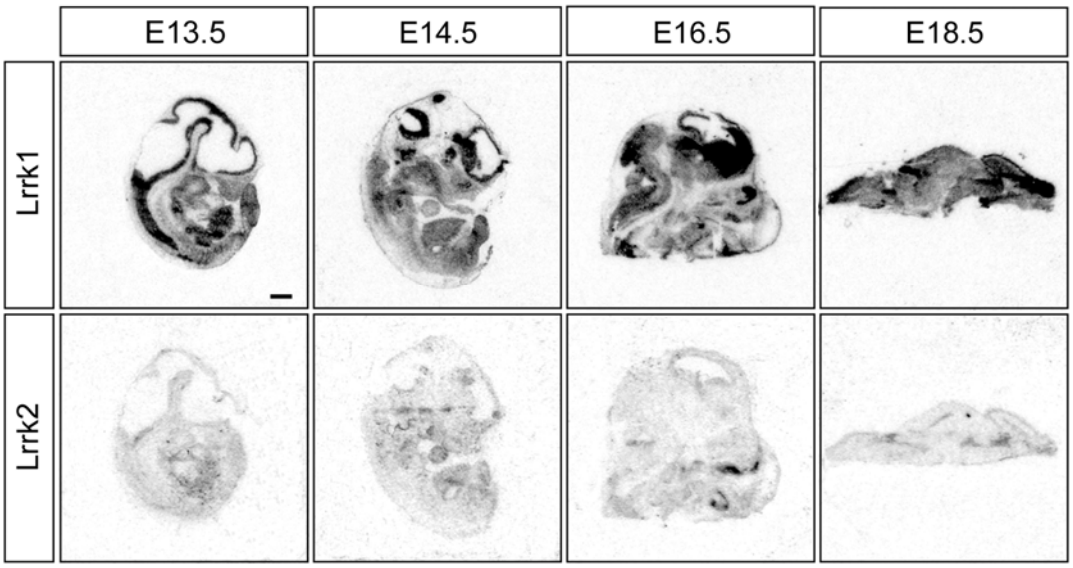


Fig. 8. Leucine-rich repeat kinase 1 and 2 (*Lrrk1* and *Lrrk2*) mRNA expression in the developing rat at embryonic day (E)13.5, E14.5 (whole embryos), E16.5 (head) and E18.5 (brain). *Lrrk1* is widely distributed in the embryo with the highest expression in the developing brain and spinal cord. *Lrrk2* is present in the area of the developing jaws at stage E16.5, whereas the brain is devoid of detectable levels of *Lrrk2* mRNA. Scale bar = 1 mm. (Modified from paper VII.)

Overall, *Lrrk2* mRNA expression in the embryonic rat was found to be low in peripheral areas and absent from the brain, whereas *Lrrk1* was expressed at moderate levels in both brain and peripheral tissues. In the developing rat brain, *Lrrk2* was first observed in striatum around one week after birth and the signal intensity gradually increased during the following weeks of development. Interestingly, *Lrrk1* levels in the postnatal rat striatum changed in the opposite direction. While *Lrrk2* mRNA expression levels increased during the first month of development, *Lrrk1* levels gradually decreased, suggesting complementary activities of the two paralogous genes in the brain. In the adult rat brain, *Lrrk2* mRNA expression was generally higher and more restricted to particular brain areas, than the expression of *Lrrk1* (Fig. 9). *Lrrk2* mRNA expression was particularly high in striatum and in piriform cortex, while lower levels were observed in cerebral cortex, hippocampus and the granular cell layer in cerebellum. *Lrrk1* on the other hand, was expressed at low levels and in almost all brain areas.

We also investigated whether *Lrrk2* levels were affected by aging. We found the *Lrrk2* mRNA levels in the brain of aged rats to be almost unchanged from 4 to 24 months of age. This suggests that Parkinson's disease-related symptoms are not likely to be a result of declining *Lrrk2* activity, but rather an effect of dysfunctional Lrrk2 protein, possibly due to mutations in the gene.

