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GENES INVOLVED IN PARKINSON'S DISEASE - FOCUS ON MITOCHONDRIAL AND DETOXIFYING ENZYMES

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The cover illustrates DNA sequences related to the genetic focus on Parkinson's disease.

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ABSTRACT

Parkinson's disease is a common progressive neurodegenerative disorder which mostly affects the elderly population, with a prevalence of more than 1.5% in the population over the age of 65 years. Clinical motor symptoms are mainly caused by degeneration of dopamine neurons in substantia nigra pars compacta. In order to identify genes with potential roles in the pathology of Parkinson's disease, the candidate gene approach has been applied. Investigated genes assumed to play a role in mitochondrial maintenance were *DJ-1*, PTEN-induced putative kinase 1 (*PINK1*), the serine-protease *OMI/HTRA2*, mitochondrial translation initiation factor 3 (*MTIF3*), DNA polymerase gamma 1 (*POLG1*), mitochondrial Ras homolog gene family, member T1 and T2 (*MIRO1*, *MIRO2*). Genes involved in detoxification including paraoxonases (*PON1*, *PON2*, *PON3*) and alcohol dehydrogenases (*ADH1C*, *ADH4*) were also studied. Association studies were performed in a Swedish case-control material consisting of 619 Parkinson patients and 1564 neurologically healthy controls. The screening resulted in identification of several potential risk or protective factors such as *DJ-1* Ala167Ala (c.501A>G), *MTIF3* rs7669 (C>T), *POLG1* CAG repeat variability and *PON1* rs854571 (G>A). *MIRO1* and *MIRO2* need further investigations before they can be excluded as contributing factors. The investigation of *OMI/HTRA2* A141S (G>T) in Parkinson and Alzheimer patients resulted in an association with Alzheimer's disease. *In situ* hybridization of human postmortem brain tissue was used to detect any alteration of *PINK1* mRNA expression in Parkinson patients and of *OMI/HTRA2* mRNA in patients with either Parkinson's or Alzhemier's disease. No differences compared to control levels were observed for the two genes. Protein quantification of OMI/HTRA2 in frontal cortex indicated reduced levels of the active enzyme form and increased protease activity in patients with Alzheimer's disease. Using quantitative real-time PCR we detected a reduction of mRNA expression from the *MTIF3* rs7669 minor allele. Based on previous report on association of genetic variants in *ADH1C* and *ADH4* with Parkinson's disease, we studied spontaneous and drug induced locomotor behavior in *Adh1* and *Adh4* knockout mice, and in *Adh1/4* double knockout mice with respect to dopamine-system-related activity and olfactory function. Neurotransmitter levels were analyzed with high-performance liquid chromatography in different brain regions. All three knockout strains displayed increased drug induced behavior, as well as alteration of levels of monoamines and their metabolites compared to wild-type littermates. *Adh4*-/- mice had a reduced sense of smell as well as reduction of dopamine in the olfactory bulb, and results from *Adh1/4*-/- pointed in the same direction. In conclusion, the findings presented in this thesis suggest genetic variability has an important role in the pathogenesis of Parkinson's disease. The disease is a multifactorial and genetically complex disorder for which the etiology is unknown in most of the cases. It needs to be resolved how different molecular pathways involving different genes individually or together, contribute to disease by causing degeneration of dopamine neurons and other neuron types.

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LIST OF ABBREVIATIONS

A	Adenine
ADH	Alcohol dehydrogenase
AREP	Autosomal recessive early-onset parkinsonism
ATP	Adenosine-5'-triphosphate
C	Cytosine
COMT	Catechol-O-methyltransferase
DA	Dopamine
ddNTP	Dideoxynucleotide triphosphate
DOPAC	3,4-dihydroxyphenylacetic acid
EBV	Epstein-Barr virus
G	Guanine
GWAS	Genome-wide association study
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
HVA	Homovanillic acid
LB	Lewy body
LDL	Low-density lipoprotein
L-DOPA	L-3,4-dihydroxyphenylalanine
LRRK2	Leucine-rich repeat kinase 2
MAO	Monoamine oxidase
MAO-B	Monoamine oxidase B
MIRO1	Mitochondrial Ras homolog gene family, member T1
MIRO2	Mitochondrial Ras homolog gene family, member T2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+	1-methyl-4-phenylpyridinium
mtDNA	Mitochondrial DNA
MTIF3	Mitochondrial translation initiation factor 3
PCR	Polymerase chain reaction
PINK1	PTEN-induced putative kinase 1
POLG1	DNA polymerase gamma 1
Poly-Q	Polyglutamine
PON	Paraoxonase
PRKN	Parkin
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
SNCA	α-synuclein
SNP	Single nucleotide polymorphism
SNpc	Substantia nigra pars compacta
T	Thymine
UCH-L1	Ubiquitin carboxyl-terminal esterase L1
WT	Wild-type

INTRODUCTION

Parkinson's disease

General background

Parkinson's disease is a neurodegenerative disorder with a prevalence between 0.1% and 0.3% in the general population and between 1% and 2% in individuals over the age of 65 years (Alves et al., 2008; Weintraub et al., 2008). The disease is a heterogeneous and genetically complex disorder and the etiology is unknown in the majority of cases. In 1817 the British neurologist James Parkinson (1755-1824) described the clinical symptoms of the progressive disease in his monograph "An Essay on the Shaking Palsy" (Parkinson, 1817). Due to the detailed description, the founder of neurology, Jean Martin Charcot, proposed in 1877 that the syndrome should be named *La maladie de Parkinson*, Parkinson's disease. However, this was not the first documentation of the disease. Probably the first descriptions of Parkinson's disease originate from the ancient Indian medical system *Ayurveda* created more than 4500 years ago. In the *Ayurveda* system Parkinson's disease was called *Kampavata* and the symptoms were treated with seeds from *Mucuna pruriens* (Manyam and Sanchez-Ramos, 1999). At that time, the active substance in the plant was unknown and it took until the 1930´s before the component L-3,4-dihydroxyphenylalanine (L-DOPA) was purified from the seeds (Damodaran and Ramaswamy, 1937). The fact that dopamine (DA) was playing an important role in Parkinson's disease was not known at this time. In 1957 Arvid Carlsson and colleagues identified the catecholamine DA (Carlsson et al., 1957; Carlsson et al., 1958) and proposed that brain DA is the key neurotransmitter in Parkinson's disease (Carlsson, 1959). It was soon discovered that there is a deficiency of striatal DA in Parkinson's disease (Ehringer and Hornykiewicz, 1960). Thanks to the development of the Falck-Hillarp fluorescence histochemical method (Falck et al., 1962), which allows direct visualization of DA, noradrenaline, adrenaline and serotonin in brain sections with chemical certainty, a thorough mapping of the monoaminergic systems throughout the entire brain was carried out (Andén et al., 1966; Carlsson et al., 1962; Dahlström and Fuxe, 1964; Olson and Seiger, 1972; Seiger and Olson, 1973; Ungerstedt, 1971). In the early 1900´s, the neurologist Frederic Lewy described inclusions in neurons in the brains of Parkinson patients (Lewy, 1912). At the same time, Tretiakoff reported loss of pigmented neurons and gliosis in substantia nigra and the inclusions were named Lewy bodies (LBs) by Tretiakoff (Tretiakoff, 1919). The LBs are eosinophilic cytoplasmic inclusions and today are considered pathological hallmarks of the disease.

In Parkinson's disease, degeneration of neurons is most pronounced in substantia nigra pars compacta (SNpc) in the brainstem. Other neuronal systems affected are the noradrenergic locus coeruleus, the cholinergic basal nucleus of Meynert and the cholinergic-glutamatergic pedunculopontine nucleus (Jellinger, 1991). The neuronal cell loss is slow and occurs over many years and when the motor symptoms arise 50-70% of the DA neurons are already lost (Barzilai and Melamed, 2003).

Today there is neither a cure nor a disease modifying treatment for Parkinson's disease; available treatment is only symptomatic. The most efficacious medication is L-DOPA, a precursor to DA. Because of severe side effects, such as dyskinesia, L-DOPA is given in combination with other drugs and avoided during the early stages of the disease (Schapira et al., 2009). Other drugs that are given alone or together with L-DOPA are enzyme inhibitors that decrease the breakdown of DA, such as monoamine oxidase B (MAO-B) and catechol-O-methyltransferase (COMT) inhibitors, or DA receptor agonists that mimic the effect of DA. An alternative option is to deliver L-DOPA directly by a pump into the small intestines, which reduces side effects due to a more even administration (Nyholm et al., 2008). Deep brain stimulation is a surgical approach used in patients with advanced Parkinson's disease. An electrode is implanted into the desirable area, usually the subthalamic nucleus or globus pallidus interna (Sydow, 2008).

Dopamine metabolism

Degeneration of DA neurons in SNpc of Parkinson patients causes loss of DA innervation in striatum, degeneration of DA neurons in the ventral tegmental area causes loss of cortical DA innervation. DA is involved in a wide variety of functions including movements, reward, motivation and cognition. DA is synthesized from the amino acid tyrosine in a two-step process in which tyrosine is first transformed into L-DOPA by the rate limiting enzyme tyrosine hydroxylase after which L-DOPA is converted into DA by aromatic L-amino acid decarboxylase. In cells other than DA neurons, DA can be converted into noradrenaline and adrenaline. DA is packed into synaptic vesicles by a vesicular monoamine transporter and stored until released from a presynaptic bouton. Diffusing across the synaptic cleft, DA binds to DA receptors on a postsynaptic site on a dendrite. The DA transporter transports unbound DA back into the presynaptic compartment where the neurotransmitter is repackaged into vesicles. Another route to take care of surplus DA is to degrade it to the inactive metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine and homovanillic acid (HVA) by monoamine oxidase (MAO) and COMT. A bi-product of DA metabolism in DA neurons is neuromelanin, which gives raise to the dark color of SNpc.

Symptoms

The clinical phenotype in Parkinson's disease is characterized by *tremor*, trembling in hands, arms, legs and head, *rigidity*, stiffness of trunk and limbs, *bradykinesia*, slowness of movements and *postural instability*, impaired balance. Symptoms are initially typically asymmetrical and then spread to involve both sides of the body (Kovari et al., 2009; Lees et al., 2009). The classical motor features are due to the progressive degeneration of DA neurons in the brainstem. These symptoms appear late, when more than half of the DA neurons are lost. Non-dopaminergic and non-motor symptoms are often present before the clinical diagnosis and progress with development of the disease. The premotor signs are important for early detection and diagnosis. Symptoms and signs that are suggested to appear early are olfactory dysfunction, sleep disturbance, depression, anxiety, apathy, low impulsiveness, constipation and other autonomic features (Abbott et al., 2001; Wu et al., 2011).

Neuropathology

The neuropathological pattern comprises primarily the degeneration of the neuromelanin positive mesencephalic DA neurons. The SNpc is a nucleus of the basal ganglia and participates in the control of movement. Depletion of nigral neurons is one neuropathological hallmark of Parkinson's disease and is accompanied by cell loss in other groups of neurons such as noradrenergic cells in locus coerulus and the motor vagal nucleus, the serotonergic raphe nuclei, the cholinergic nucleus basalis of Meynert, pedunculopontine nucleus pars compacta, the Westphal-Edinger nucleus and many peptidergic brainstem nuclei (Jellinger, 1991). The postural instability seen in Parkinson patients is suggested to be caused by degeneration of such non-dopaminergic neurons (Grimbergen et al., 2009). It should be noted that neuropathology can be detected from Auerbach's plexus of the intestines to cortex cerebri.
A second hallmark is the intracytoplasmic proteinaceous inclusions known as LBs. They consist of more than 70 different molecules and α-synuclein is a major constituent (Spillantini et al., 1997). The aggregates are located in the soma and neurites in the brainstem and cortical areas (Braak et al., 2004; Wakabayashi et al., 2007) and they also exist in 3-4% of the surviving DA neurons in the brainstem, independent of disease severity (Greffard et al., 2010). According to one theory, the pathological process and the spread of LBs progress in six stages (Braak et al., 2003) beginning in the gastric autonomic plexus of Meissner and the olfactory nerve endings (Braak et al., 2006; Braak et al., 2003) and spreading to regions of medulla oblongata and the anterior olfactory nucleus. From the lower brainstem it gradually ascends into the rostral part of the brainstem and will affect the SNpc.

