On the influence of dopamine-related genetic variation on dopamine-related disorders

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2009

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Printed by Intellecta Infolog AB, Gothenburg

ISBN 978-91-628-7938-9

http://hdl.handle.net/2077/21077

ABSTRACT

Rationale Dopamine synthesizing neurons are involved in a wide variety of functions. The most prominent dopamine pathways originate in the midbrain. The development, function and survival of these dopaminergic neurons are under the influence of numerous transcription and neurotrophic factors. Subtle differences in the genes encoding these factors may be of importance for several psychiatric and neurodegenerative disorders. *LMX1A*, *LMX1B* and *PITX3* are transcription factors that are essential for the development, specification and survival of midbrain dopaminergic neurons. *BDNF* is a neurotrophic factor involved in neurodevelopmental processes including differentiation and survival of dopaminergic neurons. Another protein of importance for dopaminergic neurotransmission is the dopamine transporter (DAT) that mediates reuptake and inactivation of extracellular dopamine and is hence of fundamental importance in regulating dopamine transmission. The specific aim of this thesis was to investigate the possible influence of polymorphisms in these dopamine-related genes on dopamine-related disorders, *i.e.* Parkinson's disease (PD), attention-deficit/hyperactivity disorder (ADHD), social anxiety disorder (SAD) and schizophrenia. **Observations** Three single nucleotide polymorphisms (SNPs) in *LMX1A* and one in *LMX1B* were associated with PD. After splitting for gender, six SNPs were associated with PD in women and four in men (Paper I). Two SNPs in *PITX3* were associated with PD in patients with an early age of onset when compared either to controls or to PD patients with late onset (Paper II). One of the *PITX3* polymorphisms was also associated with schizophrenia, as were two polymorphisms in *LMX1A*, and one SNP in *LMX1B* (Paper III). We assessed longitudinal, quantitative phenotypes of hyperactivity-impulsivity and inattention, and found that the Met allele of the Val66Met polymorphism in the BDNF gene was associated with increased persistent hyperactivity-impulsivity symptoms as well as with increased age-specific inattention symptoms (Paper IV). The amygdala, essential for detection of biologically relevant stimuli and fear generation, is under excitatory influence of dopamine. Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) were used to investigate if a variable number of tandem repeat (VNTR) polymorphism in the DAT gene (*SLC6A3*) influences amygdala function during processing of aversive emotional stimuli in SAD patients and healthy controls, respectively. The 9-repeat allele was associated with significantly increased amygdala activity, as assessed with PET, across tests (*i.e.* public speaking, processing of angry and neutral faces) in SAD patients, but with decreased amygdala activity in controls. Moreover, 9-repeat carriers, regardless of diagnosis, displayed augmented amygdala reactivity, *i.e.* a greater activation, of the left amygdala in response to angry compared to neutral faces. Blood oxygen level-dependent (BOLD) fMRI was used to assess healthy volunteers, and in line with the results from the PET study, 9-repeat carriers displayed higher reactivity of the left amygdala in response to angry faces, compared to neutral geometric shapes (Paper V). **Conclusions** All of the studies were based on *a priori* hypotheses regarding the possible relationship between the genes and the disorders under investigation. Some of the associations reported in this thesis have not been described earlier, others have been confirmed in independent samples, whereas in some cases, earlier studies have been inconclusive. In summary, our results support the notion that variation in dopamine-related genes is of importance for dopamine-related disorders and amygdala function.

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. *PITX3* polymorphism is associated with early onset Parkinson's disease. **Olle Bergman**, Anna Håkansson, Lars Westberg, Kajsa Nordenström, Andrea Carmine Belin, Olof Sydow, Lars Olson, Björn Holmberg, Elias Eriksson and Hans Nissbrandt. *Neurobiology of Aging (2008) Apr 16 (Epub. ahead of print).*

II. Do polymorphisms in transcription factors *LMX1A* and *LMX1B* influence the risk for Parkinson's disease? **Olle Bergman**, Anna Håkansson, Lars Westberg, Andrea Carmine Belin, Olof Sydow, Lars Olson, Björn Holmberg, Laura Fratiglioni, Lars Bäckman, Elias Eriksson, Hans Nissbrandt. *Journal of Neural Transmission (2009) 116:333–338.*

III. Polymorphisms in dopamine-related transcription factors *LMX1A*, *LMX1B* and *PITX3* are associated with schizophrenia. **Olle Bergman**, Lars Westberg, Lars-Göran Nilsson, Rolf Adolfsson and Elias Eriksson. Preliminary manuscript.