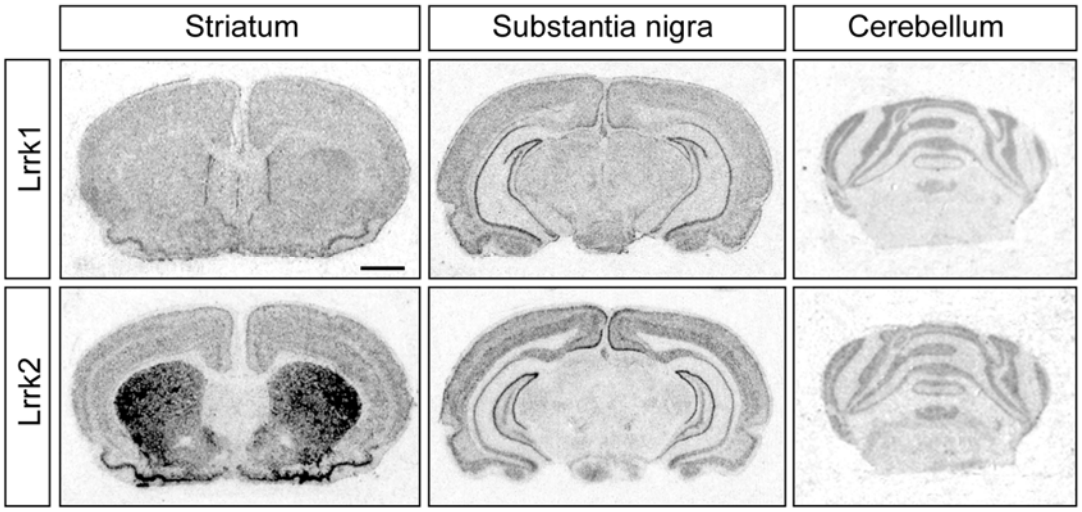


Fig. 9. mRNA expression of *Lrrk1* and *Lrrk2* at three levels of the rat brain: striatum, substantia nigra/hippocampus and cerebellum. *Lrrk2* shows high and restricted expression compared to *Lrrk1*, which is evenly distributed in most areas and expressed at lower levels than *Lrrk2*. Scale bar = 2 mm.

In addition to mapping the cellular expression pattern of *LRRK*s in the central nervous system (CNS), we explored the expression of these genes in various peripheral tissues in an attempt to find peripheral targets for LRRK2-related dysfunctions in Parkinson's disease. Like in the CNS, *Lrrk1* exhibited a wider expression pattern with activity in kidney, adrenal cortex, spleen, liver, thymus and lung. *Lrrk2*, on the other hand, was expressed in lung and the renal medulla, as well as in thymus, spleen and Peyer's patches in the GI tract, suggesting a possible role for *LRRK2* in the immune system. The *Lrrk2* gene activity in neurons of sympathetic ganglia may be related to the well described autonomic dysfunctions seen in Parkinson patients[143].

VIII. LRRK2 expression in human and rodent nervous tissues

One interesting finding in papers VII and VIII was that *LRRK2* gene activity is particularly robust in dopamine-innervated areas of the brain in humans, rats and mice. This makes *LRRK2* the first and so far only Parkinson-linked gene that has an expression pattern that relates specifically to the dopamine system. No other known Parkinson's disease-linked gene has a specific anatomical relation to the dopamine system. *α*-Synuclein, *DJ-1* and *UCH-L1* are expressed in the dopamine neurons although not at higher levels than in other brain areas not involved in Parkinson's disease. Our findings are in line with other studies describing higher levels of *LRRK2* mRNA and protein in striatum compared to substantia nigra[144-148], implicating striatum as the target area of the pathological changes in *LRRK2*-mutated patients. A hypothetic effect of mutated *LRRK2* in striatum is reduced target-derived trophic

support of the dopamine system, leading to a slow degeneration. Our findings in adult human brain and peripheral tissues were similar to our observations in rat tissues. Both species expressed *LRRK2* mRNA in striatum, cerebral cortex and hippocampus, as well as in peripheral tissues such as renal medulla and thymus.

To search for variations in *LRRK2* expression levels, we quantified the mRNA signal in post mortem caudate and putamen of five Parkinson patients and four control individuals and compared it to the expression of the dopamine D2 receptor. The analysis revealed similar levels of *LRRK2* mRNA in both caudate and putamen between the two groups, indicating that gene malfunction, rather than failing expression levels causes the disease. As was expected from earlier studies, there was a tendency towards higher D2 receptor mRNA levels in the striatum of patients with Parkinson's disease.