Detoxifying enzymes, such as alcohol dehydrogenases and paraoxonases, are involved in the protection against alcohols and aldehydes present in food or pesticide exposure to which mucous membranes of the respiratory and gastrointestinal tracts are exposed and which may lead to entry of toxic compounds into the blood. For example, alcohol dehydrogenases are expressed in the olfactory epithelium and paraoxonases exist in the blood and hydrolyzes organophosphates. A reduced sense of smell among Parkinson patients has been reported in 96% of the cases, as compared to young and healthy individuals (Haehner et al., 2009). After a correction for age-related norms this figure falls into 74.5%. Parkinson's disease is not the only neurodegenerative disorder where the olfactory system can be a marker for disease; the same has been seen in Alzheimer's disease. In olfactory epithelium and neuronal cells from Alzheimer's disease patients, markers for oxidative damage have been found to be increased (Ghanbari et al., 2004; Perry et al., 2003). Parkinson's and Alzheimer's disease are two common neurodegenerative disorders with somewhat overlapping mechanisms (Bertoli-Avella et al., 2004). Reduced mitochondrial function in both Parkinson's and Alzheimer's disease might underlie neurodegeneration (Orth and Schapira, 2001). There is also an accumulation of abnormally processed proteins in the two disorders. A neuropathological hallmark for Alzheimer's disease is the presence of intracellular neurofibrillary tangles and extracellular amyloid plaque formation (Yoshimoto et al., 1995) and in Parkinson's disease, LBs, consisting of α-synuclein. Therefore, it is of interest to study these two neurodegenerative disorders in parallel.

Etiology

The pathology of Parkinson's disease is relatively well understood, but the pathophysiological mechanisms remain poorly understood, although genetic causes of rare monogenic forms of the disease are known. One of the major risk factors is aging, although 10% of the people with Parkinson's disease are younger than 45 years of age. The incidence to develop the disease seems to decrease in the ninth decade of life (Taylor et al., 2005). Caffeine, the active substance in coffee, is an antagonist of adenosine A receptors, which are highly expressed in subpopulations of basal ganglia neurons (Ferre et al., 1991). Inhibition of the adenosine A2 receptor with caffeine or other compounds may be neuroprotective (Chen et al., 2001; Marcellino et al., 2010) and are responsible for a reduced risk for Parkinson's disease (Jankovic, 2008; Ross et al., 2000). Suggested environmental risk factors may include exposure to insecticides such as paraquat, organophosphates and rotenone (Le Couteur et al., 1999).

In recent years, it has been clear that genetic factors are very important for the etiology of Parkinson's disease. Dominantly inherited familial forms of parkinsonism were first reported by Allen in North Carolina, USA (Allen, 1937). In Sweden, Henry Mjönes described an autosomal dominant form of Parkinson's disease (Mjönes, 1949). In the genome, susceptibility genes have been mapped to loci known as PARK loci. Today, there are eighteen reported chromosomal loci (PARK1-18) suggested to be involved in the disease. For six of the loci, a specific gene implicated in the disease has been identified and results replicated in several populations (Belin and Westerlund, 2008; Hamza et al., 2010; Lesage and Brice, 2009; Satake et al., 2009).

A breakthrough discovery was made by Polymeropoulos and colleagues (Polymeropoulos et al., 1996), who mapped a Parkinson locus by linkage analysis in an Italian family with an autosomal dominant form of the disease. The disease causing gene was soon found to be α-synuclein (*SNCA*), located in the PARK1 locus on chromosome 4q21-q23 (Polymeropoulos et al., 1997). Gene dosage of *α-synuclein* is also of importance, a multiplication has been detected in familial parkinsonism (Fuchs et al., 2007; Lesage and Brice, 2009; Singleton et al., 2003) and a triplication was earlier reported at PARK4 (Singleton et al., 2003). Another locus for a dominant trait of Parkinson's disease is PARK8, which was first mapped in a Japanese family (Funayama et al., 2002). Leucine-rich repeat kinase 2 (*LRRK2*) was identified to be the disease causing gene at PARK8, a gene which spans a genomic region of 144 kb with 51 exons encoding 2527 amino acids. Mutations in *LRRK2* constitute the most common known cause of both sporadic and familial Parkinson's disease today.

Six genes in the PARK loci have been identified and the findings replicated, three autosomal dominant genes: *α-synuclein* (PARK1 / PARK4) (Hamza et al., 2010; Polymeropoulos et al., 1997; Strauss et al., 2005), ubiquitin carboxyl-terminal esterase L1 (*UCH-L1*, PARK5) (Leroy et al., 1998) and *LRRK2* (PARK8) (Funayama et al., 2002) and three autosomal recessive genes: parkin (*PRKN*, PARK2) (Kitada et al., 1998), PTEN-induced putative kinase 1 (*PINK1*, PARK6) (Valente et al., 2001) and *DJ-1* (PARK7) (van Duijn et al., 2001).

Mitochondrial impairment

Parkinson's disease has been suggested to involve aberrant mitochondrial function and possible loss of the ability to meet the energy demand in cells (Lin and Beal, 2006; Schapira, 2008; Vila et al., 2008). The mitochondrial DNA (mtDNA) is maternally inherited and the main function of the organelle is to produce energy. The circular mtDNA contains approximately 16 kb pairs, encoding two ribosomal ribonucleic acids (rRNAs), 22 transfer RNAs (tRNAs) and 13 proteins. All proteins encoded by the mitochondrial genome are hydrophobic, located to the mitochondrial membrane and take part in the oxidative phosphorylation either in the electron transfer complexes or in the ATP synthase (Chinnery and Schon, 2003). In neurons, a higher density of mitochondria is found close to the axon hillock, at the nodes of Ranvier and in synaptic terminals (Chen and Chan, 2006). Axonal transport of mitochondria takes place along the microtubules (Hollenbeck and Saxton, 2005).

In support of a mitochondrial involvement in Parkinson's disease, the metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is 1-methyl-4-phenylpyridinium (MPP^+), is a neurotoxin and inhibits the mitochondrial respiratory chain complex I, which causes degeneration of DA neurons in SNpc (Langston et al., 1983). Another inhibitor of complex I is the pesticide rotenone, which causes a selective degeneration of DA neurons, as well as other neurons, in rodents, thereby resembling the pathology of Parkinson's disease (Greenamyre et al., 1999). Complex I activity has been found to be reduced in SNpc and in platelets from Parkinson patients (van der Walt et al., 2003). Different polymorphisms in mtDNA have been reported to be associated with both increased and decreased risk of Parkinson's disease (Otaegui et al., 2004).

Several of the PARK genes identified by linkage analysis have been reported to influence mitochondria in different ways. The kinase *PINK1* (PARK6) is reported to regulate mitochondrial fission, and mutations in this gene cause early-onset familial Parkinson's disease (Valente et al., 2001). Mutations in *Parkin* (PARK2) are associated with autosomal recessive early-onset parkinsonism (AREP) (Kitada et al., 1998) and the protein is believed to be recruited by PINK1 from the cytoplasm to initiate autophagic degradation of impaired mitochondria (Matsuda et al., 2010). The PARK7 gene, *DJ-1*, causes AREP (van Duijn et al., 2001) and is suggested to operate in a parallel pathway to that of PINK1/Parkin to maintain mitochondrial function in oxidative environments (Thomas et al., 2010). A study by Wolozin et al. show that genetic modulation of *α-synuclein*, *parkin* and *DJ-1* can disrupt the function of mitochondria in a *Caenorhabditis elegans* model system (Ved et al., 2005). Dominant forms of familial Parkinson's disease are mainly caused by mutations in *LRRK2* and particularly the G2019S variant, which is located in the kinase domain of the protein. It has been demonstrated that overexpression of *LRRK2* protects *Caenorhabditis elegans* against the mitochondrial toxin rotenone, and that G2019S *LRRK2* has a less protective effect than wild-type *LRRK2* (Wolozin et al., 2008). The PARK13 gene, *OMI/HTRA2* is also targeted to the mitochondria (Strauss et al., 2005). Several additional genes, not located within known PARK loci, are of importance for mitochondrial function and have also been suggested to be candidate genes for Parkinson's disease. Four of these

genes are investigated in this thesis: mitochondrial translation initiation factor 3 (*MTIF3*), DNA polymerase gamma 1 (*POLG1*), and mitochondrial Ras homolog gene family members T1 and T2 (*MIRO1* and *MIRO2*).

Approaches to identify susceptibility genes

There are two main approaches to search for susceptibility genes that may cause or influence the risk to develop a disorder; linkage analysis and association studies. Linkage analysis is hypothesis free and enables identification of chromosomal regions (loci) coupled to the disease. Genetic information from families is used to identify the region in the genome, which is inherited together with the disease. Linkage analysis is most suitable when resolving the genetic component in monogenic disorders where the disease causing gene has a high penetrance.

Association study

A complex disorder is often polygenic and the phenotype is the result of involvement of several different genes. A case-control design is used where affected individuals are compared to controls with respect to allele and genotype frequencies of polymorphisms in the gene of interest. The candidate gene, investigated in an association study, is often chosen for its possible pathophysiological role in the disease. Typically, the candidate gene is located in a genomic region, which has been identified through linkage analysis. Other candidate genes are chosen based on a hypothesis regarding their role for maintenance of DA neurons and mitochondria as well as protein aggregation and detoxification.

A problem in association studies is the genetic heterogeneity in a population, leading to variable results. To overcome this problem and increase reliability in association analysis, one strategy is to enlarge the case-control material from geographically distinct populations in order to get a homogenous sample set. Association studies need to be replicated independently to improve statistical significance because a single study has limited power to detect true disease affecting genes. Other approaches are to carry out meta-analyses of multiple independent materials or to collaborate in multi-center studies with standardized diagnostic criteria, methodologies and techniques. Increasing amounts of information about genetic variation in public databases together with faster and cheaper genotyping techniques has opened the door to genome-wide association studies (GWAS). The GWAS are aimed to identify genetic markers of complex disorders and individual traits. The studies compare cases and controls with respect to genetic markers distributed all over the genome and without any prior hypothesis about possible candidate genes. The next generation technique is exome sequencing, which allows for the identification of rare mutations in all coding sequences of genes and is a good complement to searches for common variants with a GWAS approach.

Genetic variations

The human genome harbors variable sites in the DNA code and alterations in the genomic structure. A mutation is a change or an alteration in the DNA sequence. If a single nucleotide change is more frequent than 1% it is classified as a single nucleotide polymorphism (SNP), where one base in the DNA is replaced by another (Brookes, 1999). Instead of a substitution, a SNP can also be a deletion or insertion of a nucleotide. A polymorphism that can affect the protein function is called functional. If located within a coding exon it can lead to an amino acid shift or create a stop codon, whereas an insertion or deletion can shift the three letter reading frame. Synonymous SNPs do not alter the amino acid residues. However, these "silent" variations have received increased attention since some of them have been shown to influence splicing, mRNA stability, secondary structure, transcriptional activity or changes in protein synthesis, folding, levels, turnover and/or function (Parmley and Hurst, 2007). Repeats are another form of individual polymorphic differences, where a specific region of the genome contains a sequence that is repeated a variable number of times. There are different types of repeats; variable number of tandem repeats (Nakamura et al., 1987) and microsatellites or short tandem repeats (Weber and May, 1989). Larger insertions or deletions in a chromosomal region are referred to as copy number variations (Sebat et al., 2004). Epigenetic effects on genome function are defined as changes in gene expression, not caused by alterations of DNA sequence, but instead due to variations of DNA methylation, and changes in chromatin structure by histone modifications. Epigenetic modifications can be altered in disease and in combination with environmental factors (Marques et al., 2011). Other factors that can affect gene expression are small RNAs, they are able to either promote or silence gene activity (Grosshans and Filipowicz, 2008).

