GENETICS OF PARKINSON'S DISEASE

– WITH FOCUS ON GENES OF RELEVANCE FOR INFLAMMATION AND DOPAMINE NEURON DEVELOPMENT

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Abstract

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Introduction: The risk to be affected by Parkinson's disease (PD) is considered to be influenced by genetic factors. In some rare cases of familial PD, mutations in some specific genes are known to cause the disease, but in the more common sporadic form of PD the causes are probably environmental factors interacting with genetic vulnerability. The main objective of this thesis was to identify genes of importance for this genetic vulnerability in sporadic PD, by analysing the frequency of polymorphisms in PD patients and control subjects. The investigated genes encode proteins involved in one, or both, of two processes suggested to be of importance for the pathophysiology of PD; inflammation and development of dopaminergic neurons. ***Main observations:*** A single nucleotide polymorphism (SNP) in the gene encoding estrogen receptor beta was found to be associated with PD with an early age of onset. Furthermore, this SNP seems to interact with a SNP in the gene for the pro-inflammatory cytokine interleukin 6, potentiating the susceptibility to PD, especially among early age of onset patients. In the genes encoding the anti-inflammatory cytokine interleukin 10 and the dopaminergic transcription factor Pitx3, polymorphisms associated with age of onset were identified. ***Conclusions:*** The results indicate that several of the investigated genes might be of importance for the pathophysiology of sporadic PD. Often the polymorphisms were associated only with PD with an early age of onset, possibly explained by a more important role of genetic factors among patients with an early onset. An alternative explanation is that some of the polymorphisms affect the age of onset of PD, for example by modulating the vulnerability to disease-causing environmental factors. The relevance of the present results can only be confirmed by additional studies in other PD populations. For some of the genes the results of the present thesis have been replicated, while for others no additional studies have been published or the findings have not been confirmed.

Keywords: Parkinson's disease (PD), single nucleotide polymorphism (SNP), gene, age of onset, Pitx3, estrogen receptor beta, interleukin 6

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Paper I: Westberg L, **Håkansson A**, Melke J, Shahabi HN, Nilsson S, Buervenich S, Carmine A, Ahlberg J, Grundell MB, Schulhof B, Klingborg K, Holmberg B, Sydow O, Olson L, Johnles EB, Eriksson E, Nissbrandt H. Association between the estrogen receptor beta gene and age of onset of Parkinson's disease. *Psychoneuroendocrinology* 2004; 29: 993-998.

Paper II: **Håkansson A**, Westberg L, Nilsson S, Buervenich S, Carmine A, Holmberg B, Sydow O, Olson L, Johnels B, Eriksson E, Nissbrandt H. Interaction of polymorphisms in the genes encoding interleukin-6 and estrogen receptor beta on the susceptibility to Parkinson's disease. *American Journal. of Medical Genetics (Part B)* 2005; 13: 88-92.

Paper III: **Håkansson A**, Westberg L, Nilsson S, Buervenich S, Carmine A, Holmberg B, Sydow O, Olson L, Johnels B, Eriksson E, Nissbrandt H. Investigation of genes coding for inflammatory components in Parkinson's disease. *Movement Disorders* 2005; 20: 569-573.

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Paper V: **Håkansson A**, Carmine Belin A, Stiller C, Sydow O, Johnels B, Olson L, Holmberg B, Nissbrandt H. Investigation of genes related to familial forms of Parkinson's disease - With focus on the Parkin gene. *Parkinsonism and Related Disorders* 2008; 14: 520-522.

Paper VI: Bergman O, **Håkansson A**, Westberg L, Nordenström K, Carmine Belin A, Sydow O, Olson L, Holmberg B, Eriksson E, Nissbrandt H. PITX3 polymorphism is associated with early onset Parkinson's disease. *Neurobiology of Aging* 2010; 31: 114-117.

Paper VII: Bergman O, **Håkansson A**, Westberg L, Carmine Belin A, Sydow O, Olson L, Holmberg B, Fratiglioni L, Bäckman L, Eriksson E, Nissbrandt H. Do polymorphisms in transcription factors LMX1A and LMX1B influence the risk for Parkinson's disease? *Journal of Neural Transmission* 2009; 116: 333-338.

The papers were published in the name Anna Håkansson.

List of abbreviations

CNS	central nervous system
COX-2	cyclooxygenease 2
DNA	deoxyribonucleic acid
ER	estrogen receptor
GWAS	genome wide association study
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon gamma
IFN-γR2	interferon gamma receptor 2
IgG	immunoglobulin G
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-6	interleukin 6
IL-10	interleukin 10
iNOS	nitric oxide synthase
LD	linkage disequilibrium
L-DOPA	L-3, 4-dihydroxyphenylalanine/levodopa
LFA-1	lymphocyte function-associated antigen 1
Lmx1a	lim-homedomain factor a
Lmx1b	lim-homeodomain factor b
LPS	lipopolysaccharides
LRRK2	leucine-rich repeat kinase 2
MAPT	microtubule-associated protein tau
MHC	major histocompability complex
MHO	mid-hindbrain organiser
MLPA	multiplex ligation-dependent probe amplification
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
NSAID	nonsteroidal anti-inflammatory drug
6-OHDA	6-hydroxydopamine
PAF	platelet activating factor
PAF-AH	platelet activating factor acetylhydrolase
PCR	polymerase chain reaction
PD	Parkinson's disease
PINK1	PTEN-induced putative kinase 1
Pitx3	paired-like homeodomain 3
PRKN	parkin
RNA	ribonucleic acid
SNCA	α-synuclein
SNP	single nucleotide polymorphism
TH	tyrosine hydroxylase
TNF-α	tumour necrosis factor alpha
UCHL-1	ubiquitin C-terminal hydrolase-L1
UTR	untranslated region

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INTRODUCTION

PARKINSON'S DISEASE – GENERAL BACKGROUND

Parkinson's disease (PD) is the second most frequent neurodegenerative disorder; only Alzheimer's disease is more common. PD usually appears late in life and the prevalence is 0.2% in the general population, but 1-2% for people over 60 years of age. The disease was comprehensively described by James Parkinson in 1871 in his monograph "An essay of the shaking palsy". However, it was not until 1958 dopamine, the key neurotransmitter in PD, was identified in the brain (Carlsson *et al.*, 1958) and short thereafter suggested to be related to the disease (Carlsson, 1959).

PD is identified by the cardinal symptoms, resting tremor, rigidity, bradykinesia and loss of postural stability (Sian *et al.*, 1999), but the patients often suffer also from symptoms not associated with lack of motor performance, for example cognitive impairment, depression and olfactory dysfunction (Foley and Riederer, 1999). Pathologically the disease above all is characterised by degeneration of dopaminergic neurons and intraneuronal inclusions, so called Lewy bodies (Sian *et al.*, 1999). Although the neuronal degeneration is most pronounced in the substantia nigra, which is the brainstem nucleus where the dopaminergic cell bodies in the nigrostriatal pathway (substantia nigra to striatum) are located, also other neuronal systems, such as norepinephrine containing neurons are affected. The rate of cell degeneration is relatively slow and during several years the remaining surviving cells can compensate for the cell loss. Therefore, the clinical expression of the disease starts not until approximately 50-70% of the dopaminergic cells in the substantia nigra have degenerated (Barzilai and Melamed, 2003). Lewy bodies are cytoplasmic inclusion bodies present in degenerating neurons and considered to be the result of altered metabolism/transportation of neurofilaments or proteins (Foley and Riederer, 1999). Lewy bodies are, however, not specific for PD; they have also been found in patients with other disorders, such as dementia with Lewy bodies, subacute sclerosing panencephalitis and Hallenvorden-Spatz disease (Calne, 2000). There are also PD patients who lack Lewy bodies, for example the majority of those who suffer from PD caused by mutations in the Parkin gene (Gasser, 2005).

Parkinsonism is the general term for describing motor symptoms like tremor, rigidity and hypo-bradykinesia. One cause for parkinsonism is PD but it can also be caused by other diseases and even drug treatment. Based on careful monitoring of

symptomatology and pathological findings, subgroups of PD have emerged during the years. Parkinson-plus syndromes, such as multiple system atrophy (MSA) or progressive supranuclear palsy (PSA), are recognized as separate disease entities (Bhat and Weiner, 2005), with a quicker progress and additional symptoms as compared to typical PD. Some of the monogenic hereditary forms of PD are often, but not always, atypical with a much more early age of onset, differential progression and additional symptoms not seen in typical PD. The core of PD patients, with typical symptoms only and no signs of conspicuous familial aggregation are often referred to as sporadic PD or idiopathic PD. However, as been shown in clinicopathological studies, the clinical diagnosis of PD is not always certain (Hughes *et al.*, 1993).

Despite intensive research little is known about the aetiology of PD and the processes leading to neuronal degeneration. Some rare genetic forms of the disease are established, but for the majority of PD patients the disease is probably caused by a combination of genetic vulnerability and environmental factors, for example infections or toxins. The genetic and environmental components are assumed to trigger various pathophysiological mechanisms, such as inflammation, oxidative stress, protein misfolding, mitochondrial dysfunction or apoptosis.

The most efficacious drug used in the treatment of PD is levodopa (L-DOPA). However, when the disease proceeds troublesome adverse effects will appear, such as motor fluctuations and dyskinesias. Other available drugs are monoamine oxidase-B (MAO-B) and catechol-O-methyltransferase (COMT) inhibitors and dopamine receptor agonists, which often are used together with L-DOPA (Jankovic, 2006). Deep brain stimulation is a neurosurgical treatment where an electrode is implanted in a selected brain area, preferably in the subthalamic nucleus (Sydow, 2008).

MOLECULAR GENETICS

From gene to protein

A gene is made up by sequences of coding exons and non-coding introns (see Figure 1). During the process of transcription, the double stranded DNA of a gene is copied into single stranded RNA by the protein RNA polymerase. The introns are then removed in a process called splicing, generating a messenger RNA (mRNA). This mRNA acts as a template in the translation process, where amino acids are attached to each other in a specific order and finally form a protein.

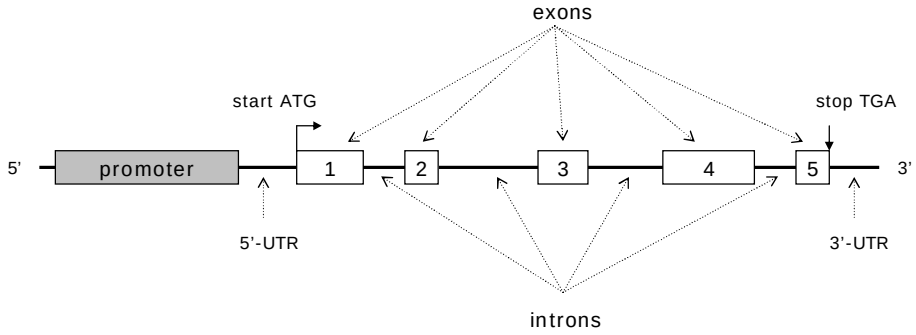

Figure 1. Schematic picture of a gene. Promoter: a regulatory region normally located upstream of a gene, containing specific DNA sequences that can be recognised by so called transcription factors, which are proteins involved in the control of gene expression. UTR: untranslated region.

Genetic variation

A mutation is defined as a change or an alteration in the DNA. If a mutation appears more frequently than 1 % in a population it is called a polymorphism. The most common type of polymorphism is named single nucleotide polymorphism (SNP), since one base in the DNA chain is replaced by another (Brookes, 1999). Today, more than 11.5 million SNPs are reported in the human genome (dbSNP: www.ncbi.hlm.nih.gov/SNP). Repeats are another form of polymorphism where the DNA in the polymorphic area contains a sequence that is repeated a variable number of times. The repeats are categorised into two classes; variable number of tandem repeats (VNTR) (Nakamura *et al.*, 1987) and microsatellites or short tandem repeats (STR) (Weber and May, 1989). Insertions and deletions are additional kinds of polymorphisms, where pieces of the DNA sequence are either multiplied or missing. When these insertions or deletions are relatively large they will give rise to copy number variation (CNV), which is a further type of polymorphism (Sebat *et al.*, 2004).

A polymorphism that can affect the protein is categorised as functional. A SNP located in a coding region, i.e. exon, may give rise to an amino acid shift or create a stop codon, whereas an insertion or deletion in the same region is able to change the reading frame for the RNA polymerase. SNPs in exons which do not alter amino acid residues are called synonymous. Such SNPs were previously considered to be silent, since they do not influence the protein by causing any of the above mentioned alterations. However, synonymous SNPs are now regarded to be of potential importance since some of them has been shown to affect splicing, stability or

structure of the mRNA, or protein folding (Komar, 2007; Sauna *et al.*, 2007). SNPs located in non-coding regions, such as the promoter, 5'- and 3'-UTR and introns, might also be of functional importance. Such polymorphisms can for example influence mRNA stability or splicing and create or interrupt binding sites for transcription factors in the DNA, thereby altering the expression of the protein (Wang *et al.*, 2006).

Studying genetic variation

To identify genes that cause or influence the risk to develop a disorder two main strategies are commonly used; linkage analyses and association studies (Risch and Merikangas, 1996). A linkage analysis uses genetic information from families to identify the region that is inherited together with the disorder, by using a large set of markers evenly distributed throughout the genome. This approach needs no prior hypothesis about which gene that might be involved and is most suitable when investigating genes with high penetrance, like those implicated in monogenic disorders. Complex diseases, such as sporadic PD, are thought to be polygenic, which implies that the phenotype depends on several genes. The genetic component in such disorders is often searched for by using the association study approach with a case-control design, where affected individuals are compared to control subjects with respect to allele- and genotype frequencies of polymorphisms in the gene of interest. The choice of a candidate gene for an association study is often based on a hypothesis regarding the pathophysiological role of the gene. Association studies are also carried out in order to study genes located in gene areas already identified through linkage analyses.

The large amount of information about genetic variations in databases, together with the genotyping techniques available today, have prompted researchers to perform genome wide association studies (GWAS). In GWAS cases and controls are compared with respect to genetic markers distributed all over the genome, and consequently no prior hypothesis about which gene or genes that are involved in the disease is needed. Still, some problems have arisen when performing these large association studies, for example reaching enough statistical power to detect variations with low effect size, but also logistical problems regarding automated but accurate genotype calling, genotype quality controls and data handling (Panoutsopoulou and Zeggini, 2009). Moreover, strategies for sequencing the complete coding region (*i.e.* whole exome), for identification of rare functional mutations of the genome, have evolved recently.