IX. LRRK2 mutations in Swedish Parkinson patients

To assess the prevalence of the three missense mutations R1441C/G/H (exon 31), G2019S, and I2020T (exon 41) in *LRRK2*, we genotyped these genetic variants in our Swedish Parkinson's disease case-control material using RFLP and pyrosequencing. The G2019S and I2020T mutations are both located in the kinase domain of the protein. The former has been associated with formation of inclusion bodies and cell death in vitro[149]. Due to its implication in disease, the mutation has been thoroughly investigated in genetic materials from different geographical areas and has been detected in European, American, Asian, African and Australian populations. We reported a G2019S mutation frequency of 1.4% in Swedish Parkinson patients which is similar to the frequencies found in other European cohorts. A heterozygous 95 year-old mutation carrier was identified among the control subjects which is in accordance with other studies reporting non-affected carriers[150-152]. Mutations in rare asymptomatic control subjects suggest an incomplete penetrance of the mutation or involvement of protective factors which are still to be identified. Reduced penetrance of the G2019S mutation has recently been reported by others[153].

X. PARK genes in rodent models of Parkinson's disease

To search for possible regulation of the Parkinson's disease-linked genes α-synuclein, *UCH-L1*, *DJ-1*, and *LRRK2*, we studied mRNA expression levels in striatum in six different rodent models including mice over-expressing human α-synuclein under the mouse α-synuclein promoter, α-synuclein knockout mice, *DARPP-32* knockout mice, mice carrying a T34A mutation in *DARPP-32*, MitoPark mice in which the *Tfam* gene is specifically deleted in dopamine neurons, and the well established 6-OHDA rat model of Parkinson's disease.

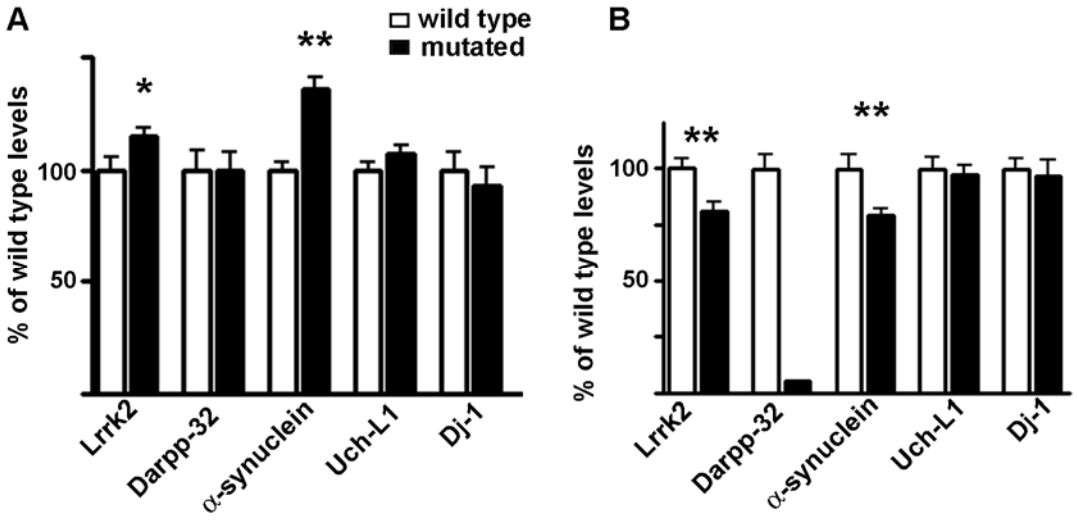


Fig. 10. Quantification of *Lrrk2, Darpp-32, α-synuclein, Uch-L1* and *Dj-1* mRNA levels in (**A**) α-synuclein over-expressing and (**B**) DARPP-32 knockout mice. *Lrrk2* is significantly up-regulated in α-synuclein over-expressing mice whereas both *Lrrk2* and α-synuclein are down-regulated in DARPP-32 knockout mice, suggesting a possible co-regulation between the two genes. * $p<0.05$; ** $p<0.01$. (Modified from paper X.)

The results indicated a possible co-regulation between Lrrk2 and α-synuclein since *Lrrk2* mRNA was up-regulated in mice over-expressing α-synuclein (Fig. 10). Moreover, *Lrrk2* and α-synuclein were both down-regulated in *DARPP-32* knockout mice whereas the other investigated genes (*Uch-L1* and *Dj-1*) remained unchanged. The parallel alterations in *Lrrk2* and α-synuclein in these transgenic mouse models seemed to be independent of dopamine since 6-OHDA lesioned rats had unchanged levels of all Parkinson's disease-linked genes. The only exception was *Dj-1*, which appeared slightly increased during the two first days after injury, suggesting increased activity during the ongoing degeneration of the dopamine fibers.