Candidate genes in Parkinson's disease

Sporadic Parkinson's disease is complex and heterogeneous and might involve a combination of genetic and environmental risk factors. The candidate genes investigated in this thesis code for proteins belonging to two main groups, proteins of importance for mitochondrial functions and detoxifying enzymes (Table 1, Figure 1). Some of the genes were initially identified through linkage analysis and subsequently also reported to be involved in sporadic Parkinson's disease, such as *DJ-1*, *PINK1* and *OMI/HTRA2*. Other genes, such as *MTIF3*, *POLG1*, *MIRO1* and *MIRO2*, have been investigated due to their function in mitochondrial maintenance or possible relation to the disease found in other populations. The group of detoxifying enzymes, paraoxonase (*PON*) and alcohol dehydrogenase (*ADH*), have been reported to be associated with Parkinson's disease.

Table 1. Susceptibility genes for Parkinson's disease investigated in this thesis.

Gene	Chromosomal location	Presumed function	Paper
DJ-1	1p36.23 (PARK7)	Protect cells against oxidative stress and cell death	I
PINK1	1p36 (PARK6)	Protect cells from stress-induced mitochondrial dysfunction	II
OMI/HTRA2	2p12 (PARK13)	Involved in mitochondrial function, autophagy, chaperone activity and apoptosis	III
MTIF3	13q12.2	Promote formation of the initiation complex on the mitochondrial 55S ribosome	IV
POLG1	15q25	Involved in replication and repair of the mitochondrial genome	V
MIRO1	17q11.2	Involved in mitochondrial trafficking	VI
MIRO2	16p13.3	Involved in mitochondrial trafficking	VI
PON1	7q21.3	Hydrolyze organophosphate substrates	VII
PON2	7q21.3	A cellular antioxidant	VII
PON3	7q21.3	Inhibit the oxidation of low-density lipoprotein	VII
ADH1C	4q21-q23	Involved in ethanol metabolism	IX
ADH4	4q23-q24	Involved in retinol metabolism	VIII, IX

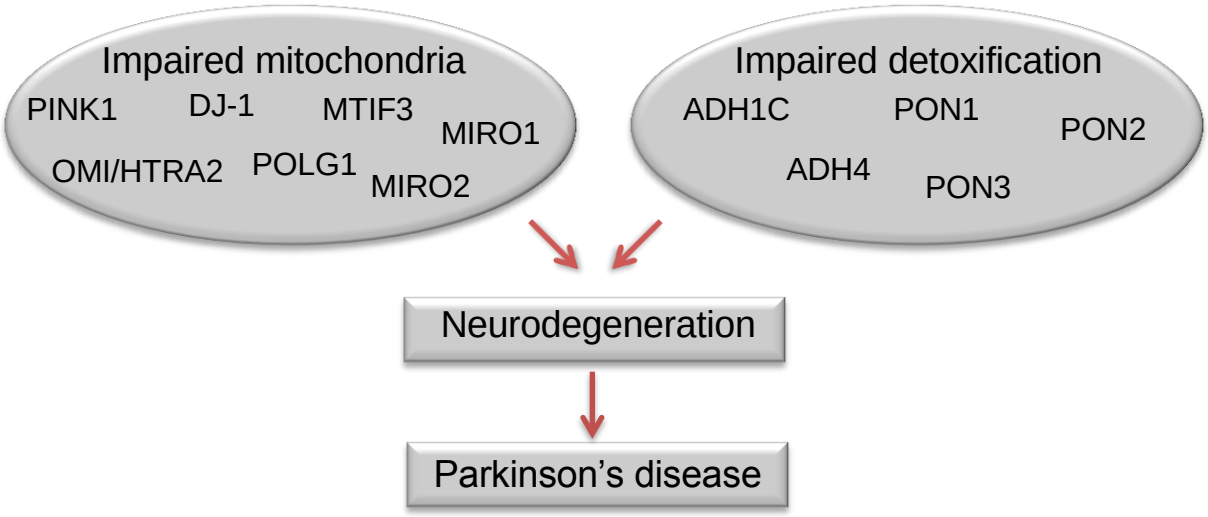

Figure 1. Candidate genes for Parkinson's disease investigated in the present thesis and how they are suggested to contribute to degeneration of dopamine neurons, which in the end might lead to disease.

DJ-1

DJ-1 is normally found in the cytoplasm and oxidative stress promotes translocation to mitochondria where it protects against mitochondrial toxins (Canet-Aviles et al., 2004; Zhang et al., 2005). This suggest that DJ-1 protects neurons against oxidative stress by acting as a redox-dependent chaperone (Zhou et al., 2006). Oxidation of cysteine at residue 106 has been shown in structural studies to be essential for DJ-1 to exert its full activities (Kahle et al., 2009). The 189 amino acid protein is conserved across species (Bonifati et al., 2003) and expressed throughout the body. In the normal human brain is it moderately expressed in neurons and astrocytes (Bandopadhyay et al., 2004; Galter et al., 2007). *DJ-1* was first recognized and defined as an oncogene because it enhanced Ras mediated transformation of cells (Nagakubo et al., 1997). In 2001 the PARK7 locus on chromosome 1p36 was linked to a family with AREP in the Netherlands using homozygosity mapping in a genetically isolated population (van Duijn et al., 2001). The following year the same locus was linked to a family with AREP in Italy (Bonifati et al., 2002). A 14 kb deletion was later identified in the Dutch Parkinson's disease family and a L166P substitution in the Italian Parkinson's disease family, which both lead to loss of gene function (Bonifati et al., 2003). Mutations in *DJ-1* are recessively inherited and cause Parkinson's disease with early-onset; overall mutations identified to date are rare and constitute ~1% of the Parkinson's disease cases world-wide (Lockhart et al., 2004). The frequency of *DJ-1* mutations in Parkinson's disease has been reported to vary between different ethnic populations, Caucasians: 0.91%, Asians: 3.03%, Arabs: 0.74% and Ashkenazi Jews: 1.96%. Copy number variations in *DJ-1* have so far only been reported in Caucasian Parkinson's disease populations (0.54%) (Nuytemans et al., 2010).

PINK1

The PTEN-induced putative kinase 1 (*PINK1*) gene encodes a 581 amino acid protein with a serine/threonine kinase and was discovered when screening for genes activated by tumor suppressor phosphatase and tensin homolog (PTEN) (Unoki and Nakamura, 2001). The PARK6 locus was mapped to chromosome 1p36 in an AREP family from Sicily (Valente et al., 2001). Three years later, mutations in *PINK1* located at PARK6 were found to be associated with AREP (Valente et al., 2004a). PINK1 contains a N-terminal mitochondrial targeting peptide involved in mitochondrial response to cellular and oxidative stress (Valente et al., 2004a) and is believed to be involved in autophagic degradation of impaired mitochondria (Matsuda et al., 2010). Mutations in *PINK1* account for 1-2% of the early-onset Parkinson cases (Hatano et al., 2004; Marongiu et al., 2008; Rogaeva et al., 2004; Rohe et al., 2004; Valente et al., 2004b).

OMI/HTRA2

OMI, also known as high temperature requirement factor A2 (*HTRA2*), was originally identified as a heat-shock-induced serine-protease in *Escherichia coli* that degrades misfolded proteins (Pallen and Wren, 1997). The serine-protease OMI/HTRA2 has an N-terminal mitochondrial targeting sequence (Strauss et al., 2005) which locates the protein to the intermembrane space of mitochondria; however, endoplasmatic reticulum and Golgi localization of OMI/HTRA2 has also been reported (Gray et al., 2000). In mitochondria, OMI/HTRA2 activates pro-apoptotic proteins when it is released from damaged mitochondria to the cytosol (Hegde et al., 2002; van et al., 2002). Misfolded or damaged proteins are recognized by the C-terminal PDZ domain, a protein interaction domain (Clausen et al., 2002). Evidence linking *OMI/HTRA2* to Parkinson's disease is the motor neuron degeneration-2 (mnd2) mouse strain which displays striatal degeneration, microglia activation and Parkinson-like features, caused by a spontaneously occurring point mutation (Jones et al., 1993; Jones et al., 2003). The *mnd2* mutation was identified as the missense mutation Ser276Cys in the protease domain of *OMI/HTRA2*. PINK1 phosphorylates OMI/HTRA2, which is suggested to alter the protease activity by increasing the resistance to mitochondrial-induced stress (Alnemri, 2007). OMI/HTRA2 phosphorylation has been reported to be decreased in brains of Parkinson patients carrying *PINK1* mutations (Plun-Favreau et al., 2007). Genetic studies have also implicated *OMI/HTRA2* in Parkinson's disease in a German Parkinson cohort (Strauss et al., 2005) and in Belgian Parkinson patients (Bogaerts et al., 2008).

MTIF3

Translation of mRNA encoded by mtDNA requires a protein biosynthetic system separate from the one in the cell cytoplasm (Sharma et al., 2003). Mitochondrial translation initiation factor 3 (*MTIF3*) is a nuclear gene and encodes a 29 kDa protein identified from the human expressed sequence tag data base (Koc and Spremulli, 2002). The MTIF3 protein promotes formation of the initiation complex on the mitochondrial 55S ribosome and has an active role in initiation of translation of mitochondrial protein synthesis (Koc and Spremulli, 2002). The rs7669 SNP in *MTIF3* has been reported to be associated with Parkinson's disease in a German cohort (Abahuni et al., 2007).

POLG1

DNA polymerase gamma 1 (*POLG1*) was first identified in human HeLa cells as an RNA-dependent DNA polymerase (Fridlender et al., 1972) and activity of the protein was found to be associated with mitochondria (Bolden et al., 1977). POLG1 is part of the heterotrimeric mitochondrial DNA polymerase which consists of a catalytic subunit encoded by *POLG1* and two identical accessory subunits translated from *POLG2* (Longley et al., 1998). The polymerase has an important function in replication and repair of the mtDNA (Kaguni, 2004). In addition to its 5' to 3' polymerase activity, POLG1 has a 3' to 5' exonuclease proofreading activity and a spacer region which mediates DNA binding and interaction with the accessory subunits (Kaguni, 2004). Mutations in the proofreading region of *POLG1* can have a domino effect on the

activity and induce accumulation of secondary defects in mtDNA during replication, which results in dysfunction of the respiratory chain, where all the 13 proteins encoded by the mtDNA play a role (Attardi, 1985; Chomyn et al., 1986; Hudson and Chinnery, 2006). Two missense mutations, G737R and R853W, have been reported in early-onset familial parkinsonism (Davidzon et al., 2006). It should be noted that *POLG1* mutations may cause a broad spectrum of symptoms. Thus the missense mutation W748S was found in a Finnish patient with parkinsonism, chronic progressive external ophthalmoplegia, ataxia, peripheral neuropathy and hearing loss (Remes et al., 2008). By contrast, no significant association with Parkinson's disease was found in two GWAS covering *POLG1* SNPs and performed in a North American, United Kingdom and German material of Caucasian origin with sporadic Parkinson's disease (Simón-Sánchez et al., 2009). In the N-terminal part of *POLG1* a polyglutamine tract (poly-Q) is located, which is encoded by a CAG repeat in exon 2 (Lecrenier et al., 1997). Normally, the poly-Q tract consists of 10Q (frequency >80%) or 11Q (frequency 6-12%) followed by CAA and two additional CAG repeats (Rovio et al., 1999; Rovio et al., 2004). Variations in length of the poly-Q tract of *POLG1* from normal 10 or 11 CAG repeats have been found to associate with Parkinson's disease by a number of groups (Eerola et al., 2010; Luoma et al., 2007).