In the early 2000 the number of SNPs available in the database dbSNP was relatively limited. At that time, the choice of SNPs was often based on descriptions in the literature, concerning for example functionality or associations with related disorders. If there were no or very few SNPs reported in the databases, the gene of interest had to be sequenced in order to find possible risk-variations for genotyping. The release of the public HapMap database (The International HapMap Consortium, 2003) offered new possibilities when choosing SNPs in association studies of candidate genes. The database is a haplotype map of the human genome, made by the international HapMap consortium through genotyping of millions of polymorphisms in 270 individuals from four human populations with different ethnicity. The HapMap resource can guide the selection of appropriate SNPs, called tagSNPs, covering almost all variation in a gene, relying on the fact that information from a small number of variants can capture most of the common patterns of variation in the genome. This is based on a concept called linkage disequilibrium (LD), which means a non-random inheritance of alleles at different loci. Statistically LD can be used to measure co-segregation of alleles in a population.

GENETICS IN PARKINSON'S DISEASE

That genetics could be of importance for the aetiology/pathohysiology of PD has been known since the early studies from the middle of the last century describing cases with autosomal dominantly inherited forms of PD (Allen, 1937; Mjönes, 1949). However, it was first in the middle of the nineties, after the first gene linked to PD was reported (Polymeropoulos *et al.*, 1996), that researchers made considerable efforts to clarify the genetic influence on the disease. The finding of the first gene was soon followed by others and to date several loci (named PARK1, and so on) and genes have been implicated in PD (Lesage and Brice, 2009). Mutations in some of the genes, which originally were linked to familial PD, have also been found to act as risk factors for sporadic PD.

Familial forms of PD

Genes in autosomal dominantly inherited PD

PARK1 and PARK4: Of the genes causing dominantly inherited PD the first discovered was α-Synuclein (*SNCA*) (Polymeropoulos *et al.*, 1997). Point mutations in *SNCA* have been found in some families (Polymeropoulos *et al.*, 1997; Lesage and

Brice, 2009; Puschmann *et al.*, 2009), but also the gene dosage is of importance, considering that gene multiplications have been detected in nine families with parkinsonism (Singleton *et al.*, 2003; Fuchs *et al.*, 2007; Lesage and Brice, 2009). A triplication of the gene was earlier referred to as PARK4 (Singleton *et al.*, 2003). Furthermore, variability in the *SNCA* promoter seems to be associated with sporadic PD according to a large meta-analysis (Maraganore *et al.*, 2006). The protein encoded by *SNCA* is thought to play a role in vesicular transmitter release and is also a major component of Lewy bodies (Spillantini *et al.*, 1997; Hardy *et al.*, 2006).

PARK5: The gene ubiquitin C-terminal hydrolase-L1 (*UCHL-1*), encodes an enzyme with ubiquitin ligase as well as peptide-ubiquitin hydrolysing activity (Liu *et al.*, 2002). A mutation in the gene was identified in a sibling pair from Germany with parkinsonism (Leroy *et al.*, 1998), but whether this alteration is pathogenic or not remains controversial (Healy *et al.*, 2004). Furthermore, the Tyr18 allele of a polymorphism (Ser18Tyr) has been reported to be inversely associated with sporadic PD (Maraganore *et al.*, 2004; Carmine Belin *et al.*, 2007).

PARK8: The gene responsible for PD at locus 8 has been recognised as leucine-rich repeat kinase 2 (*LRRK2*) (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004). Mutations in this gene are the most common genetic cause of dominant familial and sporadic PD known so far. The LRRK2 protein has been suggested to be involved in several functions including protein-protein interactions, maintenance of neurites and regulation of neuronal survival, but the precise function remains unclear (Belin and Westerlund, 2008).

Genes in autosomal recessively inherited PD

PARK2: Of the recessively inherited forms, *parkin* (*PRKN*) associated PD appears to be the most frequent. The protein encoded by the *parkin* gene acts as an E3-ubiquitin ligase that is involved in protein degradation (Shimura *et al.*, 2000). Mutations in the *parkin* gene were initially reported to cause juvenile onset (before 20 years) PD (Kitada *et al.*, 1998). Today, *parkin* mutations have been found in patients also with later onset, but it seems like the majority of the patients manifest the disease before the age of 40. Many different types of mutations have been reported, including point mutations as well as exon rearrangements with both deletions and duplications (Mata *et al.*, 2004).

PARK6: One of the genes associated with PD, supporting the theory that mitochondrial dysfunction is of pathophysiological importance for the disorder, is PTEN-induced putative kinase 1 (*PINK1*) which encodes a mitochondrial protein kinase (Valente *et al.*, 2001). Mutations in the gene were initially linked to PD in families from Italy and Spain (Valente *et al.*, 2004).

PARK7: Mutations in the *DJ-1* gene were first discovered in a Dutch and in an Italian family with parkinsonism (Bonifati *et al.*, 2003). The gene encodes a protein that has been suggested to be of importance for the cellular response to oxidative stress (Bandopadhyay *et al.*, 2004).

Other PARK- loci and possible PD genes

PARK11: It has been proposed that the gene coupled to PD at locus 11 is *GIGYF2*, which encodes the GRB10-interacting GYF protein 2, a component of the insulin signalling pathway (Lautier *et al.*, 2008). However, the finding of mutations in this gene in PD patients has so far not been replicated.

PARK13: Omi/Htra2 is a mitochondrial protease that is released into the cytosol during apoptosis (Strauss *et al.*, 2005). The gene (*OMI/HTRA2*) encoding the protein has been studied regarding PD based on the biological function of the protein and not because of results from a previous linkage study. Possible disease causing mutations have been found in a study conducted on a German PD population (Strauss *et al.*, 2005) and additional mutations have been detected in Belgian PD patients (Bogaerts *et al.*, 2008).

For three of the loci, PARK3 (Gasser et al. 1998), PARK10 (Hicks et al. 2002) and PARK12 (Pankratz *et al.*, 2003), the responsible genes have not been clearly identified. However, it has recently been suggested that the gene at PARK3 could be sepiapterine reductase (*SPR*) (Sharma *et al.*, 2006).

In families with atypical PD three different genes have been isolated; PARK9/*ATP13A2* (Ramirez *et al.*, 2006), PARK14/*PLA2G6* (Paisan-Ruiz *et al.*, 2009) and PARK 15/*FBXO7* (Di Fonzo *et al.*, 2009).

Sporadic PD and gene variants acting as risk factors

PD patients are considered to be sporadic cases if a familial aggregation for the disease is not clinically manifested. Still, it is known from epidemiological reports that patients with sporadic PD often report of relatives with the disease (Payami *et al.*, 1994; Marder *et al.*, 1996; Taylor *et al.*, 1999; Sveinbjornsdottir *et al.*, 2000). However, several twin studies of PD, including some investigations with relatively large numbers of twins (Tanner *et al.*, 1999; Wirdefeldt *et al.*, 2008), have failed to show a significantly different concordance in monozygotic as compared to dizygotic twins. One drawback is that most twin studies have been cross-sectional instead of longitudinal and it has been reported that in monozygotic twins the age of onset of the disease can be separated by up to 20 years (Dickson *et al.*, 2001). Therefore, as a complement to clinically diagnosed PD, flourodopa PET scanning can be used to indirectly assess the amount of nigrostriatal dopamine neurons in the unaffected twin of disconcordant twins. The results from one study using PET show that the concordance level for dopaminergic hypofunction was significantly higher in monozygotic than in dizygotic twins (Piccini *et al.*, 1999). In addition, two other studies, where monozygotic and dizygotic twins were pooled and compared to control subjects, indicated a decreased dopaminergic function also in the asymptomatic co-twin (Lahinen et al 2000; Holthoff et al. 1994).

Since it is believed that genetic variation also are important for the aetiology and pathophsyiology of sporadic PD, a huge amount of studies trying to identify gene variants associated with the disease have been undertaken. Apart from investigating genes located in regions identified through linkage analysis, candidate genes have been chosen by looking at genes involved in plausible pathophysiological processes, such as dopamine metabolism, mitochondrial dysfunction, protein degradation, oxidative stress or toxin induced alterations in cellular systems. However, except some of the genes related to familial PD described above, only a few genes have been established as risk-genes in sporadic PD. Some examples are the genes encoding microtubule-associated protein tau (*MAPT*) and glucocerebrosidase (*GBA*), initially implicated in frontotemporal dementia (Poorkaj *et al.*, 1998) and Gaucher disease (Sidransky, 2004), respectively. Still, the results from association studies of these genes are somewhat inconsistent (Zhang *et al.*, 2005; Toft *et al.*, 2006; Mata *et al.*, 2008).

Before any clear-cut conclusions can be drawn about a gene being a risk factor for a disease, an initial positive study must be replicated by several additional investigations. When it comes to candidate genes in the areas of interest in the

present thesis, promising results have been made in association studies of the gene encoding the pro-inflammatory cytokine interleukin 1 beta (IL-1β) (Nishimura *et al.*, 2000; Schulte *et al.*, 2002; Wahner *et al.*, 2007b), where the majority of the studies show significant association with PD.

So far six GWAS have been performed regarding PD, four in Caucasian populations and one in a Japanese population (Maraganore *et al.*, 2005; Fung *et al.*, 2006; Pankratz *et al.*, 2009b; Satake *et al.*, 2009; Simon-Sanchez *et al.*, 2009; Edwards *et al.*, 2010). Thirteen SNPs found to be associated with the disorder in the first study by Maraganore and colleagues (2005), were genotyped in a large-scale association study (Elbaz *et al.*, 2006), but none of the associations could be replicated. The results from the second GWAS by Fung *et al.* (2006), showed no overlap with the results presented by Maraganore *et al.* (2005). In the third GWAS published by Pankratz and colleagues (2009) the strongest associations were found in the regions where the *SNCA* and *MAPT* genes are located; results that were supported, by reaching a genome-wide significance level, in the GWAS made by Simon-Sanchez *et al*. (2009). Associations of SNPs in the *SNCA* gene with PD were also reported in the Japanese study (Satake *et al.*, 2009). In the most recently published GWAS (Edwards *et al.*, 2010), further support for an involvement of polymorphisms in the *SNCA* gene and *MAPT*-region was found.

Apart from finding genetic variants that influence the risk of developing a disease, it is of importance to search for variants which influence the age of onset of the disorder. The relationship between the frequency of a genetic variant and age of onset might look different for different genes, which is described in Figure 2. Identification of age at onset modifiers is of particular interest in age-related degenerative disorders with relatively late onset, like PD, because identifying pathophysiological factors that influence disease onset might make it possible to postpone the first appearance of disease symptoms so late in life that the disabling effects of the disease will be marginal. Segregation analyses of PD have shown that there is stronger evidence for major genes influencing age of onset than for genes influencing susceptibility to disease (Zareparsi *et al.*, 1998; Maher *et al.*, 2002). In a large GWAS searching for genes related to age of onset of PD (Latourelle *et al.*, 2009), a possible association with a gene (AAK1) located close to the PARK3 region (2p13) was found. This genomic region has been implicated in previous linkage studies with age at onset of PD (DeStefano *et al.*, 2002; Pankratz *et al.*, 2009b).

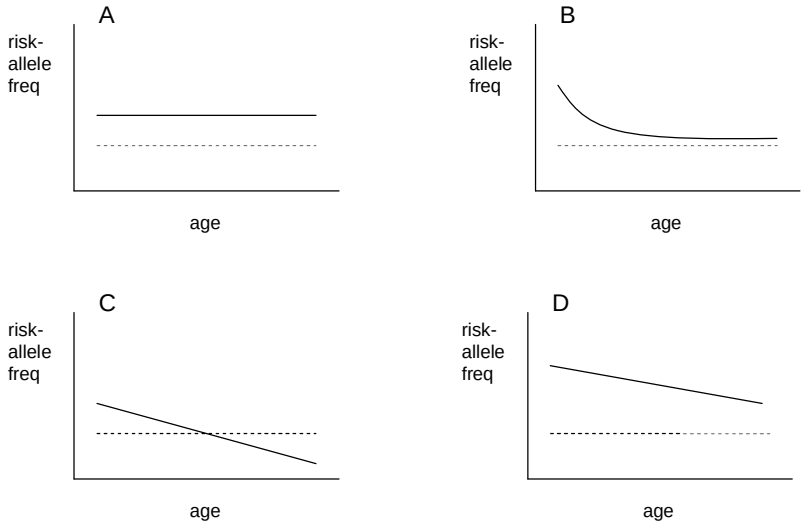

Figure 2. Hypothetical models of association with risk and age at onset of a disease. The plots show the risk allele frequency distribution as a function of age in controls (dashed lines) versus patients (solid lines). If the allele is not associated with the disease the curves for patients and controls will be superimposed (not shown). Model A describes an allele which frequency is elevated in patients uniformly across all ages. Model B describes an age-varying association where the allele is associated only with the early age at onset form of a disease. Model C describes an age at onset modifier, where there is no difference in the overall average allele frequency between patients and controls, but there is a shift in patients showing higher frequencies of the allele in younger onset cases and a subsequent depletion of the allele toward later onsets. Model D describes an allele that is associated with both risk (as shown by higher allele frequency in patients versus controls) and age at onset (as shown by the allele frequency shift in patients). Adopted from Payami et al. 2009.

ESTROGEN IN PARKINSON'S DISEASE

It is now quite established that PD is more common among men than women (Marder *et al.*, 1996; Baldereschi *et al.*, 2000; Van Den Eeden *et al.*, 2003; Wooten *et al.*, 2004). One suggested explanation for the observed gender difference is cytoprotective effects exerted by estrogen (Bourque *et al.*, 2009). Evidences speaking in favour of this hypothesis are given by studies showing an association between use of estrogen replacement therapy and reduced risk of developing PD (Currie *et al.*, 2004), and a higher frequency of women who have undergone hysterectomy among PD cases as compared to healthy individuals (Benedetti *et al.*, 2001). Furthermore, in a large epidemiological study on women's health, it was seen that a long fertile

lifespan was associated with reduced risk of PD (Saunders-Pullman *et al.*, 2009). However, divergent results regarding PD and estrogen therapy have also been presented (Marder *et al.*, 1998). The progress of PD symptoms and responses to levodopa also seem to differ between men and women, as men display more severe motor deficit symptoms (Lyons *et al.*, 1998) and women show more improvement in motor function during levodopa treatment (Growdon *et al.*, 1998). In addition, estrogen therapy has been found to be associated with less severe symptoms in women with early onset PD (Saunders-Pullman *et al.*, 1999) and improvement of motor disability by estrogen treatment has been reported in one study (Tsang *et al.*, 2000), but not in another (Strijks et al. 1999). Additional evidences for a protective role of estrogen come from experimental animal studies, since estrogen has been found to attenuate the neurotoxicity induced by both 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA) and methamphetamine (Liu and Dluzen, 2007).