IV. Association of brain-derived neurotrophic factor polymorphism with the developmental course of attention-deficit/hyperactivity disorder. **Olle Bergman**, Lars Westberg, Paul Lichtenstein, Elias Eriksson and Henrik Larsson. Submitted manuscript.

V. Amygdala function is associated with a dopamine transporter gene polymorphism in patients with social anxiety disorder and healthy controls. **Olle Bergman**, Fredrik Åhs, Tomas Furmark, Lieuwe Appel, Clas Linnman, Vanda Faria, Stephen B. Manuck, Robert E. Ferrell, Ahmad Hariri, Susanne Henningsson, Mats Fredrikson, Elias Eriksson, and Lars Westberg. Submitted manuscript.

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LIST OF ABBREVIATIONS

CNS	Central nervous system
mdDA	Mesodiencephalic dopaminergic
VTA	Ventral tegmental area
SNc	Substantia nigra pars compacta
RRF	Retrorubral field
FGF2/8	Fibroblast growth factor 2/8
Shh	Sonic hedgehog homolog
Otx2	Orthodenticle homeobox 2
Gbx2	Gastrulation brain homeobox 2
Lmx1a/b	LIM homeobox transcription factor 1 alpha/beta
Pitx3	Paired-like homeodomain 3
En1/2	Engrailed homeobox 1/2
Pax2/5	Paired box 2/5
Foxa1/2	Forkhead box A1/2
Tgfα/β	Transforming growth factor alpha/beta
Nurr1 (NR4A2)	Nuclear receptor subfamily 4, group A, member 2
PD	Parkinson's disease
SAD	Social anxiety disorder
ADHD	Attention-deficit/hyperactivity disorder
Msx1	Msh homeobox 1
Nkx6.1	NK6 homeobox 1
PET	Positron emission tomography
fMRI	Functional magnetic resonance imaging
rCBF	Regional cerebral blood flow
GABA	Gamma-Aminobutyric acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
COMT	Catechol-O-methyltransferase
5-HTTLPR (SLC6A4)	Serotonin transporter gene
SLC6A3 (DAT1)	Dopamine transporter gene
ADRB1	β_1-adrenergic receptor gene
DAO	D-amino-acid oxidase
DRD1/2/4	Dopamine receptor D1/2/4
NRG1	Neuregulin 1
AKT1	v-akt murine thymoma viral oncogene homolog 1
BDNF	Brain-derived neurotrophic factor
DISC1	Disrupted in schizophrenia 1
DTNBP1	Dystrobrevin binding protein 1
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
LD	Linkage disequilibrium
GWAS	Genome-wide association study
VNTR	Variable number of tandem repeat
SNP	Single nucleotide polymorphism

INTRODUCTORY COMMENT

"How extremely stupid not to have thought of that!" was Thomas Henry Huxley's response to Charles Darwin's theory of natural selection (1838). In hindsight the idea of natural selection and its requirements seem obvious; more individuals are born than can survive and reproduce, these individuals vary in their ability to survive and reproduce and this variety is partly heritable. Importantly, this variety also allows for traits that under certain conditions can become deleterious and cause disease.

Whereas the disorders investigated in this thesis are very different from one another, all have a large genetic component and are assumed to be associated with the neurotransmitter dopamine. Dopamine is involved in a variety of brain functions, including cognition, locomotor activity and emotion (Vallone *et al*, 2000). Because of its importance to the pathology of several brain disorders such as schizophrenia, attention-deficit/hyperactivity disorder (ADHD) and Parkinson's disease (PD), dopamine has been a major target of research ever since its function as a neurotransmitter was discovered by Arvid Carlsson and co-workers in the late 1950s (Carlsson and Waldeck, 1958; Iversen and Iversen, 2007).

Since the first human genome sequence was published in 2001 (Lander *et al*, 2001; Venter *et al*, 2001), efforts to link human genetic variation with commonly occurring disorders have been intensified. We all carry two versions of each chromosome and consequently have two versions of most genes (with the exception of genes on sex chromosomes). These two versions (alleles) are almost identical, but the devil is in the details; subtle genetic differences are the foundation of all psychiatric and neurodegenerative disorders.