MIRO1 and MIRO2

The mitochondrial Ras homolog gene family, member T1 and T2 (*MIRO1* and *MIRO2*) also, referred to as RHOT1 and RHOT2, belongs to the mitochondrial Rho GTPase family. Miro was originally identified in yeast (Frederick et al., 2004) and later described to share similarities with Rho GTPases (Fransson et al., 2003). The mitochondrial Rho GTPases are involved in axonal transport of mitochondria (Fransson et al., 2006; Macaskill et al., 2008). The Miro proteins have a C-terminal domain which locates them to the mitochondrial outer membrane, as well as two GTPase domains and two EF-hands regulating the trafficking of mitochondria along microtubules. When calcium binds to the EF-hands, the transport stops (Lewit-Bentley and Rety, 2000; Macaskill et al., 2009). Studies done of the Miro homolog in yeast, Gem1P, have shown that all domains are needed for proper mitochondrial movement (Frederick et al., 2004). Loss-of-function mutations in the Miro GTPase in *Drosophila melanogaster* lead to impaired locomotion and premature death (Guo et al., 2005). Recently, it was reported that the PARK6 gene *Pink1* might be a part of a mitochondrial multi-protein complex together with the atypical GTPase Miro and the adaptor protein Milton (Weihofen et al., 2009). This suggests that Miro might have a role in neurodegenerative disorders like Parkinson's disease.

PON

The paraoxonase (PON) gene family has three known members, *PON1*, *PON2* and *PON3* clustered within 500 kb from each other on the long arm of human chromosome 7q21.3 (Mochizuki et al., 1998; Primo-Parmo et al., 1996). The three genes show 70% similarities at the nucleotide level and share approximately 60% at the amino acid level (Mackness et al., 2002), but have different functions. *PON* gene family members are also differently expressed in human tissues (Primo-Parmo et al., 1996). *PON1* and *PON3* are exclusively expressed and synthesized in the liver, while *PON2* is expressed

in various tissues such as brain, liver, kidney and testis (Ng et al., 2001). PON1 and PON3 are secreted from liver cells into the circulation (Blatter et al., 1993), where they are anchored to high-density lipoprotein (HDL) by their hydrophobic N-terminal (Sorenson et al., 1999). PON1 activity predominates over PON3 in human serum (Blatter et al., 1993). PON2 is expressed in many tissues and is not released from cells. Instead, PON2 exposes its active site to the cells outside (Stoltz et al., 2009), whereas PON1 is localized to the cell membrane before it is secreted to the circulation and bound to HDL (Deakin et al., 2002). PON1 is an arylesterase able to hydrolyze organophosphate substrates such as pesticides, neurotoxins and arylesters (Cowan et al., 2001; Hassett et al., 1991; Kelso et al., 1994). Toxic aldehydes can be the result of lipid peroxidation, and are highly reactive (Esterbauer et al., 1991). Such aldehydes can also be hydrolyzed by PON1 (Ahmed et al., 2002). The PON1 enzyme also prevents oxidation of HDL and low-density lipoproteins (LDL) (Aviram et al., 1998). Exposure to environmental factors like pesticides, have been suggested in epidemiological studies to increase the risk for Parkinson's disease (Le Couteur et al., 1999). Organophosphoric pesticides are one example of environmental toxins that have structural similarities to the DA neurotoxin MPTP (Barbeau, 1984; Burns et al., 1983; Langston et al., 1983). There are two coding polymorphisms in the *PON1* gene, Leu54Met (rs854560) and Gln192Arg (rs662), both reported to disturb enzymatic activity (Humbert et al., 1993; Mackness et al., 1998). Individuals homozygous for the wild-type Leu-allele of the rs854560 tend to have a higher mRNA and protein level and increased PON1 activity than homozygous Met-allele carriers, whereas heterozygous individuals show intermediate protein levels and gene activity (Garin et al., 1997; Leviev et al., 1997). An association was recently found between Parkinson's disease and exposure to the organophosphates diazinon, chloropyrifos and parathion (Manthripragada et al., 2010). Individuals homozygous for the rs854560 Met-allele were reported to exhibit a two-fold increase risk in developing Parkinson's disease when exposed to diazinon and chloropyrifos compared to individuals with wild-type or heterozygous genotype and no exposure. In addition, the two genetic variants of rs662 (Gln192Arg) have substrate-dependent differences in hydrolysis kinetics (Davies et al., 1996). Homozygous Arg-allele carriers are poor metabolizers of environmental chemicals and food-derivatives similar to the neurotoxins soman and sarin, but in contrast hydrolyze the insecticide paraoxon rapidly compared to Gln-allele carriers. Recently, a negative genetic study was reported on rs854560 and rs662 in African Americans and Caucasian materials (Wingo et al., 2010). Genetic variations in the *PON1* 5'-promoter region (rs854572; c.-909G>C, rs854571; c.-832G>A, rs705379; c.-108C>T) (Leviev and James, 2000) can also have an effect on the concentration in serum and enzyme activity (Brophy et al., 2001). Two of the promoter SNPs, rs705379 and rs854572, induce an approximately two-fold increase of expression levels whereas rs854571 has only a minor effect on expression levels (Brophy et al., 2001).

ADH

Human alcohol dehydrogenases (*ADH*) are located in a cluster on chromosome 4q21-q25 (Duester et al., 1999; Yokoyama et al., 1996), consisting of seven genes grouped into five classes (Szalai et al., 2002). ADHs form an enzymatic defense barrier against the environment by being present in tissues such as epidermis and the epithelial lining of the gastrointestinal and respiratory tract (Deltour et al., 1997; Haselbeck et al., 1997; Haselbeck and Duester, 1997; Tietjen et al., 1994; Vaglenova et al., 2003; Westerlund et al., 2007). ADHs metabolize a broad spectrum of substrates such as alcohols and aldehydes present in food and air or endogenously produced during lipid peroxidation (Duester et al., 1999). ADHs are also involved in the metabolism of retinol (vitamin A) to retinalaldehyde to prevent vitamin A toxicity and detoxification of many different types of aldehydes (Pares et al., 2008). Aldehydes can react with DA and form salsolinol, a tetrahydroisoquinoline (Falck et al., 1962; Yamanaka et al., 1970). Salsolinol is a product similar to the parkinsonism-inducing product MPTP (Niwa et al., 1992). Expression of aldehyde dehydrogenase 1A1 in the mesencephalic DA system (Haselbeck et al., 1999) suggests that aldehyde metabolism may be important for normal DA function.

Location of ADHs at interfaces between the environment and the organism and suggestions that environmental factors might play a role in Parkinson's disease (Tanner, 2010) in turn suggest mutations in the ADHs should be investigated. A promoter mutation in the human class IV ADH gene, often referred to as *ADH4* (official gene name: *ADH7*), has been reported to be associated with Parkinson's disease (Buervenich et al., 2000) and induces a 25-30% reduction in transcriptional activity (Buervenich, 2002). *ADH1C* belongs to the class I ADH gene family (Duester et al., 1999). A truncating stop mutation in *ADH1C* has been reported to be significantly associated with Parkinson's disease (Buervenich et al., 2005). Notably, *ADH1C* and *ADH4* are not expressed in human or rodent brain tissue (Galter et al., 2003b). *Adh1* is mainly expressed in the lower gastrointestinal tract, from stomach to rectum in mice and in duodenum, colon and rectum in rats (Westerlund et al., 2007). On the other hand, *Adh4* shows a higher expression in the upper part of the digestive tract such as the epithelial lining of the tongue, esophagus and stomach. Additional, *Adh4* is highly expressed in the olfactory epithelia, whereas the expression of *Adh1* is lower (Westerlund et al., 2007; Westerlund et al., 2005).

AIM OF THE THESIS

The aim of the present thesis was to investigate possible candidate genes for Parkinson's disease.

Identification of genetic risk factors in Swedish Parkinson patients:

- Screening for known and new genetic variants in candidate genes.
- Determination of mutation, polymorphism and haplotype frequencies.

Investigation of gene and protein expression:

- Localization and quantification of cellular mRNA expression in human and rodent brain tissue.
- Quantification of mRNA expression.
- Quantification of protein levels in normal and pathological human brain.

Analysis of genetic risk factors for Parkinson's disease in mouse models:

- Investigation of spontaneous and drug-induced behavior in adult and aged animals.
- Quantification of monoamine and monoamine metabolite levels.

MATERIAL AND METHODS

Subjects

Human DNA and tissue samples

The DNA was obtained after oral and written information was given and each subject signed an informed consent. Approval of the relevant local ethical committee in Sweden was obtained. All subjects were unrelated Caucasians of Swedish origin diagnosed with either Parkinson's disease or Alzheimer's disease and controls without neurological disease. All Parkinson patients fulfilled the United Kingdom Parkinson's Disease Society Brain Bank Criteria for Parkinson's disease except that more than one affected relative was allowed (Daniel and Lees, 1993). Control subjects were spouses to Parkinson patients or individuals visiting hospitals or care centers for non-neurological reasons, blood donors or participants from the Swedish National Study on Aging and Care in Kungsholmen (SNAC-K) (Table 2). DNA was extracted from blood samples according to standard protocols (papers I, III-VII). Human postmortem brain tissue was provided by the Netherlands Brain Bank (Amsterdam, The Netherlands), the Harvard Brain Tissue Resource Center (Belmont, USA), the Queen Square Brain Bank for Neurological disorders (University of London, United Kingdom) and the Brain Bank at Karolinska Institutet (Stockholm, Sweden) (papers II, III).

Table 2. DNA used in Parkinson's and Alzheimer's disease case-control association studies.

Material	Origin	Female (n)	Male (n)	Total (n)	Mean age at sampling (years)	Mean age of onset (years)
Control						
	Stockholm	529	463	992	59.7	-
	Göteborg	105	66	171	69.0	-
	Linköping	203	198	401	68.6	-
Total				**1564**		
Parkinson's disease						
	Stockholm	113	189	302	67.9	60.1
	Göteborg	49	67	116	67.5	59.2
	Linköping	78	123	201	71.3	-
Total				**619**		
Alzheimer's disease	Stockholm	265	165	430	-	78.9
Total				**430**		

Epstein-Barr virus transfection

In parallel with DNA extraction from peripheral blood, transfection of B-lymphocytes with Epstein-Barr virus (EBV) (Walls and Crawford, 1987) was carried out with cells from Parkinson patients and controls collected in Stockholm. B-lymphocytes were separated from peripheral blood by standard protocols using Ficoll-Paque (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The cells were cultivated in RPMI 1640 medium (SIGMA, St. Louis, MO, USA) with 20% fetal calf serum and L-glutamine (200 mM; Invitrogen, Carlsbad, CA, USA), penicillin - streptomycin (5000 µg/ml; Invitrogen, Carlsbad, CA, USA), cyclosporine (1 µl/ml; Apoteket, Stockholm, Sweden) and filtered supernatant of EBV infected B95-8 cells. The medium was changed twice a week until the cell lines were established at which time the cells were gradually frozen and kept at -140°C until use. For mRNA quantification, the cells were thawed, cultured for approximately two weeks and harvested when cell numbers reached 4 - 5 million (paper IV).

Animal models and tissue

Animals were housed under standardized conditions with food and water *ad libitum* in a light controlled room (12 h light/dark cycle, lights on at 6:00 am) at 21°C and 60% humidity. Experiments were approved by the local Animal Ethics Board of Stockholm, Sweden. *Adh1* knockout (*Adh1*-/-), *Adh4* knockout (*Adh4*-/-) and *Adh1*/*Adh4* double knockout (*Adh1/4*-/-) mice were used to study possible disturbances of DA-system-related behavior. Generation of *Adh1*-/-, *Adh4*-/- and *Adh1/4*-/- knockouts have been described earlier (Deltour et al., 1999a; Deltour et al., 1999b; Molotkov et al., 2002). In the truncated mutant *Adh1* strain exons 7-9 have been replaced by a neo cassette. The double knockout *Adh1/4* lacks exons 7-9 plus the polyadenylation signal of *Adh1* which has been replaced with PGK-hygro and the *Adh4* gene lacks the promoter region plus exons 1-6 which have been exchanged by a neo cassette. The same has been done in *Adh4*-/-. The mice strains were backcrossed with wild-type (WT) C57BL/6BKL mice for ten generations or more before the experiments (papers VIII, IX).