DJ-1 and UCH-L1 (Papers XI-XII)

XI. DJ-1 and UCH-L1 gene activity patterns in human and rodents

UCH-LI plays a role in the ubiquitin proteasome pathway and is present in inclusion bodies in neurodegenerative disorders[99]. It is a candidate gene for Parkinson's disease, not only because of its strictly neuron-specific expression including substantia nigra dopamine neurons[154], but also because a *UCH-L1* mutation has been found in familial Parkinson's disease[100]. Converging results from genetic, biochemical and cell culture studies have also suggested a potential role for DJ-1 in Parkinson's disease

pathogenesis since the gene has been linked to autosomal recessive early onset forms of the disease[92] and to resistance to oxidative stress[86,87]. In paper XI, we mapped the cellular activity patterns of *DJ-1* and *UCH-L1* in human and rodent brain using *in situ* hybridization. Knowledge about the cellular localization of the two genes should help determine the modes in which mutations may be causally linked to Parkinson's disease. In addition, we compared the expression levels in different brain areas, as well as between Parkinson patients, controls and schizophrenia patients. *UCH-L1* and *DJ-1* mRNA expression patterns were similar in brain tissues, although the levels of *DJ-1* were found to be lower. Both genes were observed in neurons of hippocampus, amygdala, the caudate nucleus, substantia nigra and in deep layers of the frontal, temporal and entorhinal cortex, with reduced levels of *DJ-1* in temporal areas, as well as in Purkinje cells of cerebellum. In glial cells, the expression of *DJ-1* mRNA was below the detection limit of our method. However, previous studies have indicated *DJ-1* mRNA and protein in neuronal, as well as non-neuronal brain tissue of both humans and rodents[86,155-158] using other methods.

A major difference in expression pattern between the two genes was the presence of *DJ-1* in non-neuronal peripheral tissues such as liver, GI tract, adrenal and pituitary gland, while *UCH-L1* was restricted to neurons also outside the CNS, in line with known usefulness of antibodies against UCH-L1 (also known as PGP 9.5) as a specific neuronal marker. Measurement of signal intensity revealed that human frontal cortex of controls expressed *DJ-1* mRNA more abundantly than other regions such as substantia nigra. We did not detect any differences in expression levels of *DJ-1* in substantia nigra dopamine neurons between Parkinson, schizophrenia or control individuals. These findings suggest that alteration of *DJ-1* levels in substantia nigra is not a general sign of Parkinson's disease and that the levels are not changed by the pathological process in the disease. Decreased DJ-1 protein levels have however been observed in cerebrospinal fluid of sporadic Parkinson patients, in particular in the early stages of disease[159].

XII. UCH-L1 S18Y variability and Parkinson's disease

We selected the non-synonymous SNP S18Y (Ser18Tyr) of *UCH-L1* for analysis in our Swedish Parkinson's disease case-control material, since the SNP has been reported to be inversely associated with disease in a meta-analysis of eleven published association studies[29]. Another mutation in the *UCH-L1* gene, the I93M (Ile93Met) mutation, was only investigated in patients with a positive family history of disease, since this mutation has only been reported in rare familial cases.

Using pyrosequencing we genotyped the S18Y polymorphism in Swedish Parkinson patients and controls and found a significant association with disease. The Y-allele, encoding the amino acid tyrosine, was associated with a lower risk of Parkinson's disease, which is in accordance with the meta-analysis[29]. Stratification of the sample set by age of onset (≤50 or >50), revealed a greater risk reduction in the

younger onset group. This is also in line with previous studies in which the relationship between Parkinson's disease and the S18Y variant was modified by the age of onset[102,103,108]. Taken together, the results from the present study together with previously published results of the meta-analysis support the hypothesis of the Y-allele as a protective factor in Parkinson's disease.