Findings from twin studies suggest that genetic factors play an important role in the pathogenesis of three of the disorders investigated in this thesis (schizophrenia, social anxiety disorder (SAD) and ADHD), and some importance also for the fourth (PD). Unfortunately, nature is not bound to our diagnostic nomenclature, and the genetic heterogeneity of these disorders is substantial. Moreover, very few of these genetic factors result in changes in protein structure and function. More often, aspects that have relatively subtle biological effects, like gene regulation, may be implicated. Importantly, whereas patients do not inherit an illness *per se*, they may inherit variants leading to an altered brain development, which, in combination with environmental factors, may increase the risk of developing a disorder later in life. Consequently, these disorders are genetically complex and identifying robust susceptibility genes have so far proven to be very difficult. Nevertheless, identifying the underlying genetic components of these disorders will be necessary to fully understand their pathophysiology, and will hopefully lead to the development of more rational treatments than those available today.

The aim of this thesis has been to investigate how and if dopamine-related genetic variants influence the susceptibility to schizophrenia, PD, symptoms of ADHD and amygdala function in SAD patients and healthy controls, respectively.

GENETICS AND HERITABILITY

DNA, RNA and proteins

The human genome is made up of deoxyribonucleic acid (DNA), consisting of a sugar called deoxyribose, phospate groups and four different nucleotide bases: adenine (A), cytosine (C), guanine (G) and thymine (T). A and G are purines while C and T are pyrimidines. The DNA components are connected to each other with strong covalent bonds, forming a long single strand with two ends, called 5′ and 3′ (Lehman, 1974). Weaker hydrogen bonds pair A with T and C with G, connecting two single strands to form a double helix of DNA (Watson and Crick, 1953). There are normally 46 DNA molecules, called chromosomes, consisting of 22 homologous pairs of autosomes and one pair of sex chromosomes, in the cell nucleus under normal cell conditions. One chromosome in each pair is from the mother and the other from the father. Double stranded DNA is cleaved prior to replication and a complementary strand is created for each strand of DNA. Exons are parts of the DNA strand that encode genes. These are normally separated by introns, which are non-coding sequences. Regulatory sequences called promoter regions are normally located upstream of any given gene. Promoter regions contain special nucleotide sequences (*i.e.* motifs) that are recognized by a group of proteins called transcription factors. These proteins bind to promoter regions and participate in the control of gene expression. Whereas it has previously been assumed that promoter regions are usually located upstream from the 5' end of a gene, mapping studies found that in fact only 22% of transcription factor binding sites were located upstream of the 5' ends, whereas 36% actually lied within gene boundaries, often in noncoding areas (Cawley *et al*, 2004; Martone *et al*, 2003). The 3′ untranslated region (3′ UTR) is located downstream of a gene and contains regions which influence RNA stability and translation (Mill *et al*, 2002; Miller and Madras, 2002), as well as sites that are partially complementary to noncoding microRNAs. Different cell types express distinct combinations of microRNAs, which may regulate cell-specific target genes (Krek *et al*, 2005; Lai, 2002). The haploid (*i.e.* one copy of each chromosome) human genome consists of about 3.3 billion base pairs and harbours an estimated 20.000 to 30.000 protein-coding genes (2004; Stein, 2004). In fact only 1.1% of the genome is believed to consist of exons, whereas 24% is intronic, the remaining 75% is intergenic DNA (Lander *et al*, 2001; Venter *et al*, 2001). The parts of the genome encoding genes are transcribed to complementary RNA that subsequently becomes mRNA after non-translated sequences have been removed in a process called post-transcriptional processing. Mature mRNA contains so-called codons (*i.e.* nucleotide trios) that are translated into amino acids, which combine to form proteins (Burton *et al*, 2005).

Classes of genetic variation

Small individual differences in the genome are the foundation of all common hereditary disorders, including the psychiatric and neurodegenerative ones. Several different forms of genetic variation have been identified; the most common are the single nucleotide polymorphisms (SNPs), which represent variation in a single nucleotide. A SNP can be defined as a locus (unique chromosomal location) in the genome at which two (or sometimes more) nucleotide bases can occur, all at a frequency of 1% or more.