Techniques

Several different techniques have been used in this thesis and the most frequently used are listed in the table below (Table 3).

Table 3. Methods used in the different papers.

Techniques	Papers
mRNA expression and localization	
Quantitative Real-Time PCR	IV
In situ hybridization	II, III
Genotyping approaches	
Pyrosequencing	I, III, IV, VII
TaqMan	VI, VII
Fragment analysis	V
Capillary sequencing	I, V
Linkage disequilibrium	VII
In silico mRNA folding	I, IV, V
Behavioral experiments	
Locomotor activity	VIII, IX
Olfactory testing	VIII, IX
Biochemical analysis	
High-performance liquid chromatography	VIII, IX

mRNA expression and localization

Quantitative Real-Time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) is a method for quantification of mRNA expression levels. Total RNA was isolated from EBV transfected B-lymphocytes using RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified by spectrophotometry at 260 nm. Complementary DNA was generated from 1μg RNA by a modification of the manufacturer's protocol using Deoxyribonuclease I, Amplification Grade (Invitrogen, Carlsbad, CA, USA) and SuperScript^{TM} III Platinum^{R} Two-Step qRT-PCR kit with SYBR^{R} Green (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed on an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) using SYBR Green I dye (Invitrogen, Carlsbad, CA, USA). The samples were run in triplicates for the target gene and two housekeeping genes; beta-actin and cyclophilin. Amplification of a single gene product was confirmed by monitoring the dissociation curve as well as by analyzing samples using agarose gel electrophoresis. Threshold cycle values from the exponential phase of the PCR amplification plot were analyzed with ABI Prism 7000 SDS v1.2.3. Each threshold cycle value for the target transcripts was normalized to beta-actin and cyclophilin, using qBase v1.3.5 (Hellemans et al., 2007), based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The

$2^{-\Delta\Delta Ct}$ method has been extended in the qBase software to include multiple stably expressed reference genes to improve normalization (Hellemans et al., 2007) (paper IV).

Oligonucleotide probes

Oligonucleotide probes used for *in situ* hybridization were approximately 48-51 base pairs long and had a GC content of >45%. Probes with a length of 36 base pairs were designed by using the publicly available online software primer3 (http://zeon.well.ox.ac.uk/git-bin/primer3_www.cgi) and an additional number of nucleotides were added to obtain a satisfactory probe length. The publicly available online software mfold, version 3.2, was used to calculate the folding energy (Mathews et al., 1999; Zuker, 2003). Probe specificity was controlled by aligning probes against all sequences available in a public sequence data base (http://blast.ncbi.nlm.nih.gov/) and only sequences with high specificity were selected. For each gene, several probes were designed and probes generating coherent results, good signal-to-noise and specificity with respect to available knowledge were used for analysis.

In situ hybridization

Radioactive *in situ* hybridization was invented by Pardue and Gall (Pardue and Gall, 1969) and is a sensitive method for detection and localization of mRNA expression in tissue and cell cytoplasm by complementary hybridization of a radioactively labeled oligonuclotide probe to a sequence of interest. The method used in this study was based on previously published protocols (Dagerlind et al., 1992; Galter et al., 2003a; Galter et al., 2003b).

Frozen tissues were cryosectioned at 14 µm, thawed onto coated glass slides (SuperFrost, VWR, Stockholm, Sweden) and stored at -20°C until use. 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 (Fermentas, Helsingborg, Sweden). Excess radioactive nucleotides were removed by passage through a micro-column (ProbeQuant G50-Micro Columns, GE Healthcare, Uppsala, Sweden). Cryosections were air-dried 3-5 hours prior to hybridization. Radio-labeled oligonucleotide was diluted in a cocktail containing 4xSSC, 50% formamide, 1x Denhardt's solution, 1% sarcosyl, 0.02 M Na_3PO_4 (pH 7.0), 10% w/v dextran sulfate and 50 mg sheared salmon sperm DNA. 150 µl of this solution was added to each slide and slides were individually covered (Parafilm M, VWR, Stockholm, Sweden) followed by overnight incubation at 42°C in a humidified chamber. After hybridization, slides were washed for 15 min in 60°C 1 x SSC buffer five times and cooled to room temperature, followed by rapid dehydration in water and increasing percentages of ethanol (70%, 95% and 99.5%) and air-dried. Slides were analyzed by phosphoimaging (Fujix BAS 3000 system, Fujicolor Sverige AB, Skärholmen, Sweden). For further evaluation the slides were placed on ^{14}C standard autoradiographic film (Biomax, Eastman Kodak Co, Rochester, NY, USA) for 5-10 days and for microscopic evaluation of the signal, slides were dipped in NTB2 nuclear track emulsion (Kodak NTB at 1:2, Rochester, NY, USA) and exposed up to 6 weeks, counter-stained with 0.5% cresyl violet and mounted (papers II, III).

Image analysis

To quantify mRNA content, autoradiographic films were digitized and the signal intensity from defined areas was quantified using ImageJ (http://rsb.info.nih.gov/ij/). Optical density was converted to nCi/g as determined from a ^{14}C standard curve (paper III).

Statistical analysis used in mRNA quantification studies

Student´s t-test was used for quantification of mRNA expression by qRT-PCR between genotypes and measurements of the signal intensity on autoradiographic films (GraphPad Prism version 5.03, San Diego, CA, USA). Significance level was set at $p< 0.05$.

Genotyping methods

Primers

Synthetic oligonucleotide primers used in polymerase chain reaction (PCR) were approximately 15-20 base pairs long and had a GC content of >45%. The primers were designed by using the publicly available online software primer3 (http://zeon.well.ox.ac.uk/git-bin/primer3_www.cgi) and possible RNA-foldings were tested using the mfold program (Mathews et al., 1999; Zuker, 2003).

Pyrosequencing

Pyrosequencing detects SNPs by detecting the energy released when a nucleotide is incorporated into a predefined DNA strand (Ronaghi et al., 1998). The advantage of this method is that it also provides a short sequence adjacent to the SNP to make sure that the right locus has been found (Figure 2). The pyrosequencing reaction initiates when one of the four deoxynucleotide triphosphates (dNTP, A=adenine, C=cytosine, G=guanine, T=thymine) is added. If the nucleotide is complementary to the base in the template strand, DNA polymerase catalyzes the incorporation into the strand. Visible light is generated proportional to the number of incorporated nucleotides by release of pyrophosphate. In the presence of adenosine 5´phosphosulfate, pyrophosphate is converted to ATP by the enzyme ATP sulfurylase. ATP drives the luciferase-mediated oxidation of luciferin to oxyluciferin that generates visible light in proportion to the amount of ATP. The produced light is detected by a charged coupled device camera and the signal shown in the pyrogram. Apyrase degrades unincorporated dNTPs and excess ATP, and the reaction can restart with another nucleotide.

PCR was carried out with Taq DNA polymerase after which the biotinylated PCR product was immobilized on streptavidin-coated beads according to manufacturer's instructions. The immobilized DNA template was captured onto filter probes (PyroMark Vacuum Prep Tool, Biotage AB, Uppsala, Sweden). The filter probes were flushed with 70% ethanol, denaturation solution, washing buffer and the single-stranded template was annealed to a sequencing primer. All solutions were prepared according to manufacturer's instructions (Biotage AB, Uppsala, Sweden). Samples

were analyzed on an automated pyrosequencer (PSQ 96 System with SNP Software and SNP Reagent Kits, Biotage AB, Uppsala, Sweden) (papers I, III, IV, VII).

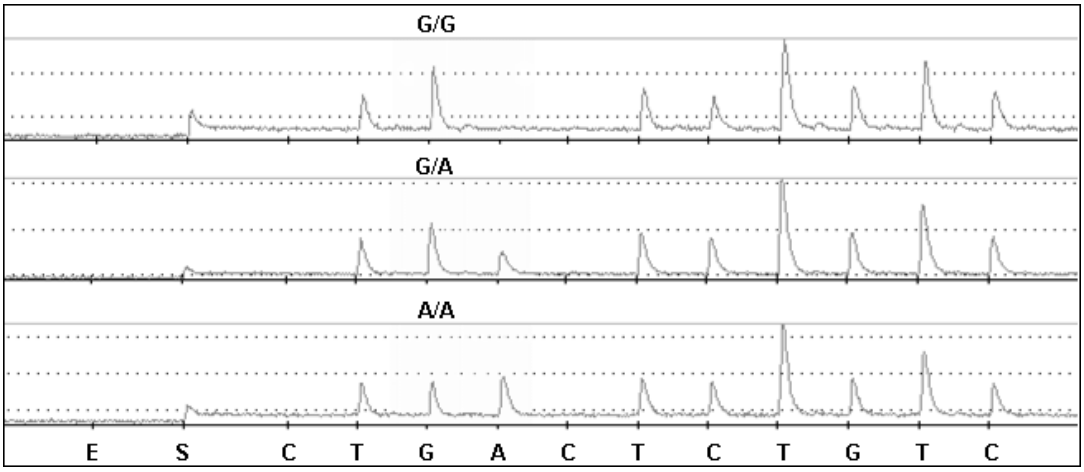

Figure 2. Pyrograms of the *MTIF3* variant rs7669, showing the three different genotypes GG, GA and AA using a reverse sequencing primer. E= Enzymes, S= Substrates.

TaqMan technique

The TaqMan technique relies on real-time PCR and allele-specific hybridization of oligonucleotide probes during PCR (Applied Biosystems, Foster City, CA, USA). The probe binds across the SNP and has a reporter at the 5´-end and a quencher at the 3´-end. During the PCR each reporter will fluoresce at a specific wavelength when they are separated from the quencher and that information gives the genotype. VIC and FAM were used as reporter colors (Figure 3).

The fast real-time PCR instrument (ABI 7500 FAST Real-Time PCR, Applied Biosystems, Foster City, CA, USA) was used for analysis. The TaqMan assay contained primers and 5´ fluorescently labeled (FAM and VIC) minor groove binding probes (20×) for detection of the SNPs. Allelic discrimination was run with pre- and custom-designed primers and probes, genotyping master mix (TaqMan®,Applied Biosystems, Foster City, CA, USA) and 10-20 ng of genomic DNA in a total reaction volume of 10 µl mixed in transparent 96-well plates. The PCR conditions followed the recommendations of default settings for the SNP assay, except that the number of cycles were set to 55 and run at 92°C for 15 s. The ramp speed was set to standard. A post-PCR read was done for allelic discrimination using appropriate software (SDS version 2.0.4) supplied with the instrument (papers VI, VII).