The main form of estrogen, estradiol, exists as two stereoisomers named 17α-estradiol and 17β-estradiol. 17β-estradiol has the highest affinity to estrogen receptors (ERs) (Kuiper *et al.*, 1997) and shows more neuroprotectivety compared to the other variants (Callier *et al.*, 2000; Jourdain *et al.*, 2005). The mechanisms behind the neuroprotective effect of estrogen have not been fully elucidated. The hormone has been suggested to be involved in the regulation of apoptotic pathways (Sawada *et al.*, 2000), to mediate antioxidative effects (Sawada *et al.*, 1998) and to have anti-inflammatory properties (Bruce-Keller *et al.*, 2000). Furthermore, in dopaminergic cells estrogen has been shown to regulate dopamine synthesis and release as well as the expression of dopamine receptors and dopamine uptake sites (Küppers *et al.*, 2008; Bourque *et al.*, 2009).

Since inflammatory processes now are regarded to be of pathophysiological importance for PD, the suggested role of estrogen as an anti-inflammatory substance is of particular interest. Except being able to attenuate microglia activation (Bruce-Keller *et al.*, 2000; Vegeto *et al.*, 2001), estrogen has been found to influence several components involved in inflammation, for example reducing lipopolysacharid (LPS)-stimulated expression of COX-2 (Baker *et al.*, 2004), inhibiting the expression of IL-6 (Ershler and Keller, 2000) and regulating the *IFN-γ* gene promoter (Fox et al. 1991).

In addition, estrogen influences the development of dopaminergic neurons, which is for example evident in studies of embryonic stem cells (Diaz *et al.*, 2009) or midbrain embryonic cells (Ivanova and Beyer, 2003) as judged by increased levels of tyrosine

hydroxylase (TH) expression after stimulation with 17β-estradiol. In addition, it has been demonstrated that mouse fetuses exposed to an aromatase (an enzyme involved in the production of estradiol) inhibitor show a robust decline in TH mRNA/protein levels at birth (Ivanova and Beyer, 2003).

The effects of estrogen are mediated by ERs. These receptors belong to a large superfamily of nuclear receptors, which act as ligand-activated transcription factors. Estrogen receptors are generally divided into two different subtypes, ERα and ERβ, localised both in the cell nucleus and in the plasma membrane (Björnström and Sjöberg, 2005). In addition transmembrane ER subtypes have been identified (Toran-Allerand *et al.*, 2002; Revankar *et al.*, 2005). Estrogen exerts so called genomic effects by nuclear receptors, but also non-genomic effects mediated by the membrane-associated ERs (Björnström and Sjöberg, 2005). A more detailed description of the ER signalling in cells is seen in Figure 3.

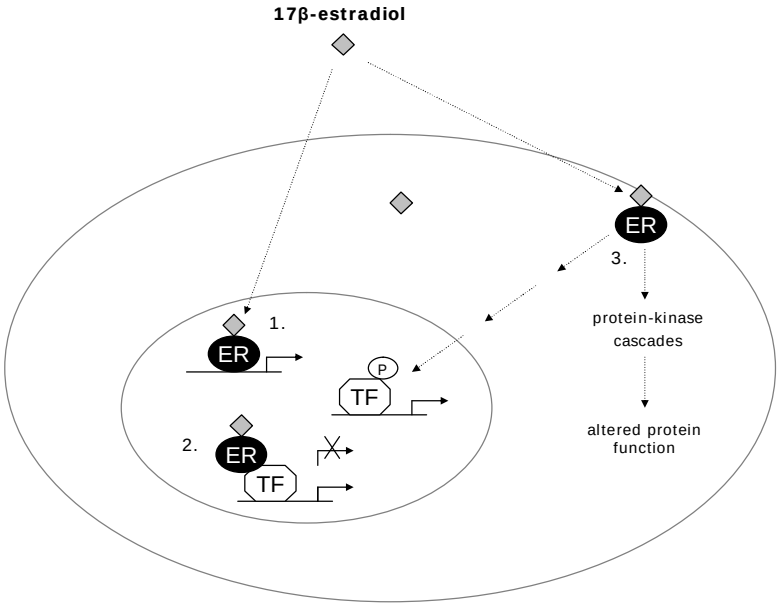

Figure 3. Schematic picture of ER signaling mechanisms.
1. The classical mechanism of ER genomic action. E2-ER complex in the nucleus binds to an estrogen responsive element (ERE) in a target gene promoter. 2. ERE-independent genomic action. Nuclear complex of E2-ER is connected to a transcription factor complex (TF) through protein-protein interactions. The TF-complex stands in contact with the target gene promoter. 3. Nongenomic actions of ERs. E2-ER complex in the membrane activates protein-kinase cascades, leading to altered functions of protein in the cytoplasm or to regulation of gene expression through phosphorylation (P) and activation of a TF. E2: 17β-estradiol. Adopted from (Björnström and Sjöberg, 2005).

Estrogen receptor beta (ERß)

The data obtained regarding distribution and expression levels of ERβ in the brain seem to be dependent on which species and experimental method that have been used, but substantial expression in the substantia nigra has been demonstrated in several studies (Zhang *et al.*, 2002; Quesada *et al.*, 2007; Yamaguchi-Shima and Yuri, 2007). On a cellular level, both neurons and astrocytes in this brain nucleus seem to express ERβ (Quesada *et al.*, 2007), but no study has so far investigated if also microglia express the receptor *in vivo*. However, it has been shown that estrogen can modulate the inflammatory action of microglial cells through binding to ERβ *in vitro* (Baker *et al.*, 2004).

ERβ knockout mice display degeneration of neuronal cell bodies throughout the brain, but the degeneration is especially evident in the substantia nigra (Wang *et al.*, 2001). No such changes could be seen in the brains of *ERα* knockout mice, although reduction of both TH and brain derived neurotrophic factor (BDNF) expression have been reported (Küppers *et al.*, 2008). However, when ER agonists were given to MPTP-treated mice, ERα was found to be the most important subtype when it comes to neuroprotection by estrogen (D'Astous *et al.*, 2004).

INFLAMMATION IN PARKINSON'S DISEASE

An inflammatory response is essential for survival in an environment where individuals are continually exposed to noxious events. However, when inflammatory signals are altered or misprocessed, an inflammation can become chronic, causing extensive damage to cells and tissues in the body. Inflammation is associated with many severe and prevalent diseases, such as atherosclerosis, rheumatoid arthritis, multiple scelrosis, psoriasis, Crohn's disease and asthma (Barreiro et al. 2010).

In PD inflammation is believed to be one of the mechanisms contributing to the cascade of events leading to neuronal degeneration. As early as twenty years ago an up-regulation of major histocompability complex (MHC) molecules and an increased number of activated microglia in the striatum and substantia nigra was found in PD patients (McGeer *et al.*, 1988). Microglia is the most important immunocompetent cell type within the CNS, acting as macrophages with phagocytic properties. The cells are activated in response to neuronal damage and several environmental stimuli, for example toxins (Block and Hong, 2007). Their activation is initiated by expression of MHC class two (Barcia *et al.*, 2003) and when activated they up-regulate or start to

express several types of receptors and other molecules involved in inflammation. In addition, activated microglia produce and release reactive oxygen and nitrogen species. It has been demonstrated by PET scanning and immunohistochemistry that activated microglia in PD preferentially are localised to the midbrain area (Ouchi *et al.*, 2005). The highest concentration of microglial cells in the brain is found in the substantia nigra (McGeer *et al.*, 1988), making neurons in this area particularly vulnerable to signals from these cells. Another cell type taking part in inflammatory reactions in the brain are astrocytes, which in their reactive state can release neurotrophic factors, pro-inflammatory components and reactive oxygen and nitrogen species. Astrocytes seem to be responsible for more specific phagocytic processes as compared to microglia (Wyss-Coray and Mucke, 2002). However, in contrast to the large amount of data published on the pathophysiological role of microglia in PD, studies investigating reactive astrogliosis are sparse.

Microglia and astrocytes both take part in the innate immunity, but also cells belonging to the adaptive (or accuired) immunity are supposed to have a pathophysiological role in PD. As early as in 1988 $CD8^+$ T cells (killer T cells) could be detected in the substantia nigra from a PD patient (Mceer et al, 1988), and a recent study reported a significant increase in the density of both $CD8^+$ and $CD4^+$ T cells (helper T cells) in the substantia nigra from PD patients as compared to control subjects (Brochard *et al.*, 2009). Peripheral activation of lymphocytes has also been proposed to be associated with PD, based on changes in the ratio between naive and activated T cells in serum (Bas *et al.*, 2001). The involvement of antibodies in PD has also been suggested, since immunoglobulin G (IgG) binding to dopamine neurons has been demonstrated (Orr *et al.*, 2005). In the same study, an increased number of microglia expressing the IgG receptor FcγRI, were found. The microglial cells also contained pigmented granules, consistent with a phagocytic attack on the IgG-immunopositive pigmented neurons (Orr *et al.*, 2005). Furthermore, the concentration of antibodies against neuromelanin, a component usually present in dopaminergic neurons, has been found to be higher in serum from PD patients as compared to control subjects (Double *et al.*, 2009). In summary, to date data indicates a pathophysiological role for the innate immune system in PD, but the importance of the adaptive immune system is ambiguous.

Investigations of inflammation in PD patients have also been performed on a molecular level. Increased concentrations of the inducible enzymes nitric oxide synthase (iNOS) and cyclooxygenease 2 (COX-2) (Knott *et al.*, 2000), as well as elevated levels of different cytokines, such as interleukin 1β (IL-1β), interleukin 2 (IL-

2), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α), transforming growth factor beta 1 (TGF- β1) and interferon gamma (IFN-γ) (Mogi *et al.*, 1994a; 1994b; 1995; 1996; 2007), have been detected in affected brain areas in post-mortem analyses of PD patients. Changes in the concentrations of cytokines in serum and cerebrospinal fluid from individuals with PD have also been demonstrated (Mogi *et al.*, 1994b; Blum-Degen *et al.*, 1995; Brodacki *et al.*, 2008). Other molecular components that seem to be involved in an inflammatory process in PD are complement proteins. Elevated levels of mRNA encoding complement proteins have been observed in affected brain areas in PD (McGeer and McGeer, 2004) and components of complements have been detected in Lewy bodies in several studies (Yamada *et al.*, 1992; Loeffler *et al.*, 2006).

Further support for the importance of inflammation in PD are the results from an epidemiological study, indicating that regular use of non-aspirin NSAIDs (nonsteroidal anti-inflammatory drugs) would act as a protective factor against the disease (Chen *et al.*, 2005). Results from subsequent studies, both confirmed (Ton *et al.*, 2006) and opposed (Wahner *et al.*, 2007a) the original finding. Furthermore, a vast amount of studies using animal models of PD have been performed with respect to inflammation. Some of them will be referred to below, in the sections considering inflammation-related molecules.

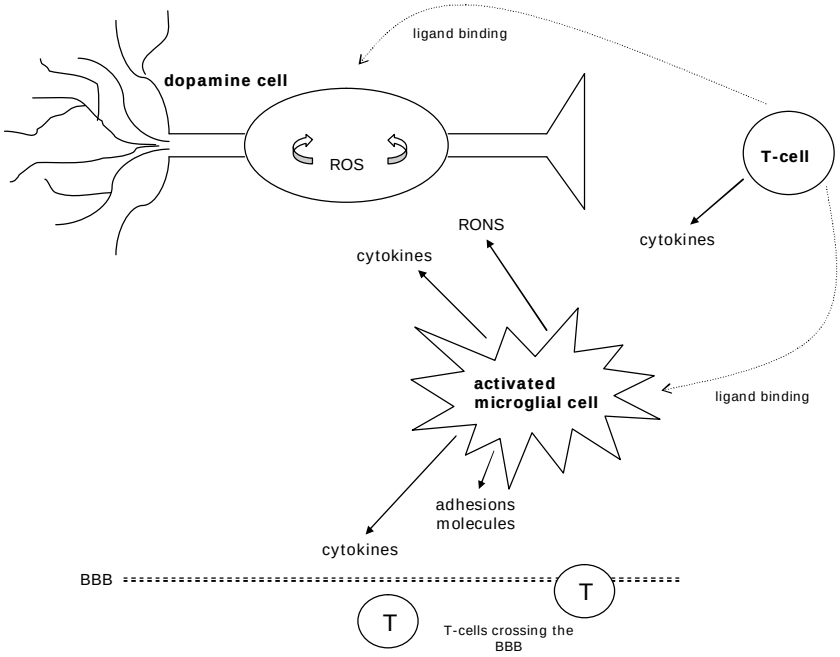

Figure 4. Simplified schematic illustration of possible inflammatory mechanisms in PD. Activated microglia release inflammatory mediators such as cytokines which can stimulate the production and release of reactive oxygen and nitrogen species (RONS) from adjacent microglial cells, through induction of the enzymes iNOS, COX-2 and NADPH oxidase. The cytokines can also stimulate the induction of COX-2 within the dopamine cells, generating additional reactive oxygen species (ROS). Further, the production of cytokines together with the increased expression of adhesion molecules on microglial cells can facilitate the recruitment of T lymphocytes from the blood vessels across the blood brain barrier. The T lymphocytes also release cytokines and might in turn influence microglia and dopaminergic neurons through ligand binding. Altogether these processes create a harmful environment, possibly contributing to dopaminergic neurodegeneration. BBB: blood brain barrier; NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase.

Interleukin 6 (IL-6)

IL-6 belongs to the family of neuropoietic cytokines, participating in many different biological processes (Gadient and Otten, 1997). The cytokine is an important inflammatory mediator both within the CNS and in the periphery. It was first thought of only as a pro-inflammatory cytokine, but it is now assumed to have anti-inflammatory activities as well (Sredni-Kenigsbuch, 2002). While acting as a mediator of inflammation IL-6 can contribute to a cascade of processes, such as synthesis of acute-phase proteins, increase of leukocytes and activation of lymphocytes. Under normal conditions IL-6 is expressed at a low level by some neurons and glial cells.