Mitochondrial transcription factor A (Paper XIII)

XIII. Genetic analysis of TFAM in Parkinson's and Alzheimer's disease

Based on the suggested involvement of mitochondrial dysfunction in Parkinson's disease, we performed an association study of the two SNPs IVS2+113A>G (rs2306604 A>G) and S12T (rs1937G>C) in the *TFAM* gene in our Swedish Parkinson's disease case-control material. *TFAM* has also been implicated in Alzheimer's disease (AD) since the gene is located on a chromosomal locus (10q21.1) linked to late-onset forms of the disease and carries a polymorphic site associated with AD[160]. Therefore, we also included a second sample set, consisting of Alzheimer patients and control individuals in the analysis. The results showed significant genotypic and allelic association of the SNP rs2306604 with AD, with the A-allele coupled to an increased risk of disease, in line with a recent meta analysis[161]. The polymorphism rs1937 showed no trend towards association. Positive association of SNP rs2306604 with diseae in the present study and of SNP rs1937 in a previous study[160] suggests polymorphisms in the *TFAM* gene as a possible risk factor for AD, although disease-associated variants may differ between populations. In the Parkinson's disease sample set however, we did not find any association of the two SNPs with disease. Our findings were recently supported by a study in which the authors also reported a lack of association of *TFAM* polymorphisms with Parkinson's disease[162]. Taken together, these finding suggest involvement of *TFAM* polymorphisms in Alzheimer's, but not in Parkinson's disease.

GENERAL DISCUSSION

Humans differ between each other at approximately 0.1% of the genome, and some of these differences determine the susceptibility to disease. To understand how genetic variability may lead to Parkinson's disease, it is important to analyze the occurrence of genetic variants in different populations across the world, and also to decipher precisely when and where these genes are active. Characterization of gene expression patterns is valuable in order to promote understanding of the pathways in which the genes are involved and also to identify their interacting players. As Parkinson's disease is a heterogeneous disorder, it seems likely that multiple factors individually or in concert operate to cause neurodegeneration. In support of this hypothesis, several putatively pathogenic genetic variants have been implicated in familial and sporadic forms of the disease. However, a genetic susceptibility factor found to be strongly associated with disease in a certain geographically or genetically confined population can be present at another frequency or completely absent in another population. These differences make genetic analyses extremely complex, and they also point at the importance of mapping genetic variations in different populations.

A desirable outcome of a genetic study would be, for instance, identification of significant biomarkers which can be used for early and reliable diagnosis. However, identification of new genetic markers will lead to the demand for individual genetic testing. Genetic testing of healthy individuals is still controversial and it requires careful consideration of the potential risks and benefits before being introduced. Another important goal of genetic studies is identification of new drug targets for improved therapy with less side effects. The pharmacological and surgical treatments used in Parkinson's disease today are effective in improving disease symptoms and reducing motor complications. Unfortunately, despite the long history of research, there is currently no treatment that stops the progressive loss of dopamine neurons or modifies the rate of progression. Increased knowledge about susceptibility genes may therefore aid in the identification of pathogenic mechanisms, which can be used as targets for therapeutic intervention, such as modulation of LRRK2 function or interference with the expression and accumulation of α-synuclein.

CONCLUSIONS

- High expression in epithelial linings and the known physiological functions indicate a possible role for ADH and ALDH in a defense system protecting against endogenous and environmental alcohols and aldehydes.

- Genetic association together with high expression in the blood-brain barrier and tissues involved in excretion and absorption, suggest a role for MDR1 in the interface between the genetic and environmental factors affecting disease susceptibility.

- Polymorphic association and altered brain protein levels strengthen the role of α-synuclein as a risk factor for sporadic Parkinson's disease.

- Expression of LRRK2 in key dopamine target areas and presence of gene mutations in a substantial number of Parkinson patients, indicate a particular relevance of LRRK2 in the pathogenesis of Parkinson's disease.

- Neuron-specific expression and involvement of UCH-L1 in the ubiquitin-proteasome system emphasize the need for efficient handling of defective proteins to prevent neurodegeneration in the nervous system.

- Genetic variations in DJ-1 in Parkinson's disease, the protective role in oxidative stress conditions and the abundant expression in the nervous system including substantia nigra, indicate involvement of DJ-1 in the pathogenesis of the disease.

- The suggested involvement of mtDNA mutations and mitochondrial dysfunction in aging, strengthen the case for TFAM as a susceptibility gene in neurodegenerative disease.

In summary, the findings presented in the present thesis together with previous work suggest genetic risk factors to be important in the pathology of Parkinson's disease. It is hoped that further understanding of susceptibility genes, will help advance knowledge about the pathogenesis, and that discovery of the true causes hopefully in the near future, will lead to improvements of disease therapy and prevention.

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Mormor – MIN IDOL

Birgitta, Thore and Jenny

Filip, for making me the happiest person in the world!

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