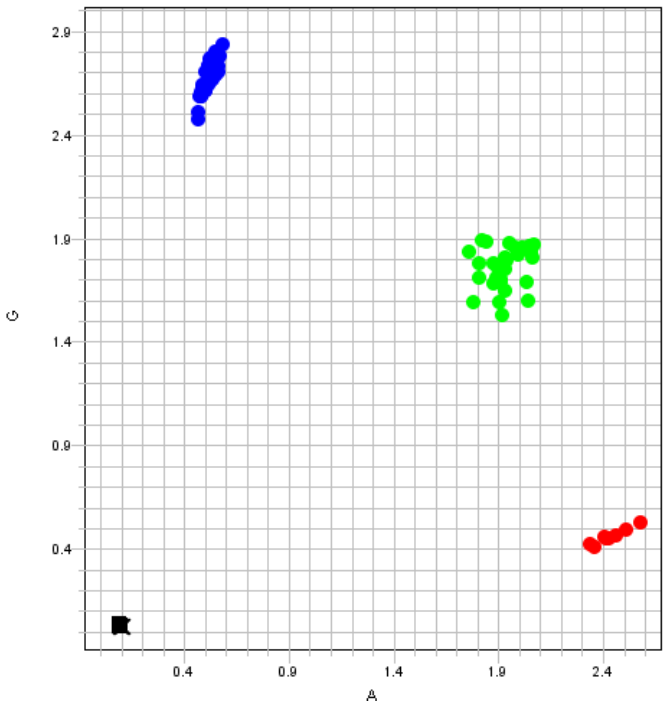

Figure 3. Results from a TaqMan run showing an allelic discrimination plot of the *MIRO2* rs1139897. In this example the blue dots are samples which are homozygotes for the wild-type genotype (G/G) labeled with FAM, the green dots are heterozygotes (G/A) consisting of a mixture of FAM and VIC labeled probes and the red dots are homozygotes (A/A) labeled with VIC. The collection of spots in the lower left corner are difficult to distinguish in this plot when illustrated simultaneously and represents, black boxes = H_2O controls and black crosses = undetermined.

Fragment analysis

Fragment analysis (3730 DNA Analyzer, Applied Biosystems, Foster City, CA, USA) is a high-throughput system to screen for length variation in equally amplified PCR fragments. Taq DNA polymerase PCR was run, the forward primer was labeled with the fluorophore VIC® at the 5´ end and the reverse primer was tailed with a 7 base pairs tail attached at the 5´ end in order to promote the non-template adenosine addition by the Taq DNA polymerase at the 3´ end of the PCR products (Brownstein et al., 1996) (Applied Biosystems, Foster City, CA, USA). For detection of the length variation in the fragments a mixture of Hi-Di formamide and a size standard (GeneScan-500 LIZ, Applied Biosystems, Foster City, CA, USA) was added to the PCR products, following the provider's instruction. Data were analyzed with appropriate software (GeneMapper® Software version 4.0 and/or Peak Scanner™ Software version 1.0, Applied Biosystems, Foster City, CA, USA) (paper V).

Capillary sequencing

Automated capillary sequencing is preferred when searching for new mutations. The method provides the entire DNA sequence of the PCR fragment. PCR was carried out to amplify a fragment of genomic DNA using Taq DNA polymerase and forward and reverse primers specific for the region of interest (Figure 4). The amplified fragments were purified (QIAquick® PCR Purification kit, Qiagen, Hilden, Germany). The isolated DNA fragments were further amplified by a sequencing PCR with a single primer and fluorescent labeled dideoxynucleotide triphosphates (ddNTP), which are incorporated randomly and terminate the elongation process using a DTCS kit (CEQ 2000 system, Beckman Coulter Inc., Fullerton, CA). This will result in DNA fragments of different lengths, all terminating with a fluorescent ddNTP. The samples were mixed thoroughly with stop solution (3 M NaOAc, 0.5 M EDTA, 20 mg/ml glycogen) and 95% ethanol (-20°C). To wash the DNA, samples were centrifuged followed by removal of the supernatant and the addition 70% ethanol (-20°C) twice. To dry the DNA, samples were vacuum centrifuged and resuspended in 40 µl deionized formamide. Purified DNA fragments were separated using automated capillary gel electrophoresis (CEQ 2000 system, Beckman Coulter Inc., Fullerton, CA, USA) according to the manufacturer's instructions (papers I, V).

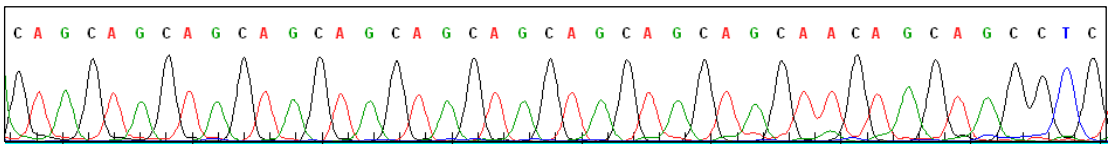

Figure 4. Example of a sequence from a capillary sequencing run of the CAG repeat in *POLG1*. All the four letters (A, C, G and T) in the DNA code are represented.

Genotyping errors

To test for genotyping and sequencing errors water was used as a negative control and random samples were reanalyzed to confirm the results.

Linkage disequilibrium

Haplotype analysis was used to identify the haplotype window with the strongest association to disease. Pairwise linkage diseqilibrium was examined with the program GOLD (Abecasis and Cookson, 2000). Haplotype analyses of the SNPs were performed in the sliding window model using appropriate software (Unphased version 3.1.3) (Dudbridge, 2008). Statistical significance was defined as $p< 0.05$. Bonferroni correction was used for the number of studied SNPs in all single marker analyses and permutation tests with 10,000 permutations were performed in the sliding window analysis (paper VII).

In silico predication of mRNA secondary structure

To evaluate the effect on the mRNA structure, the secondary structure was predicted using the publicly available online mfold program, version 3.2 (Mathews et al., 1999; Zuker, 2003). Parts of the mRNA sequences of 141 nucleotides including flanking sequences (70 nucleotides) on either side of the polymorphism were analyzed and compared to the wild-type sequence (papers I, IV, V).

Statistical analysis used in genotyping studies

Genotype distribution and allele frequencies were compared between the different groups using the Chi-square (χ^2) test (Sham and Curtis, 1995) or Fisher's exact test (GraphPad Prism version 5.03, San Diego, CA, USA). Statistical significance levels were set at $p< 0.05$. Distribution of genotypes in controls was tested for consistency with the Hardy–Weinberg equilibrium (Stern, 1943).

Animal studies

Behavior

A computerized multicage infrared-sensitive motion detection system was used to study animal behavior. Horizontal movement, total distance and vertical activity were measured in mice for 5 minute periods (Accuscan Instruments, Columbus, OH, USA). Horizontal movement was measured as total number of horizontal beam breaks and total distance was defined as the total distance travelled in centimeters. Vertical activity was the parameter defined as the total number of beam breaks measured by the vertical sensor. Locomotor boxes were squared 42x42 cm and 32 cm tall, equipped with 16x16x16 infrared beams (x, y, z-direction) were spaced 2.5 cm apart. The mice were habituated to the dimly lit, low-noise and ventilated experimental room for approximately 30 min prior the experiment. All experiments took place between 8 am and 1 pm (papers VIII, IX).

For olfactory testing mice were habituated to a hole poke cage (same size as the locomotor cage) for 60 min prior to the recording period of 10 min. A novel odor was introduced to one of the 16 grid-covered holes in the floor. The time required by the animal to find the odor and number of times the odor site was visited by the animal were recorded during a period of 10 min with the hole poke module (Versamax Activity Monitor, Accuscan Instruments, Columbus, OH, USA) (papers VIII, IX).

High-performance liquid chromatography

Concentrations of monoamines and their metabolites in rodent postmortem brain tissue samples were determined by high-performance liquid chromatography (HPLC) with electrochemical detection. All tissues were weighted and homogenized by sonication in 100 µl of 0.1 M per-chloric acid with bisulphate followed by centrifugation. The supernatant was transferred to a column and centrifuged a second time. The endogenous levels of noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were determined in the supernatants by

comparison to standards as follows: dl-arterenol-hydrochloride for NA, 3-hydroxytyramine-hydrochloride for DA, 3,4-dihydroxyphenylacetic acid for DOPAC, 4-hydroxy-3-methoxyphenylacetic acid for HVA, 5-hydroxy-tryptamine creatinine sulfate for 5-HT and 5-hydroxyindole-3-acetic acid for 5-HIAA.

The separations were performed using a reverse-phase column (Reprosil-Pur, C18-AQ, 150 mm X 4mm, 5 µm particle diameter). The mobile phase consisted of a 0.05 M sodium phosphate/0.03 M citric acid buffer containing 0.1 mM EDTA, with various amounts of methanol and sodium-l-octane sulphonic acid, at a flow rate of 0.7 ml/min. Monoamines and metabolites were detected (ESA Coulochem III electrochemical detector system with a High Sensitivity Analytical Cell, ESA, Dalco Chromtech AB, Sweden), with the detector cell set at 50/350 mV and the resultant peak areas were measured with appropriate software (EZ Chrom Elite from ESA). Tissue levels were expressed as ng/g wet weight. Levels of monoamines and metabolites were compared to those of wild-type animals set to 100% (papers VIII, IX).

Statistical analysis used in behavior and biochemical studies

Data from the three parameters collected over time in the locomotor study (horizontal activity, total distance and vertical activity) were analyzed using two-way ANOVA to detect group differences. Possible weight differences, olfactory testing and HPLC data from measurements of monoamines and metabolites were analyzed using one-way ANOVA (GraphPad Prism version 5.03, San Diego, CA, USA).

RESULTS AND COMMENTS

DJ-1 at PARK7 locus (paper I)

I. DJ-1 mutations in Parkinson's disease

The PARK7 gene, *DJ-1*, has been linked to a family with autosomal recessive early-onset parkinsonism (AREP) in the Netherlands (van Duijn et al., 2001) and the same locus has also been linked to a family with AREP in Italy (Bonifati et al., 2003). In the Caucasian population, the majority of *DJ-1* mutations leading to changes in the amino acid sequence have been found in exons 2, 5 and 7 (Nuytemans et al., 2010). Therefore, these exons and the exon/intron borders were sequenced using capillary sequencing in a Swedish Parkinson material consisting of 144 Parkinson patients. 77 of the Parkinson patients had an early disease onset (≤50 years old) and 67 had a self-reported positive family history of Parkinson's disease in one or more first-, second- or third-degree relatives. 66 of the early-onset cases have previously been investigated for copy number variations of exons 1, 3, 5 and 7 of *DJ-1* although no aberrations were detected (Håkansson et al., 2008). The synonymous mutation Ala167Ala (c.501A>G) was identified in two patients. One of the patients had early-onset Parkinson's disease and was later diagnosed with Alzheimer's disease and the other had reported a brother with Parkinson's disease. Pyrosequencing was used to specifically screen for the mutation in a control material consisting of 213 neurologically healthy controls of Swedish origin. No Ala167Ala carriers were identified among the controls. To predict the effect of the synonymous mutation on the mRNA secondary structure of *DJ-1*, an *in silico* study was performed. The results indicated a small decrease in mRNA folding energy (1.3%) in the A>G substituted sequence compared to the wild-type sequence. The number of *DJ-1* mutations described today is low and they account for only a few Parkinson cases in different populations (Nuytemans et al., 2010). The observed *DJ-1* mutation frequency (1.4%) in the Swedish Parkinson cohort is in agreement with findings in other Caucasian populations (Nuytemans et al., 2010).

PINK1 at PARK6 locus (paper II)

II. PINK1 mRNA expression in brain

Mutations in PTEN-induced putative kinase 1 (*PINK1*), account for 1-2% of the early-onset sporadic Parkinson cases as heterozygous mutations (Marongiu et al., 2008). To determine which cells express PINK1 we applied *in situ* hybridization to human postmortem brain tissue from five cases with sporadic Parkinson's disease and five neurologically healthy control subjects, and to rodent brains from C57BL/6BKL mice and Sprague-Dawley rats.

The results show an abundant neuronal *PINK1* expression in all three species. No evidence for altered expression of *PINK1* mRNA in surviving neurons in sporadic Parkinson's disease compared to controls was detected. The highest levels of expression were observed in hippocampus, SN and cerebellar Purkinje cells.