During pathological conditions in the brain, the concentration is elevated due to production of IL-6 by activated microglia and astrocytes, but possibly also by infiltrating T-cells, macrophages and blood vessel endothelial cells (Gruol and Nelson, 1997). Consequently, several studies investigating the levels of IL-6 in PD have shown increased concentrations, both in nigrostriatal regions of postmortem brain and in the CSF (Mogi *et al.*, 1994a; Blum-Degen *et al.*, 1995; Nagatsu *et al.*, 2000). However, the results from investigations of plasma or serum levels of IL-6 have been more inconsistent (Blum-Degen *et al.*, 1995; Stypula *et al.*, 1996; Brodacki *et al.*, 2008).

In 1993, the first transgenic mouse model over-expressing IL-6 in the CNS, directed into astrocytes, was presented (Campbell *et al.*, 1993). The animals showed a neurologic syndrome, which developed into a severe neurodegenerative disease. However, when the expression was directed into neurons, no histological or behavioural signs of neuronal damage could be seen (Fattori *et al.*, 1995). Still, the latter mouse model developed reactive astrocytosis and showed an increased number of ramified microglial cells. The studies using *IL-6* knockout mice and that were more specifically related to PD showed divergent results; an attenuated metamphetamine-induced toxicity against dopaminergic neurons was noticed in one investigation (Ladenheim *et al.*, 2000), but the same type of neurons seemed to be more sensitive to MPTP in another (Bolin *et al.*, 2002).

Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine, in the brain expressed by activated microglia, astrocytes and monocytes (Johnston *et al.*, 2008). Initially, IL-10 was described as a cytokine inhibitory factor and exerts its anti-inflammatory properties by reducing the synthesis of pro-inflammatory cytokines, such as TNF-α, IL-1β, IFN-γ and IL-6, as well as the expression of their corresponding receptors (Strle *et al.*, 2001). In addition it inhibits activation of the receptors, by inducing expression of SOCS proteins, which in turn reduce the signalling of the receptors (Krebs and Hilton, 2000). Additionally, IL-10 is also able to inhibit proliferation of T lymphocytes, reduce the expression of MHC class two molecules as well as of cell surface markers necessary for stimulation of cellular immunity, and attenuate induction of neuro-apoptosis (Strle *et al.*, 2001).

IL-10 has been found to be increased in serum from PD patients (Brodacki *et al.*, 2008; Rentzos *et al.*, 2009). In a study using the 6-OHDA animal model of PD,

intrastriatal delivery of IL-10 by an adeno-associated viral vector was reported to decrease dopaminergic cell death (Johnston *et al.*, 2008). Furthermore, infusion of IL-10 into the substantia nigra of rats seems to protect against LPS-induced neuronal degeneration (Arimoto *et al.*, 2006).

Interferon gamma and Interferon gamma receptor 2 (IFN-γ and IFN-γ R2)

In CNS, the proinflammatory cytokine IFN-γ can be expressed by microglia and astrocytes (Dafny and Yang, 2005), but most likely also by infiltrating T-cells (Hirsch and Hunot, 2009) since these cells are the main source of this cytokine in the periphery. IFN-γ possesses several immunomodulatory activities of importance for CNS inflammation, such as activation of macrophages and promotion of leukocyte adhesion. It also shows a more direct effect on microglia by influencing CD23-dependent expression of inducible nitric oxide synthase (iNOS) and possibly also NADPH oxidase (Hirsch and Hunot, 2009). IFN-γ can bind to a receptor complex consisting of the ligand-binding chain interferon gamma receptor 1 (IFN-γR1), and the signal-transducing chain interferon gamma receptor 2 (IFN-γR2), also called accessory factor of the receptor complex (Rhee *et al.*, 1996). IFN-γ receptor mRNA expression has been observed in rat nigral dopaminergic neurons (Lindå *et al.*, 1999).

Studies of post-mortem brains from PD patients have found elevated concentrations of IFN-γ in nigrostriatal dopaminergic structures as compared to control brains (Hunot *et al.*, 1999; Mogi *et al.*, 2007) and increased levels in plasma and serum as well (Mount *et al.*, 2007; Brodacki *et al.*, 2008). Inconsistent results concerning the exact role of IFN-γ in the process of dopaminergic cell death are reported in studies using the MPTP animal model of PD. Mount and co-workers (Mount *et al.*, 2007) showed that IFN-γ contributes to the death of dopaminergic neurons by activation of microglia. However, it has recently been suggested that IFN-γ is not of crucial importance for dopaminergic neurodegeneration mediated by T cells (Brochard *et al.*, 2009). In the latter study it was also found that mice deficient in IFN-γ and wildtype mice were equally sensitive to MPTP treatment.

Cyclooxygenase 2 (COX-2)

Cyclooxygenase (COX) is an enzyme that catalyses the synthesis of prostanoids (prostaglandins (PGs), prostacyclin and thromboxanes). The synthesis is made in two steps, first formation of PGG_2 from arachidonic acid and then conversion of PGG_2

to PGH_2, the final substrate for the synthases that make other PGs, prostacyclin and thromboxane. COX exists in two main isoforms, COX-1 and COX-2. In general, COX-1 is constitutively expressed in numerous cell types and primarily involved in the production of prostanoids during physiological processes, whereas the expression of COX-2 is mainly induced by pathological stimuli (Teismann *et al.*, 2003a). However, COX-2 is expressed in some brain neural cells even during normal physiological conditions, where it seems to be of importance for synaptic plasticity (Minghetti, 2004). Still, the brain concentration of the enzyme can be up-regulated during pathological processes, such as inflammation, and synthesize prostaglandin PGE_2, which is linked to inflammation. Apart from neurons, there are several other cell types that express COX-2 in the brain, such as microglia, astrocytes, endothelial cells and infiltrating leukocytes (Minghetti, 2004).

Whether COX-2 is constitutively expressed in neurons located in the human substantia nigra or not is unclear (Knott *et al.*, 2000; Teismann *et al.*, 2003b). Elevated concentrations of COX-2 and PGE_2 in human post-mortem substantia nigra in PD patients, as compared to controls, have been demonstrated (Teismann *et al.*, 2003a), but how COX-2 exerts a potential pathogenic effect is debated. It has been proposed that neurotoxicity might be caused by reactive oxygen species generated during the formation of PGH_2 from PGG_2, rather than from inflammatory properties of PGE_2 (Teismann *et al.*, 2003a). The majority of COX-2 positive cells in the substantia nigra in PD patients seems to be neurons (Teismann *et al.*, 2003b). However, in the study by Knott and colleagues (2000), COX-2 was shown to be expressed by both microglia and astrocytes. Microglia also seem to be responsible for the induction of COX-2 (Teismann *et al.*, 2003b).

Some results from animal studies suggest that COX-2 activity might be of pathophysiological importance for PD. Thus, inactivation of COX-2 through genetic modification (knockout) in mice was found to reduce MPTP-induced damage to dopaminergic neurons (Feng *et al.*, 2003). Furthermore, pharmacological inhibition of COX-2, through selective COX-2 inhibitors, has been shown to be cytoprotective in the MPTP, 6-OHDA and LPS (lipopolysaccharides) animal models of PD (Teismann and Ferger, 2001; Sanchez-Pernaute *et al.*, 2004; Reksidler *et al.*, 2007; Aguirre *et al.*, 2008; Sui *et al.*, 2009), although contradictive results also have been presented (Przybylkowski *et al.*, 2004; Gören *et al.*, 2009). The results from epidemiological studies investigating a possible protective effect of NSAIDs are somewhat inconsistent with respect to the risk of developing PD (see above).

Intercellular adhesion molecule 1 (ICAM-1)

Adhesion molecules are known as cell surface structures mediating interactions between cells and also between cells and the extracellular matrix. Those involved in immune responses are classified into three families depending on their structure: selectins, immunoglobulin (Ig) supergene family and integrins. ICAM-1 belongs to the Ig supergene family and is known to be expressed in the brain by blood vessel endothelial cells and most likely also by activated microglia and astrocytes (Lee and Benveniste, 1999; Miklossy *et al.*, 2006; Sawada *et al.*, 2006). In addition infiltrating lymphocytes and monocytes are probably capable of expressing ICAM-1. ICAMs are ligands for the integrin receptors lymphocyte function-associated antigen 1 (LFA-1) and Mac-1, which are present on leukocytes, but also on microglial cells in the CNS (Lee and Benveniste, 1999). Through binding to these receptors, ICAM-1 plays an important role in endothelial-leukocyte cell interaction and leukocyte extra-vasation. Additionally, ICAM-1 acts as an accessory protein for antigen receptor activation on B and T cells (Lee and Benveniste, 1999).

Two studies have investigated the presence of ICAM-1 in post-mortem brain of PD patients, showing activated microglia in the putamen (Sawada et al. 2006) and reactive astrocytes in the substantia nigra (Miklossy et al. 2006) positive for this adhesion molecule. In the latter study it was seen that ICAM-1 is expressed constitutively on endothelial cells in the brain, since it was found on capillaries both in the substantia nigra of control individuals and in unaffected regions in PD cases. Further, an increase of ICAM-1 molecules has been noticed in animals (mice and monkeys) treated with MPTP (Kurkowska-Jastrzebska et al. 1999; Mikolssy et al. 2006).

Platelet activating factor acetylhydrolase (PAF-AH)

PAF-AH, also known as phospholipase A2 group VII (PLAG7), was identified as an enzyme that hydrolyses an acetyl ester at the sn-2 position of PAF, thereby inactivating it to lysoPAF. PAF is a phospholipid with diverse physiological and pathological effects, in part depending on its intracellular or extracellular location. Extracellular PAF participates in inflammation and immune responses since it can activate pro-inflammatory cells, for example macrophages (Prescott *et al.*, 1990), and possibly also microglia (Farooqui *et al.*, 2006) in the brain. PAF is synthesised by several types of cells central to inflammation, such as different leukocytes and endothelial cells (Castro Faria Neto *et al.*, 2005). Microglial cells in the CNS are probably also capable cells, since production of PAF from such cells, after

stimulation with TNF-α and LPS, has been seen *in vitro* (Jaranowska *et al.*, 1995). Furthermore, human neurons in culture have been shown to produce, but not release, PAF (Sogos *et al.*, 1990), indicating that this PAF will not interact with plasma PAF-AH.

Similar to the distribution of PAF, PAF-AH can be found both intracellularly and extracellularly (plasma). Plasma PAF-AH mRNA has been found to be expressed in all parts of the brain (Cao *et al.*, 1998), and it is proposed that developing macrophages are the main cellular source of the enzyme (Elstad *et al.*, 1989). No investigations about PAF or PAF-AH in PD patients have been conducted.

DOPAMINE NEURON DEVELOPMENT

The dopaminergic neurons in the CNS are located in several different cell groups, designated A1-A17. Three of these groups of cell nuclei, containing approximately 75% of the total number of dopamine cells in the brain, are located in the ventral mesencephalon (midbrain); the retrorubral field (A8), the substantia nigra pars compacta (A9) and the ventral tegmental area (A10).

The organisation of the mesencephalon during brain development is initiated by positioning of key signalling centres, such as the floor plate at the ventral midline and the mid-hindbrain organiser (MHO), also named isthmus (Rhinn and Brand, 2001). The floor plate is situated almost throughout the entire length of the neural tube, while the MHO is a more specific centre involved in the process of controlling the size and location of the mesencephalic dopaminergic neurons. The MHO is established by mutual repression of the transcription factors Otx2 (homolog of drosophila orthodenticle) and Gbx2 (gastrulation brain homeobox 2) (Millet *et al.*, 1999), thereby defining a sharp border. At this time, before the induction of mesencephalic dopaminergic neurons, further transcription factors participating in the regionalisation of mid- and hindbrain appear. This second wave of signals includes the transcription factors Pax2/5 (paired box 2 and 5) (Urbanek *et al.*, 1997), Lmx1b (lim-homeodomain factor b) (Adams *et al.*, 2000), and En1/2 (engrailed 1 and 2) (Alavian *et al.*, 2008), but also the diffusible glycoprotein Wnt1 (Prakash *et al.*, 2006). These factors are considered to be the first ventral midbrain markers to be expressed, but are not specific to dopaminergic cells. The induction of mesencephalic dopaminergic precursor cells is made by an interaction between the diffusible factors

Shh (sonic hedgehog), secreted by the floor plate, and Fgf8 (fibroblast growth factor 8), released at the MHO (Ye *et al.*, 1998).

The next transcription factors that will appear in the proliferating dopaminergic precursor cells are Lmx1a (lim-homedomain factor a) and Msx1 (msh homeobox 1), functioning as key determinants of midbrain dopamine neurons (Andersson *et al.*, 2006). Subsequently, the proliferating cells enter into a postmitotic differentiation stage and receive further signals from other transcription factors, such as Nurr1 (NR4A2: nuclear receptor subfamily 4, group A, member 2) (Zetterström *et al.*, 1996), Pitx3 (paired-like homeodomain transcription factor 3) (Smidt *et al.*, 1997), En1/2 (Alberi *et al.*, 2004) and Lmx1b (Smidt *et al.*, 2000) during their development to mature dopamine neurons. At the same stage, expression of early phenotypic markers of dopaminergic neurons, such as TH, is induced (Wallen *et al.*, 1999). Later during the maturation process the dopamine transporter (DAT) and other key components of the dopamine phenotype, like the vesicular monoamine transporter 2 (Vmat2), are expressed (Burbach and Smidt, 2006; Abeliovich and Hammond, 2007). Several of the transcription factors induced during the postmitotic differentiation state continue to be expressed throughout life and seem to be of importance for the maintenance of the dopamine neurons in the midbrain, although their role in the fully developed brain is less explored.

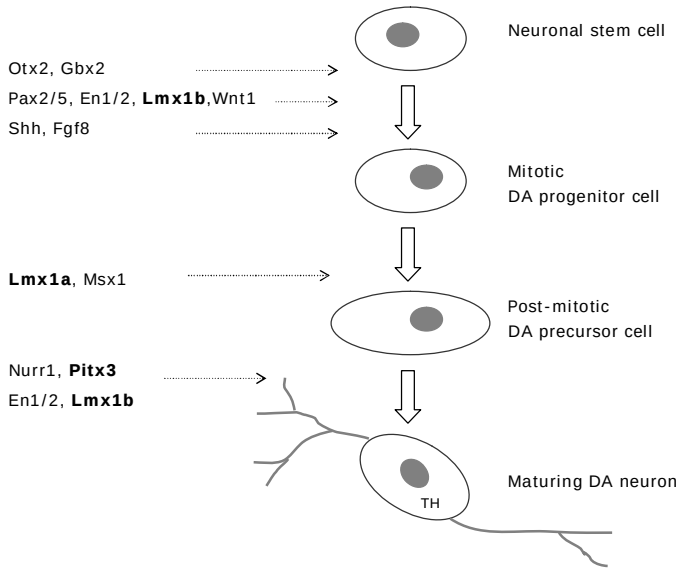

Figure 5. Simplified illustration of midbrain dopamine neuron development. The arrows represent times at which transcription factors or diffusible factors are induced. Factors highlighted in bold are those investigated in the present thesis.