OMI/HTRA2 at PARK13 locus (paper III)

III. Altered enzymatic activity and allele frequency

The serine-protease OMI/HTRA2 has been implicated in Parkinson's and Alzheimer's disease. Involvement of *OMI/HTRA2* in Alzheimer's disease was suggested when the enzyme was identified as a presenilin-1 interacting factor in a 2-hybrid system (Gray et al., 2000). Genetic studies of *OMI*/*HTRA2* in Parkinson's disease (Plun-Favreau et al., 2007; Strauss et al., 2005) discovered two variants which result in defective OMI/HTRA2 protease activity (Strauss et al., 2005). Western blot quantification of OMI/HTRA2 in frontal cortex of patients with Alzheimer's disease (n=10) and control subjects (n=10) from each of the two separate materials indicated reduced levels of active OMI/HTRA2 whereas inactive enzyme levels were not significantly different between the groups. The protease activity of OMI/HTRA2 was found to be significantly increased in frontal cortex in Alzheimer's disease compared to matched control subjects in the same material, using an enzyme activity assay. *In situ* hybridization was used to compare expression levels in frontal cortex and hippocampus in patients with Alzheimer's disease and control subjects. No difference in the *OMI/HTRA2* mRNA levels was observed. In addition, the occurrence of the *OMI/HTRA2* variants A141S and G399S were investigated in a Swedish case-control material for Alzheimer's disease (n=351, non-demented controls=348) and Parkinson's disease (n=281, neurologically healthy controls=308) using pyrosequencing. Chi-square analysis test shows an association of A141S with Alzheimer's disease (P=0.0453), but not with Parkinson's disease. The lack of association of *OMI/HTRA2* with Parkinson's disease is in agreement with recent reports (Kruger et al., 2009; Ross et al., 2008; Simón-Sánchez and Singleton, 2008).

Mitochondrial translation initiation factor 3 (paper IV)

IV. MTIF3 polymorphism causing decreased mRNA levels

Mitochondrial translation initiation factor 3 (*MTIF3*) promotes formation of the initiation complex on the mitochondrial 55S ribosome and has an active role in initiation translation (Koc and Spremulli, 2002; Shastry, 2001). Recently, a synonymous SNP in *MTIF3*, rs7669, was reported to be associated with Parkinson's disease in cohorts with different geographical origin (Abahuni et al., 2007; Behrouz et al., 2010). Pyrosequencing was used to screen for the polymorphism rs7669 (C>T) in *MTIF3*. The material consisted of 381 Parkinson patients (familial and sporadic) and 322 neurologically healthy controls. All cases and controls were of Swedish origin from Stockholm or Göteborg. Furthermore, qRT-PCR was used to investigate the consequence of rs7669 using RNA prepared from EBV transfected B-lymphocytes from individuals representing the three different genotypes (CC, CT and TT). Genotype and allele frequencies were compared between the different groups using Chi-square test. When comparing the less common genotypes TT/CT versus the common genotype CC a significant association was found with sporadic Parkinson's disease (P=0.0473). Quantification of expression was compared between the three different genotypes using Student's t-test. Interestingly, the qRT-PCR results showed a significantly lower level of *MTIF3* mRNA expression in cells from individuals that were homozygous for the TT genotype compared to the CC genotype (P=0.0163). To predict the effect of rs7669

on the mRNA secondary structure an *in silico* study was performed. The results indicated a slightly higher energy for rs7669 mRNA folding (dG) compared to the wild-type sequence. The results suggest that the TT genotype affects the *MTIF3* mRNA levels. By dividing the samples into patients and controls without respect to genotype we could not show an association with the decrease in *MTIF3* mRNA expression and Parkinson's disease (P=0.0795). However, it cannot be fully excluded that individuals with this genotype also carry additional variants affecting mRNA levels. Recently, rs7669 was found to be associated with Parkinson's disease in a Norwegian, but not in an Irish or US material (Behrouz et al., 2010).

DNA polymerase gamma 1 (paper V)

V. Variations of the polyglutamine tract in POLG1

DNA polymerase gamma 1 (*POLG1*) is important for mtDNA replication and repair (Kaguni, 2004). In the N-terminal area of *POLG1* a poly-Q polyglutamine tract is located, encoded by a CAG repeat in exon 2 (Lecrenier et al., 1997). Deviations in length of the poly-Q tract of *POLG1* from the normal 10 or 11 CAG repeats have been found to associate with Parkinson's disease by a number of groups (Eerola et al., 2010; Luoma et al., 2007). Fragment analysis was used to study the poly-Q extension of *POLG1* in a Swedish Parkinson case-control material from Stockholm. A total of 243 patients and 279 controls were screened. Results from the fragment analysis were confirmed by sequencing 80 randomly picked samples using capillary sequencing.
The results showed a variation of the poly-Q tract ranging from 5Q-15Q repeats. For statistical analysis the alleles were divided into two groups; 10/11Q, which are stated as normal, and non-10/11Q. The Fisher's exact test shows a significant association of the non-10/11Q with Parkinson's disease (P=0.002). An *in silico* analysis of possible effects of the poly-Q variations on the folding properties of the *POLG1* mRNA was performed. The analysis showed a very small difference between mRNA sequences of 10Q and 11Q, an increase of 0.6% concerning secondary structure and energy for mRNA folding (dG). A small decrease (0.5%) in mRNA folding energy was observed between 10/11Q and non-10/11Q. However, when comparing the shorter (<10/11Q) and longer (>10/11Q) alleles separately with 10/11Q they show folding energy differences in opposite directions. Less than 10/11Q show a lower energy (4.6%) and >10/11Q a higher energy (4.8%). These results are in agreement with a study in a Finnish population which showed significant association with the non-10/11Q (Luoma et al., 2007). In a North American Caucasian material a significant association was found with non-10Q (Eerola et al., 2010). Our findings indicate that both extension and reduction of the poly-Q length in *POLG1* may influence gene function.

Mitochondrial Rho GTPases (paper VI)

VI. Genetic screening of MIRO1 and MIRO2

The atypical GTPase Miro and the adaptor protein Milton have been shown to be part of a mitochondrial multi-protein complex together with Pink1 (Weihofen et al., 2009). Mutations in the kinase *PINK1* (PARK6) are reported to regulate mitochondrial fission and cause early-onset familial Parkinson's disease (Valente et al., 2004a). The TaqMan technique was used to analyze SNPs in *MIRO1* and *MIRO2* (mitochondrial Ras homolog gene family, member T1 and T2) also referred to as RHOT1 and RHOT2. Three SNPs in *MIRO1* (rs16967164, rs28630420, rs34538349) and two in *MIRO2* (rs1139897, rs3743912) were investigated in a Swedish Parkinson case-control material consisting of 241 patients and 307 neurologically healthy controls from the Stockholm area. The SNPs were genotyped using predesigned TaqMan SNP genotyping assays or custom designed assays. The polymorphisms are located in functionally important regions of the sequence. Case-control analyses between individual sequence variants in *MIRO1* and *MIRO2* were performed using Chi-square tests. We did not find an association with Parkinson's disease between any of the analyzed genotypes or alleles of *MIRO1* or *MIRO2*. To investigate whether any of the SNPs had a possible effect on age of onset, we stratified the material into early (≤50 years) or late (>50 years) disease onset and compared genotype and allele frequencies, but did not find any significant association. Genetic variation in *MIRO1* and *MIRO2* might influence transport of mitochondria along microtubules, leading to reduced local energy production which can lead to degeneration of DA neurons. Although we did not find a significant association with Parkinson's disease of any of the selected polymorphisms in *MIRO1* and *MIRO2* in our Swedish case-control material, we cannot exclude these Rho GTPases as candidate genes for Parkinson's disease or other neurodegenerative disorders.

Detoxifying enzymes (paper VII, VIII, IX)

VII. Association of a paraoxonase 1 promoter polymorphism

Epidemiological studies suggest that pesticide exposure may increase the risk to develop Parkinson's disease (Le Couteur et al., 1999). Pyrosequencing and the TaqMan technique were used to investigate the occurrence of SNPs in the paraoxonase (PON) cluster, which are detoxifying enzymes. In *PON1*, three SNPs in the promoter region (rs854572, rs854571, rs705379) and two in the coding region (rs854560, rs662) were screened. In *PON2*, we screened two coding region SNPs (rs12026, rs1058131) (Mochizuki et al., 1998) and in *PON3* two coding region SNPs (rs1132727, rs1132729) identified by us *in silico*. The material consisted of >500 Parkinson patients and >500 neurologically healthy controls of Swedish origin from Stockholm, Linköping or Göteborg. Chi-square tests was used to compare genotype and allele frequencies. Pairwise linkage disequilibrium was examined with the program GOLD and haplotype analyses of the SNPs were performed in the sliding window model using Unphased version 3.1.3 and Haploview. We found that the minor allele of the *PON1* promoter rs854571 variant was more common among healthy controls than Parkinson cases, which suggest a protective effect. Interestingly, a strong linkage disequilibrium was found between the associated rs854571 and rs854572 (D'=0.97 and r^2=0.387), which has been reported to increase the *PON1* gene expression. In *PON2* a significant association was found between one of the non-synonymous polymorphisms, rs12026, and a decreased risk for Parkinson's disease. The two investigated SNPs in *PON3* seemed to be very rare since they were absent in the Stockholm Parkinson case-control material and therefore they were not further investigated. We find that the *PON* gene family contains genetic variants that are associated with Parkinson's disease, and the possibility for interaction with pesticide exposure remains.

VIII. Modeling Parkinson's disease in Adh4 knockout

A promoter mutation in the human class IV ADH gene, often referred to as *ADH4* (official gene name: *ADH7*), has been reported to be associated with Parkinson's disease (Buervenich et al., 2000) and induces a 25-30% reduction of transcriptional activity (Buervenich, 2002). Based on these findings, *Adh4* knockout (*Adh4*-/-) and heterozygous mice (*Adh4*+/-) were investigated for changes in DA system-related activity by monitoring locomotor responses to drugs acting on the DA system, biochemical parameters and olfaction compared to wild-type (WT) mice. The three groups were similar with respect to spontaneous locomotor activity. Administration of *d*-amphetamine or apomorphine significantly increased horizontal activity in *Adh4*-/- compared to WT mice. There was an increase of DA and DOPAC in substantia nigra of *Adh4*-/- mice. Investigation of olfactory function revealed a reduced sense of smell in *Adh4*-/- mice accompanied by alterations in DA metabolite levels in the olfactory bulb. The results suggest that lack of *Adh4* gene activity induces alterations of DA metabolism and DA-mediated functions, as well as a reduced sense of smell.

IX. Adh1 and Adh1/4 as models for Parkinson's disease

To further model the role of alcohol dehydrogenases in Parkinson's disease, behavioral studies were performed on two knockout strains lacking *Adh1* or *Adh1* together with *Adh4*. The human *ADH1C* belongs to the class I ADH gene family (Duester et al., 1999) and a truncating stop mutation in *ADH1C* has been reported to be significantly associated with Parkinson's disease (Buervenich et al., 2005). As mentioned above, a promoter mutation in the human *ADH4* has also been reported to be associated with Parkinson's disease (Buervenich et al., 2000) and induces a 25-30% reduction in transcriptional activity (Buervenich, 2002). *Adh1* knockout (*Adh1*-/-) and double knockout *Adh1/4* mice (*Adh1/4*-/-) were therefore investigated with respect to possible disturbances of DA system-related activity. Seven (adult) and >15 months old mice were used to better reflect the age of onset of Parkinson's disease. *Adh1*-/- and *Adh1/4*-/- mice showed significantly increased spontaneous locomotor activity compare to WT mice. Amphetamine increased locomotor activity significantly more in *Adh1*-/- and *Adh1/4*-/- mice then in WT mice and the same was seen after apomorphine injection (Figure 5). We note that increased spontaneous, as well as drug-induced locomotion was a general phenotype, except that responses of >15 months old double knockout mice to apomorphine or *d*-amphetamine did not differ from controls. The olfactory test suggested a reduced sense of smell in >15 months old *Adh1/4*-/- male mice (Figure 6). We found decreased DOPAC/DA and HVA/DA ratios in the olfactory bulb, frontal cortex and striatum of *Adh1/4*-/- mice (Figure 7). This suggests that lack of *Adh1* and *Adh1/4* enzyme activity alters DA related functions.

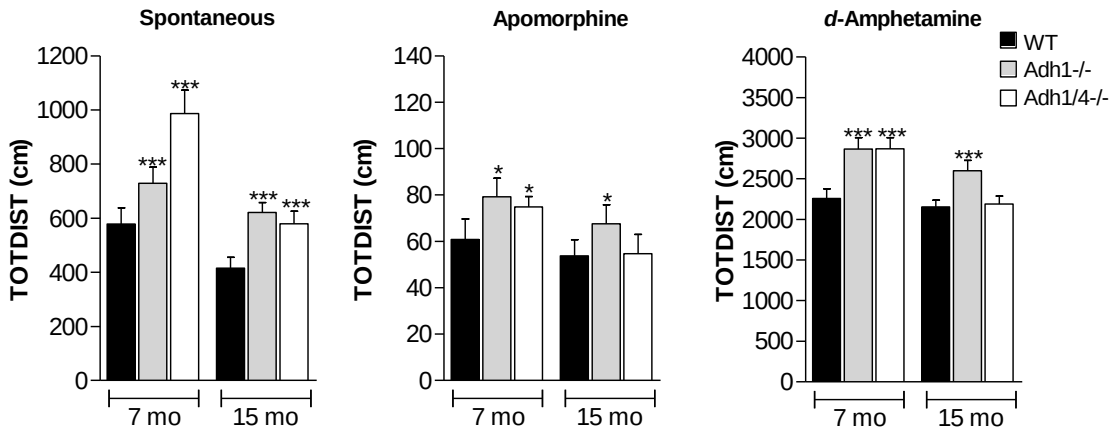

Figure 5. Total distance (TOTDIST) measured in centimeters (cm) spontaneously and after injection of apomorphine (1mg/kg) or *d*-amphetamine (3mg/kg) in *Adh1*-/-, *Adh1/4*-/- and wild-type (WT) male and female mice combined at 7 and 15 months (mo) of age. Number of animals analyzed at 7 months of age: WT (n=22), *Adh1*-/- (n=28) and *Adh1/4*-/- (n=21). Number of animals analyzed at 15 months of age: WT (n=20), *Adh1*-/- (n=28) and *Adh1/4*-/- (n=20). Data is presented as mean ± SEM, * = p< 0.05, *** = p< 0.001.

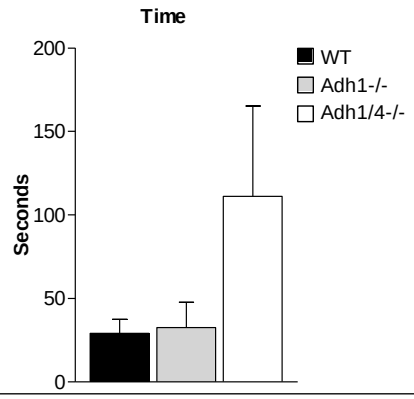

Figure 6. Olfactory function in *Adh1*-/- (n=12), *Adh1/4*-/- (n=8) and wild-type (WT) (n=9) male mice at 15 months of age. Following habituation to the experimental cage, a novel odor (0.1 g peanut butter) was introduced, and the time required by the animal to reach the odor was recorded. Data are presented as mean ± SEM.

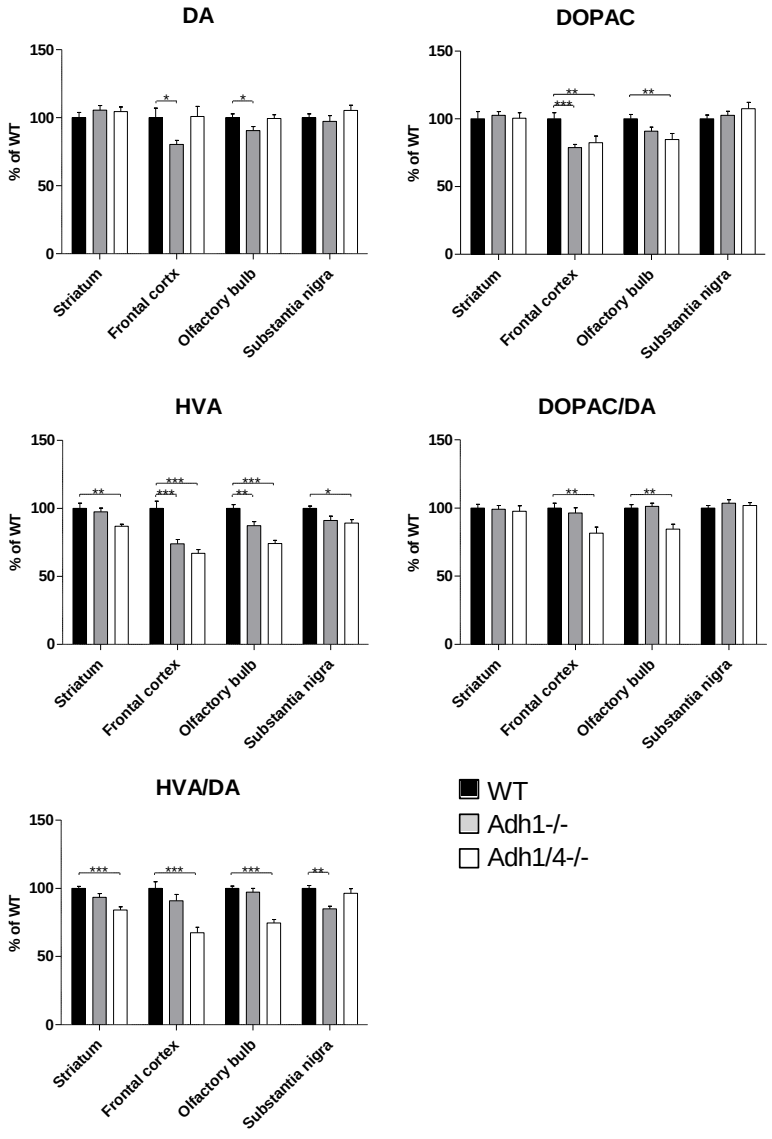

Figure 7. High-performance liquid chromatography (HPLC) of monoamines and their metabolites in *Adh1*-/- (n>27), *Adh1/4*-/- (n>19) and wild-type (WT) (n>19) mice. Each group contains both females and males. Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), DOPAC/DA, homovanillic acid (HVA) and HVA/DA levels are shown ± SEM as percent of wild-type. * = $p<0.05$, ** = $p< 0.01$, *** = $p< 0.001$.

CONCLUSIONS

- The synonymous Ala167Ala (c.501A>G) mutation in *DJ-1* was identified in two Parkinson patients, one with early-onset Parkinson's disease and Alzheimer's disease, and one with a family history of Parkinson's disease. Mutations in *DJ-1* appear to be a rare cause of familial and early-onset Parkinson's disease.

- The mRNA expression of *PINK1* is similar in sporadic Parkinson's disease compared to healthy controls. Evidence for altered expression of *PINK1* in surviving neurons was not detected.

- A141S in *OMI/HTRA2* was found to be associated with Alzheimer's, but not with Parkinson's disease. There is no difference in *OMI/HTRA2* mRNA expression in frontal cortex or hippocampus in Alzheimer's disease, but reduced levels of the active OMI/HTRA2 enzyme and increased protease activity of OMI/HTRA2 in frontal cortex were detected. This indicates that *OMI/HTRA2* might be a candidate gene for neurodegenerative disease.

- The genetic variant rs7669 (C>T) in *MTIF3* is associated with sporadic Parkinson's disease. The TT genotype leads to decreased mRNA expression of *MTIF3* in human lymphoblastoid cell lines. Variations of the *POLG1* CAG repeat in exon 2 are associated with sporadic Parkinson's disease. This supports the hypothesis of mitochondrial dysfunction in Parkinson's disease.

- No significant association was found for any of the selected polymorphisms in *MIRO1* and *MIRO2* with Parkinson's disease in our Swedish case-control material. Due to the important function of *MIRO1* and *MIRO2* in mitochondrial transportation, further studies are needed before excluding these Rho GTPases as candidate genes for Parkinson's disease.

- The promoter polymorphism rs854571 in *PON1*, was significantly associated with healthy controls. This suggests a protective effect of the *PON1* promoter variant. rs854571 was further reported to be in strong linkage disequilibrium with another *PON1* promoter polymorphism, rs854572, reported to increase the *PON1* gene expression. The findings support involvement of *PON1* in the etiology of Parkinson's disease and suggest that higher levels of PON1 reduce the risk for Parkinson's disease.

- *Adh1*-/- and *Adh1/4*-/- show higher spontaneous locomotor activity compared to WT mice, which is not seen in *Adh4*-/- mice. Administration of *d*-amphetamine increases locomotor activity in all three transgenic mice strains compared to WT mice; similar effects are seen after apomorphine injection. *Adh4*-/- mice have a reduced sense of smell and *Adh1/4*-/- show a similar tendency. All three strains have alterations of monoamine and metabolite levels in olfactory bulb, frontal cortex, striatum and substantia nigra. Hence, lack of *Adh1* and/or *Adh4* cause functional alterations of the DA system.

GENERAL DISCUSSION

Parkinson’s disease is a complex neurodegenerative and heterogenous disorder for which there is no cure. More than 1.5% of the population over the age of 65 years is affected and the world-wide incidence is the same with an increased prevalence in proportion to population longevity. The pathology of the disease is partly understood, while less is known about the etiology, except for some monogenic forms of the disorder for which genetic causes are now known. Discoveries during the last 15 years have nevertheless dramatically changed our understanding of Parkinson's disease by unravelling the roles of genes, both as causes for monogenic disease and as risk factors. The present work provides additional information concerning the possible contribution of several different genes to the risk of developing disease. It is striking that none of the genes hitherto linked to, or associated with, Parkinson's disease are directly related to DA neurons. Instead they are often generally expressed in cells, or at least in most neurons. In some cases, implicated genes are not even expressed in the brain. Moreover, it is difficult to find a common denominator for all implicated genes, although most of them can be grouped as being important for (i) mitochondrial function, (ii) protein degradation, ubiquitination and proteasome mechanisms, (iii) lysosomal function, or (iv) detoxification.

The Human Genome Project shows that humans share approximatley 99.9% of the DNA code while the remaining 0.1% is diverse, and may carry variants that determine disease vulnerability. To understand how genetic variability may lead to disease, candidate susceptibility genes need to be investigated in geographically defined populations to resolve how different mechanisms, individually or together, contribute to disease. Genetic variants that increase or decrease vulnerability significantly in one geographically isolated or confined population may be less frequent or absent in another population. Such population differences make genetic analysis complex. While results need to be replicated in different cohorts within a given area/population, regionally important genetic risk or protective factors may no longer be detected when very large and geographically diverse materials are analyzed.

The diversity of implicated genes suggests Parkinson's disease will have to be subclassified in further detail than is currently done. The general expression of many of the implicated genes suggests most forms of Parkinson's disease should be viewed as systemic diseases. The widespread expression of the genes also helps explain why many other neuronal systems than the DA neurons are also affected, from the innervation apparatus of the gastrointestinal tract to cortex cerebri.

Continued characterization and localization of gene and protein expression patterns in disease and controls are needed to understand pathways were implicated genes are involved and to identify interacting factors. To this effect, studies of cells, tissues and genetic animal models, as well as studies of transcriptional activity patterns and other parameters in human tissues are all needed. The high vulnerability of DA neurons remains enigmatic, not the least because implicated genes do not specifically locate to DA neurons relative to less vulnerable neuron types. Further research, including studies of how combinations of risk factors together may become particularly damaging to DA neurons, are needed if neuroprotective treatments are to be found.

At the time of a clinical diagnosis of Parkinson's disease, the majority of the SNpc DA neurons have already been lost. A challenge for the future is therefore to identify new markers, which can be used for early, presymptomatic diagnosis of Parkinson's disease as needed for future neuroprotective treatments to become effective.

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