Lmx1a

Lmx1a belongs to a family of LIM-homeodomain transcription factors and was originally identified as a factor activating insulin gene transcription (German *et al.*, 1992). The role of Lmx1a as a key determinant in the development of mesencephalic dopamine neurons was suggested by Andersson *et al.* (2006). The authors found that inhibiting Lmx1a expression by RNA interference resulted in loss of dopaminergic neurons in the midbrain but over-expression of Lmx1a in mouse embryonic stem cells generated dopaminergic cells. Later, it has been shown that over-expression of LMX1A also promotes generation of dopaminergic cells in human embryonic stem cells (Friling *et al.*, 2009) and increases the yield of TH expressing cells in human embryonic cells derived from the ventral midbrain (Roybon *et al.*, 2008). Furthermore, dreher mice, carrying a spontaneously generated mutation in the *Lmx1a* gene (Millonig *et al.*, 2000), show a reduced number of mesencephalic dopamine neurons as compared to wild type mice (Ono *et al.*, 2007).

On a molecular level, Lmx1a has been proposed to activate another transcription factor, Msx1 (Msh homeobox 1), resulting in an induction of the pro-neural protein Ngn2 (Neurogenin 2). Nevertheless, it seems as if Msx1 acts as a complement to Lmx1a, since the latter but not the former is able to generate dopamine neurons on its own (Andersson *et al.*, 2006).

Lmx1b

The protein Lmx1b is structurally related to Lmx1a and is important for development of the skeleton, eyes, kidneys and limbs (Chen *et al.*, 1998), but also as a regulator of neuronal cell fate (Smidt *et al.*, 2000; Ding *et al.*, 2003). Early in the developing brain (from around E7.5 in mouse), Lmx1b can be found in a broad band reaching from the ventral to the dorsal surface of the mesencephalon, a region including the MHO (Smidt *et al.*, 2000). It has been suggested that Lmx1b acts as an effector of Fgf8 in the regulation of Wnt1 within the MHO (Adams *et al.*, 2000). The initial expression of Lmx1b is subsequently downregulated, but later during the development the factor will be found in the postmitotic dopaminergic neurons of the mesencephalon (Smidt *et al.*, 2000). *Lmx1b* knockout mice express Nurr1 and TH during early development, but fail to express Pitx3, and at birth the mesencephalic dopaminergic neurons seem to be lost (Smidt *et al.*, 2000). In the same study, a reduction of LMX1B expressing neurons in the substantia nigra of PD patients correlating with the loss of dopaminergic cells was seen.

Pitx3

The transcription factor Pitx3, also named pituitary homeobox 3, belongs to the same family of paird-like homeodomain transcription factors as Pitx1, initially identified as a component of pituitary development and function (Lamonerie *et al.*, 1996). The expression of Pitx3 in the brain is restricted to mesencephalic dopaminergic neurons (Smidt *et al.*, 1997). Additionally, Pitx3 has been found to be of importance for the development of the eye lens (Semina *et al.*, 1998). A naturally occurring blind mouse mutant, called aphakia, was linked to a deletion in the promoter region of the *Pitx3* gene (Semina *et al.*, 2000), resulting in a loss of expression of the protein (van den Munckhof *et al.*, 2003). The aphakia mice were later found to be preferentially devoid of substantia nigra dopaminergic neurons as well as axonal projections to the striatum (Hwang *et al.*, 2003; Nunes *et al.*, 2003; van den Munckhof *et al.*, 2003; Smidt *et al.*, 2004a).

Pitx3 begins its expression around E11.5 in mice, but the exact time might differ between cells in the substantia nigra where the expression was seen prior to TH, and the ventral tegmental area, where TH appeared before Pitx3 (Maxwell *et al.*, 2005). It has been shown that Pitx3 up-regulates the expression of AHD2 (aldehyde dehydrogenase 2) (Chung *et al.*, 2005; Martinat *et al.*, 2006), an enzyme highly expressed in the substantia nigra. AHD2 generates retinoic acid which is involved in the patterning, differentiation and survival of these dopaminergic neurons (Jacobs *et al.*, 2007). Retinoic acid administration has been found to counteract the developmental defects caused by Pitx3 deficiency *in vivo* (Jacobs *et al.*, 2007). Furthermore, results from cell culture studies have shown that aphakia embryonic stem cells deficient in Pitx3, generates 50% fewer mature dopaminergic neurons, which could be partially restored by addition of retinoic acid (Papanikolaou *et al.*, 2009). Furthermore, the expressions of the vesicular monoamine transporter 2 and the dopamine transporter were found to be greatly reduced in aphakia mice, but forced expression of Pitx3 could up-regulate the transporter proteins in mouse embryonic stem cells (Hwang *et al.*, 2009).

AIMS

The overall aim of the thesis was:

…to investigate if variations in genes of relevance for inflammation, development of dopaminergic cells, and estrogenic effects, are of importance in sporadic PD.

The specific aims of the included papers were:

I. …to investigate if SNPs in the $ER\beta$ gene are associated with sporadic PD.

II. …to investigate if a SNP in the *IL-6* gene is associated with sporadic PD, and to analyse its possible interaction with an $ER\beta$ SNP, found to be associated with early onset PD in Paper I.

III. …to search for possible associations between SNPs in the genes encoding *IL-10*, *I*$IFN\gamma$, $IFN\gamma$*-R2*, *ICAM-1* and *PAF-AH*, and sporadic PD.

IV. …to assess whether SNPs in the gene coding for *COX-2* are of importance in sporadic PD.

V. …to screen for mutations in the *parkin* gene in a subgroup of patients with early onset PD, thereby investigating the existence of possible cases with familial PD in the used patient-material.

VI. …to search for genetic variation in the *PITX3* gene and subsequently analyse if this variation influences the risk of developing sporadic PD and/or the age of disease onset.

VII. …to study if genetic variation (SNPs) in the genes encoding *LMX1A* and *LMX1B* is associated with sporadic PD.

MATERIAL AND METHODS

SUBJECTS

Ethics

All individuals participating in the studies included in this thesis have provided an informed consent, and the studies were approved by the ethical committees at University of Gothenburg and Karolinska Institutet, Stockholm.

Populations

To date, the populations used in the studies included in this thesis consist of 362 patients with Parkinson's disease and 333 control subjects (see Table 1), recruited from hospitals and care centres in Göteborg, Stockholm, Skövde and Falköping. The number of individuals can vary from one paper to another due to continuous collection of samples from additional individuals. All patients fulfilled the PD Society Brain Bank criteria (Daniel and Lees, 1993), except that presence of more than one relative with PD was not an exclusion criterion. Age at disease onset was defined as the time when the patients noticed the first PD symptoms. The commonly used definition of having "an early age at onset" of PD if the disease begins at or before 50 years of age was used (Krüger *et al.*, 2000b; Mizuta *et al.*, 2001; Wang *et al.*, 2002). Patients and control subjects are all of Caucasian origin, and additional information about relatives with PD and age at the time of blood collection was documented if possible.

In Paper VII, one part of the controls belongs to the above mentioned control population (however those recruited in Stockholm were not included). The other part consists of 1240 individuals recruited in the Kungsholmen-project (Fratiglioni *et al.*, 1992). This project is a population-based study, including individuals living in the Kungsholmen district in Stockholm, where data concerning different aspects of aging have been gathered during a period of 12 years. The study started in 1987 and individuals born before 1913 were invited to participate. Besides DNA collection, examinations of physical, neurological as well as psychiatric status were made, and cognitive functions such as memory were assessed.

Table 1. Demographic data describing the studied populations.

	Patients n=362 (%)	Controls n=333 (%)	Kungsholmen* n=1240 (%)
Gender:			
Male	217 (59.9)	125 (37.5)	304 (24.5)
Female	145 (40.1)	179 (53.8)	936 (75.5)
Unknown	0	29 (8.7)	0
Relatives with PD	88 (24.3)	18 (5.4)	---
No information about relatives	---	142 (42.6)	---
Age at disease onset:			
≤50 years of age	69 (19.1)	---	---
>50 years of age	292 (80.7)	---	---
Unknown	1 (0.3)	---	---
Mean age at disease onset	60 years	---	---
Mean age at study initiation	68 years	58 years	81 years

* Used as an additional control material in Paper VII.

GENOTYPING METHODS

During the last fifteen years there has been a fast development of genotyping methods, from looking at single variations using different gel electrophoresis-based techniques, to automatic high-throughput systems based on biochip technology (Butler and Ragoussis, 2008; Nishida *et al.*, 2008), investigating up to 1.8 million genetic markers in large GWAS. If the early genotyping techniques, for example restriction fragment length polymorphism (RFLP) analysis, are low-throughput systems as compared to the biochip based systems, there are also systems with medium-throughput like Taqman (De la Vega *et al.*, 2005), Pyrosequencing (Nordfors *et al.*, 2002) and Sequenom (a technique based on MALDI-TOF MS) (Gabriel and Ziaugra, 2004), suitable for association studies of candidate genes.

Pyrosequencing (Paper I, II, III, IV and VI)

Pyrosequencing is a method for analysing predefined DNA sequences, based on detection of energy released during incorporation of nucleotides into a DNA strand (Nordfors *et al.*, 2002). A sequencing primer is hybridized to a single stranded PCR (polymerase chain reaction)-amplified DNA template and incubated with the

enzymes and substrates that will be used in the process. The pyrosequencing reaction is initiated when one of the four possible deoxynucleoside triphosphates (dNTPs) is added. If the nucleotide is complementary to the base in the template strand, DNA polymerase catalyzes the incorporation into the DNA strand. The incorporation of nucleotides is followed by release of pyrophosphate (PPi), in a quantity equimolar to the amount of incorporated nucleotide. PPi is converted to ATP by the enzyme ATP sulfurylase in the presence of adenosine 5´phosphosulfate. The ATP then drives the conversion of luciferin to oxyluciferin, mediated by luciferase, a process generating visible light. The light signal, detected by a charge coupled device camera (CCD), is proportional to the number of nucleotides incorporated and can be seen as a peak in a pyroprogram (see Figure 6). An enzyme named apyrase finaly degrades unincorporated dNTPs and excess amount of ATP.

Apart from using pyrosequencing for genotyping individual samples, it is also possible to use the method for estimation of allele frequencies in pooled DNA samples. In this case the peak height represents the frequency of a specific base in a whole population.

The genotype and allele frequency data in the studies included in the present thesis were analysed using the PSQ 96 or PSQ 96MA software. Primers used in the pyrosequencing reaction or in the preceding PCR amplification were made with Biotage Assay Design Software 1.0.6, Oligo 6.7 and PyrosequencingAB version 1.0Beta6 (software for design of sequencing primers only).

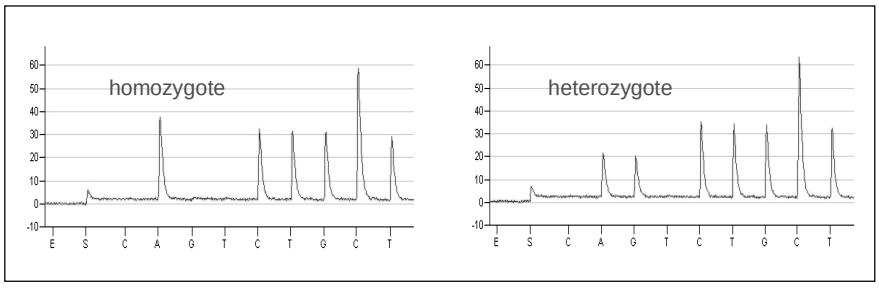

Figure 6. Example of pyrograms generated from a pyrosequencing run. To the left a homozygous (AA) sample, represented by a single peak in the A position, and to the right a heterozygous (AG) sample, represented by two lower peaks; one in the A- and one in the G position. The other peaks represent nucleotides positioned after the SNP in the DNA strain.

Sequenom (Paper VII)

Sequenom is a massARRAY platform, which relies on primer extension followed by detection with MALDI-TOF mass spectrometry (Gabriel and Ziaugra, 2004). By using the iPLEX Gold assay it is possible to make multiplexes with primers for analyses of up to 36 SNPs at a time. Following a PCR reaction, a locus-specific primer extension reaction takes place, where the primer and amplified target DNA are thermo-cycled together with mass-modified nucleotides. During this process the primer anneals to its specific position in the DNA chain and is subsequently elongated by one of the nucleotides, which terminates the extension of the primer. The extension products are allele-specific, depending on their different masses, and can therefore be analysed by mass spectrometry. The results from the mass spectrometry runs in our study were translated into genotypes by the TyperAnalyzer software 1.0.1.46. Primers used in the iPLEX Gold assay were designed with the Sequenom massARRAY Designer software.

METHODS FOR MUTATION SCREENING

DNA sequencing (Paper V-VI)

Sequencing is used to identify the specific order of the nucleotides in a DNA fragment. The DNA sequencing methods used today are refinements of the basic "dideoxy" method introduced by Sanger and collegues (1977). In the present thesis automated capillary sequencing was carried out in order to detect possible mutations in some of the investigated genes. Prior to the sequencing procedure, the DNA fragment of interest was amplified by PCR. This step is followed by a sequencing PCR, where terminator nucleotides named dideoxynucleotides are included in addition to the ordinary nucleotides. The dideoxynucleotides are labelled with a nucleotide-specific fluorescent dye. When a dideoxynucleotide is added to the growing DNA strand, the elongation stops because there is no 3'-OH group for the next nucleotide to be attached to. The termination step is a random process, generating DNA fragments of different sizes, which are separated by capillary electrophoresis. Since the terminator nucleotides are labelled, the DNA fragments can be detected by a laser beam just before reaching the end of the capillary. The fluorescence data were analysed using the SeqScape software version 2.5 (Applied Biosystems).

Multiplex Ligation-dependent Probe Amplification (MLPA) (Paper V)

MLPA is mainly used for detection of exon rearrangements, and up to 40 different nucleic acid sequences can be quantified in a single reaction. Generally, denaturated genomic DNA is hybridized with a mixture of different probes, consisting of two parts. One of the parts consists of a sequence where a PCR-primer can bind and a target specific hybridisation sequence. The other part consists of the same sequences as the first part, but it also has a "stuffer" sequence, with different length for each probe, in between. The two parts of the probe hybridise to adjacent DNA sequences and are ligated by a ligase. All the probe ligation products are then amplified by PCR, generating products with a unique length for each probe. The amplification products are separated by electrophoresis, in our case by an ABI 3730 capillary DNA sequencer. Comparison of the relative amounts of probe amplification products, seen as peaks in an electropherogram, with a control DNA sample, reflects the relative copy number of a target sequence. In the present work the resulting peaks were analysed visually using the Gene Mapper software version 3.7 (Applied Biosystems), and by further calculations after exporting the data into an excel file.

Table 2. Genes investigated in the present thesis.

Gene	Biological function	Chromosomal position	Paper
ERβ	Estrogen signaling	14q22-24	I-II
IL-6	Inflammation	7p21	II
IL-10	Inflammation	1q31-32	III
IFN-γ	Inflammation	12q14	III
IFN-γR2	Inflammation	21q22	III
ICAM-1	Inflammation	6p12-21	III
PAF-AH	Inflammation	19p13	III
COX-2	Inflammation	1q25	IV
PRKN	Protein degradation	6q25-27	V
PITX3	DA neuron development	10q25	VI
LMX1A	DA neuron development	1q22-23	VII
LMX1B	DA neuron development	9q34	VII

STATISTICAL ANALYSES

Hardy-Weinberg principle

The Hardy-Weinberg principle (Stern, 1943) states that in an "ideal" population, where no evolutionary mechanisms like mutations or selection exist, the allele and

genotype frequencies will remain constant through generations and is said to be in equilibrium. Deviations from the Hardy-Weinberg equilibrium (HWE) in a population can be analysed by Chi-square statistics. Since, in almost all populations, there are forces working against this equilibrium, testing for HWE in the present studies was mainly to check for large deviations in the control-populations, indicating for example genotyping-errors.

Chi-square test and Fisher's exact test

When investigating categorical data (such as the distribution of genotypes in patients and control subjects), the number of different observations, i.e. one for each of two categorical variables, can be arranged in a so called contingency table, where each cell represents a group (for example PD patients with the AA-genotype). Analysing the contingency table with a chi-square test of independence determines if a relationship exists between the two categorical variables. This is done by calculating if combinations of groups have more or less observations than would be expected if the variables were independent. If the contingency table is in 2*2 format, another test of independence, the Fisher's exact test, can be used. The significance level calculated from this test is exact, in contrast to the approximated significance level derived from the chi-square test. Fischer's exact test was used in the present thesis for analysing the distribution of alleles (except during application of the Haploview software in Paper VII), while chi-square analysis was performed for analysis of genotype distribution.

Linear regression and Logistic regression

Linear regression analysis models the relationship between an independent variable (usually continuous) and a dependent variable (continuous). In our studies linear regression was sometimes used to investigate if a SNP influenced age at onset of PD. When there is a dependent variable that is categorical and not continuous, logistic regression analysis has to be used instead of linear regression. This test examines the relationship between the dependent categorical variable and one or more independent variables (continuous or categorical). The result from a logistic regression analysis is presented as an odds ratio. An odds ratio is a relative measure of risk; in the present studies demonstrating how much more likely it is that those who posses a specific allele, genotype or genotype-combination will develop the disease compared to those who do not. Logistic regression was performed to test for

gene-gene interactions in Paper II, and to asses the possible impact of genotype, gender and home city on the risk of developing PD in Paper VII.

Kaplan-Meier analysis

In medical statistics a Kaplan-Meier analysis (or survival analysis) often presents data in the form of "time-to-death". The test generates survival curves showing the fractions still alive (or already dead) for each X-value, in two or more groups. A Kaplan-Meier analysis was performed in Paper VI, to confirm that the difference in age at onset of PD was dependent on the genotype of a SNP under investigation.

Significance-level, Power and Correction for multiple testing

The significance level, meaning the cut-off level, used for declaring differences in a statistical test is usually set to 0.05. This means that if the null hypothesis (stating that there are no differences) is true, it will be rejected incorrectly in 5% of the experiments. An incorrect rejection of the null hypothesis is called a type I error (or false positive) and this should be limited by setting an appropriate significance level. Another type of error called type II error (or false negative) occurs if the null hypothesis is not rejected, even though it is false. The probability of type II errors depends on the power of a study. The power of a genetic association study is dependent on the effect size of the studied relationship, the sample size and the significance level used.

When several SNPs are included in a study, there is often necessary to correct for multiple testing, for example by Bonferroni correction. A Bonferroni corrected p-value is generated through multiplication of the original p-value by the number of tests performed. When the analysed SNPs are in LD with each other (which they often are to a high or less extent when positioned in the same gene), Bonferroni correction is a too conservative method to use, because the SNPs are not independent of each other. In this case it might be more appropriate to use a permutation test. This method of correction for multiple testing is based on shuffling of observed data to determine how unusual an observed outcome is. More specifically it means running many tests (often 10 000) which are just like the original one, except that the relationship between the included variables is shuffled (or permuted) each time. The permuted p-value will be the fraction of permuted tests that are more significant than the original test.

RESULTS AND DISCUSSION

PAPER I: Association between the estrogen receptor beta gene and age of onset of Parkinson's disease.

Estrogen seems to be of pathophysiological importance in PD, according to retrospective studies in humans (Benedetti *et al.*, 2001; Currie *et al.*, 2004), experimental animal studies (Liu and Dluzen, 2007), and several studies reporting of PD being more common in men than in women (Wooten *et al.*, 2004). The fact that the hormone exerts its effects through ERs, and that mice missing the gene encoding the $ER\beta$ subtype develop brain abnormalities and display neurodegeneration, particularly in the substantia nigra, encourage us to investigate SNPs located in this gene in relation to PD.

The $ER\beta$ gene is positioned at chromosome 14q22-24 (Enmark *et al.*, 1997). The gene contains eight exons and the encoded protein has 485 amino acids. The $ER\beta$ SNPs most frequently analysed in genetic association studies are located in the 3'-UTR (G1730A: rs4986938), in exon 5 (A1082G: rs1256049) and in the promoter region (G/T: rs1271572) of the gene.

None of the investigated $ER\beta$ SNPs in Paper I, G1730A or A1082, were found to influence the risk for PD as judged by analysing all PD patients, but the G1730A SNP was associated with the subgroup of PD patients with early age of onset. The G-allele of the SNP was more common among patients with an early age of onset than among those with a late age at onset of PD (p=0.006). To analyse if the SNP is related to age of onset of PD a linear regression was performed. The statistical analysis just failed to reach significance (p=0.054) indicating that one can not, based on the present data, exclude such an effect. The 3'-UTR is of importance for post-transcriptional regulation by influencing mRNA stability and translational efficiency (Day and Tuite, 1998) and it can be speculated that polymorphisms located in this region might influence these processes. However, recently made functionality studies of the G1730A SNP (Putnik *et al.*, 2009) indicates that it is more likely that this SNP is in LD with another SNP, of functional importance, located elsewhere in the *ER*β gene.

So far, this paper is the only study investigating polymorphisms in the $ER\beta$ gene in PD. Support for the suggestion that SNPs in the *ER* genes might be of importance in neurodegenerative disorders came from earlier studies of these genes in Alzheimer's disease, finding an association with a repeat polymorphism in intron 5 of the $ER\beta$

gene (Forsell *et al.*, 2001) and with a combination of the G1730A SNP and a SNP in the *ERα* gene (Lambert *et al.*, 2001). Later studies including the G1730A SNP in Alzheimer's disease have, however, been contradictive (Pirskanen *et al.*, 2005; Luckhaus *et al.*, 2006).

It has been debated whether it is the ERβ or the ERα subtype that is most important for neuroprotection in the brain. Nevertheless, studies considering a possible relationship between SNPs in the *ERα* gene and PD have all failed to show a positive association (Isoe-Wada *et al.*, 1999; Maraganore *et al.*, 2002; Mattila *et al.*, 2002), except for PD with dementia (Isoe-Wada *et al.*, 1999). However, no data regarding age at onset of PD was included in these studies.

The association between an *ERβ* SNP and early age of onset of PD, seen in the present paper, is of additional interest in the view of a study showing an age related decrease in the level of expression of the *ERβ* gene in several parts of the rat brain, including substantia nigra, (Yamaguchi-Shima and Yuri, 2007). This might be one explanation as to why the gene seems to be related to PD in young but not in old patients.

PAPER II: Interaction of polymorphisms in the genes encoding interleukin-6 and estrogen receptor beta on the susceptibility to Parkinson's disease.

A huge amount of data indicates a role for inflammation in the pathophysiology of PD (see Introduction). Elevated concentrations of the inflammatory cytokine IL-6, which might stimulate the inflammation process, has been demonstrated in the brains of patients with PD (Mogi *et al.*, 1994a). In Paper II we analysed a SNP (G-174C, rs13447446) in the gene encoding IL-6, known to influence the expression level of the protein (Fishman *et al.*, 1998; Burzotta *et al.*, 2001; Hulkkonen *et al.*, 2001), for a possible association with PD. Interestingly, estrogen has been found to influence several components involved in inflammatory processes, *inter alia* inhibiting the expression of IL-6 by stimulating ER:s (Ershler and Keller, 2000). Encouraged by the findings in Paper I, we also investigated a possible interaction between the G1730A SNP in the *ERβ* gene and the *IL-6* SNP (G-174C).

The gene encoding IL-6 has been localised to chromosome 7p21 (Sehgal *et al.*, 1986) and consists of five exons and four introns, giving rise to a sequence of 212 amino acids. The G-174C SNP in the promoter region of the *IL-6* gene, has been extensively studied in relation to different disorders and longevity. In the present

paper, an association was found between the G-allele (p=0.02) or the GG- genotype (p=0.03) of the *IL-6* G-174C SNP and PD. The association was stronger when only the early age at onset group of patients was compared to the controls (p=0.009, for the GG genotype). Linear regression analysis indicated, however, that the SNP was not related to age of onset of PD (p=0.38). Combining the high-risk GG-genotype of the G-174C SNP in the *IL-6* gene with the high-risk genotype (also GG) of the $ER\beta$ G1730A SNP revealed an association with PD *per se* (p=0.001), but an even stronger association with early age of onset PD (p=0.0003) (see Figure 7). The existence of an interaction between the two genes was confirmed by logistic regression analysis, showing that the increase in risk of the GG/GG-genotype combination was higher than the additive risk of respective GG-genotype.

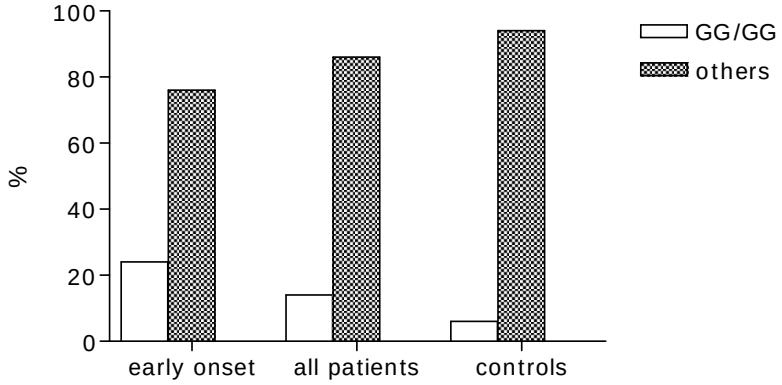

Figure 7. Frequency of high-risk genotype combination of the $ER\beta$ G1730A and *IL-6* G-174C SNPs.

Genetic association studies of the *IL-6* gene in relation to PD, generating negative results, have been performed by other research-groups (Krüger *et al.*, 2000a; Ross *et al.*, 2004; Maraganore *et al.*, 2005; Infante *et al.*, 2008). Two of the studies investigated the G-174C SNP (Ross *et al.*, 2004; Infante *et al.*, 2008), but none of them analysed the relationship with subgroups of PD patients with different age of onset.

Since the functional importance of the $ER\beta$ G1730A SNP is not yet known and different results concerning alternative genotypes of the G-174C SNP related to high expression of IL-6 are present, the effect of the combination of these SNPs is hard to interpret. However, one could speculate that a reduced ERβ-mediated IL-6 inhibition, in people already showing high levels of IL-6, might be the result of such a combination, causing even higher concentrations of IL-6 and, consequently more intense neuroinflammation.

PAPER III: Investigation of genes coding for inflammatory components in Parkinson's disease

The studies performed in Paper III were aimed at investigating thoroughly described polymorphisms in additional genes encoding factors related to inflammation, which were not previously studied in Caucasian PD-populations. In summary, none of the investigated SNPs were found to be associated with the diagnosis of PD, but the SNP located in the *IL-10* gene, was associated with the age at onset.

The *IL-10* gene maps to chromosome 1q31-32 (Kim *et al.*, 1992), and includes five exons generating a 178 amino acid sequence. Genetic studies of polymorphisms in the *IL-10* gene have been mainly focused on three SNPs in the promoter region; A-1082G (rs1800896), T-819C (rs1800871) and C-592A (rs1800872), which together represent three common haplotypes. Since it has earlier been shown that the SNP at position 1082 is associated with the expression level of IL-10 (Turner *et al.*, 1997), this polymorphism has been of major interest in genetic association studies. In the present paper the A-allele of the A-1082G SNP was related to a significantly earlier age of onset; 5 years as compared to the GG-genotype (p=0.048 after Bonferroni correction). However, several later studies including the *IL-10* A-1082G SNP, analysing age of onset of PD and/or the risk of developing the disease, have been unable to replicate this finding (Fung *et al.*, 2006; Bialecka *et al.*, 2007; Infante *et al.*, 2008; Latourelle *et al.*, 2009; Pankratz *et al.*, 2009b).

The cytokine IFN-γ is made up of a 166 amino acid sequence, built by information from the four exons of a gene mapped to chromosome 12q14 (Zimonjic *et al.*, 1995). A repeat polymorphism within intron 1 has been found to be related to the expression level of the protein (Pravica *et al.*, 1999); i.e. individuals homozygous for the allele that correspond to 12 repeats produce the highest levels of IFN-γ. The 12 repeat allele is in linkage with the T allele of a T874A (rs2430561) SNP, also located within the first intron (Pravica *et al.*, 2000), which were genotyped in the present paper. In addition we analysed a common SNP, Gln64Arg (rs9808753) at position 839 in the gene encoding the accessory factor of the IFN-γ receptor complex (IFN-γR2) located on chromosome 21q22. In contrast to the results of the present paper, the repeat polymorphism in the *IFN-γ* gene (and thereby indirectly the T874A SNP) has been associated with age at onset of PD in a Japanese population (Mizuta *et al.*, 2001). In addition, GWAS made after the publication of the present paper have investigated another SNP (rs2069718) located in intron 3 of *IFN-γ* together with the Gln64Arg SNP in *IFN-γR2*, but no associations with PD were found (Fung *et al.*, 2006; Pankratz *et al.*, 2009b).

ICAM-1 is encoded by a gene located on chromosome 19p13 (Greve *et al.*, 1989). The seven exons of this gene are translated to a protein made up by 532 amino acids. Most of the genetic association studies concerning *ICAM-1* have been focused on a common SNP in exon 6 of the gene, Lys469Arg (rs5498), which is thought to influence the binding of ICAM-1 to the LFA-1 receptor (Miklossy *et al.*, 2006). During the completion of the present paper, results from a study of this SNP in a small PD-population from Finland were published (Mattila *et al.*, 2003). The findings of this study as well as the results from several GWAS (Fung *et al.*, 2006; Pankratz *et al.*, 2009b) confirmed our negative findings.

Molecular studies have shown that differentially located forms of PAF-AH are encoded by separate genes. The gene coding for the plasma form of PAF-AH can be found at chromosome postition 6p12-21 (Stafforini *et al.*, 1996). The twelve exons included in this gene decode a protein consisting of 441 amino acids. Loss-of-function mutations in the plasma *PAF-AH* gene have been reported in Japanese individuals, and found to be associated with inflammatory diseases, such as asthma, stroke and cardiovascular diseases (Hiramoto *et al.*, 1997; Yamada and Yokota, 1997; Yamada *et al.*, 1998; Stafforini *et al.*, 1999). In the present paper we genotyped a common SNP, Val379Ala (rs1051931) located in exon 11, which have been shown to influence the substrate affinity of PAF-AH (Kruse *et al.*, 2000). This SNP almost reached statistical significance in a previous association study in schizophrenia (Bell *et al.*, 1997), but in line with the result in our paper, Val379Ala was not associated with PD in two different GWAS (Fung *et al.*, 2006; Pankratz *et al.*, 2009b).

Since none of the investigated SNPs in the present study were associated with risk of developing PD when analysed individually, we also explored potential interactions between the SNPs (results not published), but were unable to find any significant associations. In conclusion, our results do not support an influence of the investigated genes in Paper III on the risk to develop PD as juged by analysing all PD patients, but possibly an effect of the *IL-10* A-1082G SNP on age of onset. However, since inflammatory cytokines and factors act together in networks it is still possible that the genes analysed in the present paper interact with other inflammatory genes not analysed in the present thesis. In addition, our studies have only analysed the potential influence of common SNPs in these genes, we can hence not exclude that other types of variations (CNVs and rare variations) may be of importance.

PAPER IV: Cyclooxygenase-2 polymorphisms in Parkinson's disease.

Several characteristics of the COX-2 enzyme make its gene an interesting candidate gene considering PD; the enzyme is up-regulated during inflammatory processes and furthermore reactive oxygen species are generated during the COX-2-mediated conversion of PGG_2 to PGH_2. The gene encoding the 604 amino acid long protein of COX-2 is mapped to chromosome 1q25 (Kosaka *et al.*, 1994) and includes ten exons. The G926C (rs20417, also named G-765C) SNP in the promoter region has been linked to the expression level of the protein (Papafili *et al.*, 2002).

Since several common SNPs have been identified in the *COX-2* gene, the intention in study IV was to perform an association study covering more of the variation in this gene than was done for the candidate genes in the previous studies included in this thesis. It should be noted that also this study was initiated before the release of the public HapMap data base. Using an approach of allele quantification in pooled samples as a first step, it was possible to analyse very few samples for several SNPs and then continue investigating those that seemed to generate positive associations. Accordingly, we analysed pooled DNA samples for the SNPs A496G (rs689466), G3050C (rs5277) and C8473T (rs5275), while the SNP G926C (rs20417) had to be analysed separately because of its location in the DNA strand. The results from the allele quantification indicated positive results for the C843T SNP and when analysed individually it was confirmed that patients differed when compared to the control subjects with respect to the allele frequency ($p=0.01$). However, when separated into men and women the difference was only statistically significant in men ($p=0.007$).

The C843T SNP is located in the 3'-UTR of the *COX-2* gene (which is a functionally important region) characterised by multiple repeats of AU-rich sequences, common for inflammatory mediators (Caput *et al.*, 1986). This SNP has been associated with diseases in several studies, although there is a disagreement concerning which of the alleles that is harmful (Campa *et al.*, 2004; Cox *et al.*, 2004; Hu *et al.*, 2005; Sorensen *et al.*, 2005; Langsenlehner *et al.*, 2006; Park *et al.*, 2006). In the present paper we found a gender specific association between the C843T SNP and PD. Differences in the estrogen effects of have been suggested to be an underlying cause of the gender difference in the prevalence seen in PD. The hormone has been reported to reduce LPS-stimulated expression of COX-2 in microglial cells (Vegeto *et al.*, 2001; Baker *et al.*, 2004), making possible a gender specific association of polymorphisms in the *COX-2* gene with PD. However, in opposite to what one might expect, we found an association in men and not in women. This might be explained by the fact that in men who have relatively low levels of estrogen, different variants of the C843T SNP

could induce a more diverse COX-2 expression in response to estrogen, than in women. Furthermore, sex specific associations have been seen in other studies of *COX-2* polymorphisms (Szczeklik *et al.*, 2004; Ali *et al.*, 2005). With the exception of the present study, the possible involvement of *COX-2* SNPs in PD has not been extensively investigated, but three of the SNPs (including the one associated with PD in men) analysed in Paper IV, have been analysed in one GWAS, generating negative results (Fung *et al.*, 2006).

PAPER V: Investigation of genes related to familial forms of Parkinson's disease – with focus on the parkin gene

The gene named *parkin* (PRKN) is located on chromosome 6q25.2-27 (Matsumine *et al.*, 1997). The gene has 12 coding exons with a corresponding protein composed of 465 amino acids. Initially it was identified as a gene responsible for autosomal recessive juvenile parkinsonism (AR-JP) (Kitada *et al.*, 1998). The parkin mutations found in patients with AR-JP are either homozygous or compound heterozygous (two different heterozygous mutations, one on each chromosome), suggesting that loss of parkin protein function results in a diseased state. Later, *parkin* gene mutations were also found in sporadic PD, with both late- and early onset (Foroud *et al.*, 2003; Periquet *et al.*, 2003; Poorkaj *et al.*, 2004; Bertoli-Avella *et al.*, 2005; Clark *et al.*, 2006), although mainly among individuals with early onset.

Since our Parkinson population includes a relatively large number of patients with an early age of onset (defined as $\leq$50 years of age) we were interested in identifying if any of these patients (n=63) carry mutations in the *parkin* gene. This was partly because the results from association studies might be biased by having such individuals (at least if numerous) in the patient population. Since exon rearrangements are common types of mutations in the *parkin* gene, it is of importance to screen for exon dosage as a complement to conventional DNA sequencing when analysing this gene. In the present study investigation of exon rearrangements in the *parkin* gene was performed by MLPA-analysis, which also made it possible to identify variations in exon dosage of other genes related to familial PD, such as *SNCA*, *UCHL-1*, *DJ-1* and *PINK-1*. In this analysis we included 10 additional patients with reported family history of PD, but with an older age of onset, since mutations in some of the familial-PD-genes (in this case *SNCA* and *UCHL-1*) have been identified in individuals with an older age of onset than 50 years.

The identified *parkin* gene variations are summarised in Table 1 in Paper V. The two not earlier reported variations found [C938T (His279His) in exon 7 and IVS10 G+73T] are probably not able to change the protein function in a way that could contribute to PD. The allele frequencies of the identified SNPs were in line with previous studies (Kay *et al.*, 2007). No deletions or duplications of any of the investigated exons in this gene or in the other genes related to familial PD were found in any patient in the present study.

Several previously published *parkin*-studies, reporting a mutation frequency of between 18 and 49%, have been based on investigations of subpopulations of PD, such as consanguineous families, sib-pairs and early onset recessive families (Lücking *et al.*, 2000; Foroud *et al.*, 2003; Oliveira *et al.*, 2003a), thereby increasing the probability of finding mutations. Even studies investigating patient-materials selected in the same way as in the present study (Kann *et al.*, 2002; Poorkaj *et al.*, 2004), or by using "no first-degree family history of PD" as an inclusion criterion (Periquet *et al.*, 2003), found a number of *parkin* mutations. The main difference between these last mentioned studies and the present one is the relatively large number of individuals with an age of onset between 40 and 50 years of age included in the present study. This is probably the main explanation to the low mutation rate found, since it seems as if the frequency of *parkin* mutations decrease with increasing age of onset (Lücking *et al.*, 2000).

It has been discussed weather *parkin* mutations cause disease in a heterozygous state (dominant). By screening the gene in control subjects it has now became clear that heterozygous point mutations are as common in controls as in PD patients (Kay *et al.*, 2007; Pankratz *et al.*, 2009a), indicating that presence of this type of mutation should not be taken as a cause of disease, unless a second mutation is also identified. Heterozygous deletions or duplications of *parkin* exons have also been reported in control subjects (Lincoln *et al.*, 2003), but they seems to be more common in patients (Pankratz *et al.*, 2009a). In addition, PET-scan studies have demonstrated that clinically healthy individuals carrying heterozygous *parkin* deletions had significantly lower F-DOPA uptake in the striatum as compared to control subjects, possibly indicating the presence of a preclinical disease process (Hilker *et al.*, 2001).

Moreover, most of the association studies between *parkin* polymorphisms and sporadic PD have been negative (Oliveira *et al.*, 2003b; Kay *et al.*, 2007), although there are some positive results regarding a polymorphism in the promoter region (West *et al.*, 2002; Sutherland *et al.*, 2007).

PAPER VI: PITX3 polymorphism is associated with early onset Parkinson's disease

The transcription factor PITX3 consists of 302 amino acids and its gene, which contains 4 exons, maps to chromosome 10q25 (Semina *et al.*, 1998). The fact that Pitx3 is exclusively expressed in midbrain dopaminergic neurons (Smidt *et al.*, 1997) and that it seems to be of importance for the development and maintenance of these neurons, especially in the substantia nigra (Smidt *et al.*, 2004b), makes it a suitable candidate for genetic studies in PD.

The studies in Paper VI were undertaken in order to investigate if genetic variation in the *PITX3* gene could be associated with PD. Three SNPs were genotyped; one detected in a relatively large number of patients during prior sequencing (rs2281983) (which was reported in the NCBI dbSNP data base, but not genotyped in the HapMap project), one selected by Hapmap tagging (rs4919621) and one (rs3758549) located in the promoter region and found to be associated with PD in a previously published study by Fuchs *et al*. (2009). In the present study none of the analysed SNPs were found to be associated with risk of developing PD as judged by analysing all PD patients. However, both the A-allele of the rs4919621 SNP and, as a consequence of a nearly complete LD (r^2=0.96), the C-allele of the rs2281983 SNP were found to be related to the subgroup of PD patients with early age of onset (p=0.002 and p=0.004, respectively). Furthermore, when analyzing the date with linear regression the rs4919621 SNP was significantly related to age of onset (p = 0.005).

Interestingly, the finding in the present study was replicated in a recently published study by Le and co-workers, who found the same SNPs (rs4919621 and rs2281983) to be associated with PD, but particularly with early onset PD (Le *et al.*, 2009). Furthermore, the rs2281983 SNP was also analysed in the study by Fuchs and colleagues, and found to be associated with PD in one of the two studied PD populations and a tendency in the same direction was seen in the other (Fuchs *et al.*, 2009).

The previously reported association between the rs3758549 SNP and PD (Fuchs *et al.*, 2009), could not be confirmed in the present study. However, this association was supported in a recent published paper, but surprisingly with the opposite allele (Haubenberger *et al.*, 2009). The authors speculate that this contradictory finding might be explained by an overdominant effect model, where heterozygous individuals are at advantage compared to homozygous individuals of both types. The

associations reported in the present paper were not observed in the study by Haubenberger and co-workers. Moreover, results concerning the above mentioned *PITX3* SNPs can not be found in public available data from any GWAS focusing on PD.

An influence of variations in the *PITX3* gene on PD onset is further supported by the results of an earlier performed linkage study, showing an association with the genetic region in which *PITX3* is located and age at onset of PD (Li *et al.*, 2002). An explanation for the present findings could be that *PITX3* polymorphisms influence the density of dopaminergic neurons. Approximately 50-70% of the dopaminergic neurons in the substantia nigra must have degenerated before the occurrence of any PD symptoms. It is possible that PD patients displaying a relatively low number of dopamine cells after birth have a lower threshold and therefore more rapidly reach the phase where the degeneration-induced dopamine deficiency causes symptoms.

So far, no SNPs causing amino acid shifts have been reported in the *PITX3* gene. However, as emphasized earlier (see Introduction), also SNPs located in gene parts other then exons, like those in the present study, may influence the regulation of protein expression.

PAPER VII: Do polymorphisms in transcription factors LMX1A and LMX1B influence the risk for Parkinson's disease?

The finding of a possible influence of the dopaminergic transcription factor PITX3 on the age of onset of PD encouraged us to investigate genetic polymorphism in additional transcription factors involved in the development of dopamine neurons.

The 382 amino acid long transcription factor LMX1A is made by information from the eight coding exons of a gene located at chromosome 1q22-23 (German *et al.*, 1994). The gene encoding its analogue LMX1B maps to chromosome 9q34 (Iannotti *et al.*, 1997) and includes eight exons generating a protein composed of 379 amino acids. Although not specifically expressed in dopaminergic neurons (as for Pitx3) results from studies of knockout animals (Smidt *et al.*, 2000; Ono *et al.*, 2007) and overexpression in embryonic stem cells (Lmx1a) (Friling *et al.*, 2009) indicate that both factors are of importance for the development of dopamine cells.

Initially, 46 SNPs in the *LMX1A* gene and 22 SNPs in the *LMX1B* gene were tested. Due to genotyping problems, several SNPs had to be excluded before the statistical

analyses and consequently 33 SNPs in *LMX1A* and 11 SNPs in *LMX1B* could be included in the association tests. Positive associations were found for 3 SNPs in the *LMX1A* gene (rs4657411, rs4657412, rs6668493) and 1 SNP (rs10987386) in the *LMX1B* gene, with respect to PD risk. After splitting for gender some SNPs were associated with PD in women but not in men (*LMX1A*: rs4657411, rs6668493, rs12136019, rs10753668 and *LMX1B*: rs4836551, rs10448285), while the opposite was true for other SNPs (*LMX1A*: rs1123821, rs11809911 and *LMX1B*: rs12555176, rs10987386). Furthermore, the associated SNPs were found to interfere with several predicted binding sites for other transcription factors expressed in the human brain. However, after correction for multiple testing using permutation (10.000 permutations), none of the associations remained significant. In addition, no associations between the investigated SNPs and early age at onset of PD were seen.

To our knowledge, this is the only association study performed of SNPs in the *LMX1A* gene. The T-allele of the rs10987386 SNP in *LMX1B* has been reported to be associated with PD in an earlier study (Fuchs *et al.*, 2009). However, since the authors were unable to assess this SNP in an independent population they refrained from reporting the association as a major finding. In GWAS, no SNPs in *LMX1A* or *LMX1B* have been associated with PD, but only two of the SNPs significantly associated before correction for multiple testing in the present study were included in those studies (Maraganore *et al.*, 2005; Fung *et al.*, 2006; Pankratz *et al.*, 2009b). Yet, in the GWAS made by Maraganore and collegues (Maraganore *et al.*, 2005), three out of six studied SNPs in the *LMX1B* gene showed a p-value of 0.01 or less.

Needless to say, the present findings have to be evaluated with caution, since after correction for multiple testing none of the associations remained significant and several of them have not yet been investigated in other PD-populations. However, like the transcription factor PITX3, LMX1A and LMX1B are of importance for the development of dopaminergic neurons, possibly making polymorphisms in their genes able to modify the density of these neurons in adult brain. The gender differences seen might be explained by interactions between LMX1A/B and estrogen or gender-specific transcription factors.

GENERAL DISCUSSION

Data obtained in twin studies do not give much support for a genetic influence on the pathophysiology of sporadic PD. However, it is important to realise that twin studies are underpowered to exclude a genetic influence in a not fully penetrant homogenous trait. The risk alleles often detected in PD, could reflect gene-by-environment interactions, and such genetic influence, could be of importance for the risk to be affected by sporadic PD, but still escaping detection by a twin study. It has also been shown in epidemiological studies that patients with sporadic PD often have relatives with the disease, although not always of first degree (Marder *et al.*, 1996; Sveinbjornsdottir *et al.*, 2000). Furthermore, the age of onset, symptoms and end-stage pathology of PD might be quite variable, even within families that are known to be affected by monogenic PD, supported by the above mentioned (see Introduction) possibility of the presence of a subclinical disease in the non-diseased twin, as indicated by PET-scan studies (Piccini *et al.*, 1999).

Most of the findings in the present thesis indicate that the gene of interest influences age of onset of PD, but not the risk for PD as juged by analysing all PD patients. Although impossible to exactly interpret from our data, the relationship between the risk alle frequency and age of onset was often seen to be linear (*PITX3*, *IL-10* and a trend for $ER\beta$), implying that the gene variants modify the age of onset over the whole age interval, indcating that the investigated SNPs will fit in somewhere between model C and D (for $ER\beta$ model B might be more correct), according to Figure 2 (see Introduction). Model D implies that the SNP influences age of onset and also the risk for PD. The reason that we failed to obtain any association with PD risk for the SNPs in *PITX3* and *IL-10* when analysing all PD patients could be that the power of our studies was insufficient.

The distinction between gene variants influencing the risk to be affected by PD and variants that modify age of onset might be biologically significant; risk variants point to the initial cause, whereas onset modifiers implicate the process that begins after the initial insult and leads to the threshold for development of clinical signs (Payami *et al.*, 2010). However, studies showing the existence of Lewy Bodies in brains of neurologically healthy individuals without clinical PD (Schneider *et al.*, 2007) indicate that a significant proportion of, if not all, humans theoretically might get PD if they grow old enough. If this is true, it is only reasonable to, at least theoretically, speak about gene variants that modifies the age of onset. On the other hand there are data demonstrating that age-related changes in the human brain really differ from those

seen in the PD brain. For example, the regional and sub-regional pattern of striatal dopamine loss in normal aging is not the same as observed in sporadic PD (Kish *et al.*, 1992), indicating distinct pathophysiological mechanisms in PD, dissimilar from what is seen during ordinary aging. It is important to realize, however, that from an individual's perspective, the distinction between gene variants influencing the risk to get PD and influencing the age of onset might be academic; both variants modify the risk to ever get PD symptoms.

It is also possible that in PD patients with an early age of onset there are subgroups of PD, with different pathophysiology as compared to later age of onset PD. Such a notion is supported by findings that genetic factors seems to be more important in this patient group, as indicated in the twin study by Tanner and collegues (1999), who reported a difference in concordance rate among monozygotic twins as compared to dizygotic twins, diagnosed with PD before 51 years of age, but no difference in concordance rate among older twin pairs with PD. Additional support comes from the observation that the incidence and prevalence of PD after the age of 50 increase almost exponentially in contrast to early onset PD (Tanner and Goldman, 1996), which is the basis for using this age as a cut-off while defining early age of onset PD.

There is a huge amount of data indicating that inflammation is present in affected brain parts and that it might be of significance for the pathophysiology of PD, but when or how the inflammatory processes starts are uncertain. Both theories claiming that inflammation is of primary importance and starts prior to the dopaminergic cell degeneration and that inflammation is a secondary process that begins after the cell degeneration is initiated have been put forward. The latter propose that when the dopaminergic cells start to degenerate (under the influence of for example oxidative stress and/or a toxins) surrounding microglia are activated and express inflammatory factors, which in turn activate additional microglial cells and so forth, resulting in an inflammatory process that augments the degenerative process. However, it is also possible that an hitherto unknown initiating factor, like viral or bacterial infections or repeated head trauma (Tansey and Goldberg, 2009), triggers a self-perpetuating cycle of chronic neuroinflammation, which promotes clustering of activated microglia, which starts the cell degeneration and secondary induce oxidative stress/protein aggregation as a result of impaired cell function. In the present thesis we found and association between a polymorphism in the *IL-6* gene and PD in the whole patient group. A polymorphism in the *IL-10* gene seems to be more related to the age at onset of the disease, which might be more plausible, since polymorphisms in these genes may influence the expression of the mediators, which in turn affect the

intensity of the inflammatory process and thereby the rate of cell degeneration, rather than influencing weather there will be an inflammation or not.

Estrogen has been suggested to exert cell protective effects in PD. Exactly how this protection might be exerted is not clear, but the hormone can potentially affect several different processes which all could influence the viability of dopaminergic neurons. Since the possible involvement of inflammation in PD pathophysiology is highlighted in this thesis, the previously suggested anti-inflammatory effect of estrogen is of particular interest and lends support by the present result pointing at an interaction between SNPs in the genes encoding ERβ and IL-6. In addition, estrogen seems to be of importance during the development of the dopaminergic neurons. A polymorphism in a receptor, through which the hormone exerts its effects, might modulate early developmental effects of estrogen on dopaminergic neurons, and thereby the number or function of this neurons.

A SNP in the gene *PITX3* was associated with age at onset of PD. Since this transcription factor is of fundamental and exclusive importance for the DA neuron development, it is reasonable to assume that genetic variations in this gene affect the density or function of the neurons. The notion that the dopamine systems are somewhat altered already at an early age in individuals who later will develop PD, might be supported by studies showing less intake of coffee and nicotine among these persons as compared to control subjects (Ross and Petrovitch, 2001), possibly explained by a lower preference for these substances, due to less dopaminergic neurons in reward neuronal networks.

Several of the recessively inherited forms of PD in which the gene causing the disease is identified show a relatively early age of onset. These individuals are sometimes hard to epidemiologically and symptomatologically distinguish from sporadic PD patients with an early onset age, as they might have healthy parents and therefore creating the assumption that the disorder is sporadic in nature. Including individuals with masked genetic forms of PD in a material intended to use for investigating risk genes in sporadic PD might of course be a source of bias, something that we have tried to avoid in the present studies through testing of several of the mutations known to cause PD.

There are, however, also other problems while trying to collect a well-defined group of PD patients to use in genetic studies, such as the above mentioned observation that Lewy Bodies, a hallmark for PD, also exist among healthy individuals, suggesting

that there is a substantial burden of subclinical disease in the aging population, making it hard to classify individuals as patients or control subjects. In addition, other factors disturbing the homogeneity of populations used in association studies, which can limit the power to detect genetic associations, are ethnical heterogeneity and non-random sampling (maybe most relevant for control individuals). Furthermore, to small sample sizes and genotyping errors are troublesome drawbacks. Altogether, these factors might be some explanations for the difficulty of replicating findings in genetic association studies.

Each discovery of a gene mutation causing a rare inherited form of PD has opened a new window into the pathogenesis of the disease. Together, the genetic findings have implicated a limited number of convergent protein pathways and these insights provide the rationale for new therapeutic approaches. Despite, as mentioned above, that there are some problems to overcome while trying to identify genetic risk variants influencing sporadic PD, studying possible candidate genes are of importance since the results from such studies may indicate pathophysiologic mechanism/mechanisms that are of importance in this, more common, form of the disease.

In conclusion, the most important finding in the present thesis is the association between early onset PD and a SNP in *PITX3*, since it has been replicated in another population. Furthermore, associations with other SNPs in this gene and PD have also been found by other investigators. Another intriguing finding are the interaction between SNPs in *IL-6* and $ER\beta$ regarding the risk of PD, and early onset PD in particular, although further association studies of these genes are needed to confirm the result. In addition the possible involvement of a SNP in *LMX1B*, found to be associated with PD before correction for multiple testing in the present thesis, which were associated with PD in a previous study needs to be clarified. On the other hand, the associated SNPs in the genes *IL-10* and *COX-2* might not be as important; since the findings have either showed a gender-specific association that is somewhat hard to interpret (*COX-2*) or have not been replicated in other studies (*IL-10*).

Although we did not present clear evidence of associations between the gene SNPs studied in this thesis and PD diagnosis, at least not in patients with late age of onset, the selected genes may still be interesting candidate genes for the disease. In addition to the polymorphisms presented to be associated with early onset PD and/or age at onset of PD, the investigated genes may harbour other variations; *i.e.* CNVs and rare mutations, associated with PD. Although some efforts have been made trying to

study interactions between genes and environmental factors (McCulloch *et al.*, 2008), further studies, including for example the genes studied in the present thesis, are warranted.

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SUMMARY IN SWEDISH / SVENSK SAMMANFATTNING

Parkinsons sjukdom är den näst vanligaste så kallade neurodegenerativa sjukdomen i världen och man vet idag att ärftliga faktorer kan ha betydelse för uppkomsten. För vissa patienter, som har familjära former av sjukdomen, är det enbart genetik, dvs. någon form av mutation, som ligger bakom sjukdomen. I andra fall, de som brukar kallas sporadiska, är det troligtvis en genetisk sårbarhet tillsammans med olika omgivningsfaktorer som orsakar sjukdomen. För att försöka fånga några genetiska faktorer som kan ligga bakom den genetiska sårbarheten, har vi i denna avhandling studerat polymorfismer (vanligt förekommande mutationer) i s.k. kandidatgener som valts p.g.a. sin eventuella koppling till sjukdomens patofysiologi. Frekvensen av dessa polymorfismer har jämförts mellan en patientpopulation med sporadisk Parkinsons sjukdom och en kontrollpopulation.

Målsättningen i avhandlingen har varit att undersöka om en polymorfism är vanligare hos personer med Parkinsons sjukdom än hos individer som ej har sjukdomen, eller om den är relaterad till andra faktorer hos patienterna såsom t.ex. insjuknandeålder. De polymorfismer som studerats är s k "single nucleotide polymorphisms" (SNP), vilka består av förändringar i enskilda baspar i vårt DNA. De SNPar som vi undersökt är belägna i gener involverade i neuroinflammation eller gener som styr utveckling av dopamin-nervceller från omogna stamceller, två processer som tros kunna vara av betydelse för utvecklingen av Parkinsons sjukdom, samt en gen som är viktig för hormonet östrogen, vars effekter delvis påverkar de ovan nämnda processerna.

Dessutom var vi intresserade av att kontrollera om någon av patienterna i vårt material bar på mutationer i Parkin-genen, en gen kopplad till recessivt nedärvd familjär Parkinsons sjukdom. Just recessivt nedärvda former av sjukdomen kan vara svåra att skilja från sporadiska fall, speciellt när det gäller en sjukdom som är åldersrelaterad.

Huvudfynden i avhandlingen var följande:

- En SNP (A1730G) i genen som kodar för tillverkningen av proteinet östrogen-receptor beta (ERβ) var associerad till Parkinsons sjukdom bland patienter med tidig debutålder.
- Den ovan nämnda SNPen i *ERβ*-genen verkar interagera med en SNP (G-174C) i interleukin-6-genen och bidra till en ökad risk för sjukdomen om

man bär på högrisk-varianterna (GG/GG) av båda SNParna. Detta var allra tydligast bland de yngre patienterna.

- En SNP (G1082A) i genen för den anti-inflammatoriska cytokinen interleukin-10 var kopplad till debutåldern av sjukdomen.
- En SNP (C8473T) i genen för COX-2 var associerad till sjukdomen, men enbart hos män.
- En association påvisades mellan en SNP i genen för transkriptionsfaktorn PITX3 och debutåldern för Parkinsons sjukdom, vilket delvis bekräftade fynd gjorda av andra forskargrupper.
- I en storskalig studie av genetisk variation (i form av SNPar) i generna för två dopaminerga transkriptionsfaktorer (faktorer viktiga för utvecklingen av dopamin-nervceller), visade sig tre SNPar i *LMX1A*-genen och en SNP i *LMX1B*-genen vara associerade med sjukdomen, men efter korrigering för multipla test försvann dessa associationer.
- Ytterst få av patienterna i vår studie bar på mutationer i Parkin-genen och dessa tros inte vara direkt sjukdomsframkallande.

Resultaten från studierna i denna avhandling visar att SNPar i flera av de studerade generna skulle kunna påverka risken att få sporadisk Parkinsons sjukdom, men inget kan säkerställas förrän fynden har replikerats (upprepats) av andra kommande studier. I flera fall finner vi en koppling till insjuknandeålder snarare än sjukdomsrisken i sig, vilket kan vara ett tecken antingen på att genetiken är viktigare bland unga patienter eller att just de gener vi tittat på modifierar sjukdomsdebutåldern.

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