Since we have two versions of each autosomal chromosome and consequently two versions of every gene, a polymorphic locus with two potential alleles (*A* and *a*) has three

possible genotypes: two homozygous (*AA* and *aa*) and one heterozygous (*Aa*). The Hardy-Weinberg principle states that both allele and genotype frequencies in a population are in equilibrium, which can be calculated with the equation $p^2+2pq+q^2=1$, where p and q indicate frequency of allele *A* and *a*, respectively. However, this may not be true if the studied population is influenced by factors like selection, limited population size, random genetic drift, non-random mating, mutations etc. Since one or more of these factors are always present in real life, Hardy-Weinberg equilibrium simply provides a baseline against which genetic variation can be measured.

It has been estimated that the human genome contains more than ten million SNPs, about seven million of these occurring with a minor allele frequency (MAF) of over 5% (2003; Kruglyak and Nickerson, 2001). In addition to these common SNPs there are an inestimable number of rare single nucleotide mutations, in some cases occurring in only one family or individual. However, the greater part of the genetic variation that occurs between any two individuals is located at positions with variants that are common in the population as a whole (Frazer *et al*, 2009).

SNPs have until recently been thought to affect disease susceptibility mainly by altering the DNA sequence in an exon so that the amino-acid coding is changed. So-called non-synonymous SNPs give rise to novel codons that specifies an alternative amino-acid or changes the code for an amino-acid to that for a stop signal. Most susceptibility SNPs are however noncoding, and while some may function as markers for nonsynonymous variants, others are likely to alter the expression of a gene by themselves. Numerous studies have shown that promoter polymorphisms can influence transcriptional activity of a gene (Caspi *et al*, 2003; Greenwood and Kelsoe, 2003; Laws *et al*, 2002; Wilson *et al*, 1997). Similarly, intronic SNPs can also affect transcription, or alter splicing or mRNA stability, and hence change the relative quantity and proportions of isoforms (Greenwood *et al*, 2003; Tokuhiro *et al*, 2003; von Ahsen and Oellerich, 2004). In addition, SNPs in the 3' UTR have also been shown to increase the susceptibility of certain disorders (Ueda *et al*, 2003) by altering mRNA stability or by being located in motifs for microRNAs that inhibit translation of a gene (Mill *et al*, 2002; Miller *et al*, 2002). Synonymous (conservative) exonic SNPs alter DNA sequence but do not change the amino-acid sequence. Synonymous SNPs are usually considered functionless, but some are under natural selection, indicating that they may cause disease, most likely by altering mRNA structure and translation (Chamary *et al*, 2006; Duan *et al*, 2003; Kimchi-Sarfaty *et al*, 2007; Komar, 2007; Shen *et al*, 1999).

Other forms of polymorphisms include repeats. Microsatellite repeat polymorphisms include mono- di-, tri-, tetra-, and pentanucleotide tandem repeats that are dispersed in most chromosomes. There are on average one dinucleotide repeat occurring on every 30.000 bases in the human genome (Stallings *et al*, 1991). Minisatellite repeats are also known as variable number tandem repeats (VNTRs). They contain a repeating unit that is usually around 30 to 50 bp in length with a conserved core sequence of 10 to 15 bp, and occur at around 1000 sites in the genome (Doggett, 2001).

The parts of the human genome sequence that do not encode genes sometimes contain transposons (also called insertion sequences), believed to be the remains of intracellular parasites from our evolutionary history. These elements are excised from one site in the human genome sequence and integrated into another site, usually located less than 100

kb from the original site (Muotri *et al*, 2007). Retrotransposons constitute another form of genetic variation in humans, which are similar to retroviruses, and are elements that have been transcribed into RNA, reverse transcribed and subsequently reintegrated into the genome, thus duplicating the element (Kazazian, 2004). Genetic structural variation, including large (more than 1 kilobase pair (kbp)) insertions, deletions, inversion of sequences, block substitutions and duplications (also called copy-number variations) are common in the human genome (Feuk *et al*, 2006; Kidd *et al*, 2008). When the human genome sequence was published in 2001, it was thought that genetic differences accounted for approximately 0.1% of the sequence and consisted mostly of SNPs (Lander *et al*, 2001; Venter *et al*, 2001). Structural variation is today believed to encompass around 1% of any given persons genome and have received increasing interest in later years after it has become increasingly clear that complex disorders cannot be easily explained by SNP variation alone (Frazer *et al*, 2009; Kidd *et al*, 2008). As a result, appropriate methods of detecting structural variants and their association with complex traits have started to appear (Conrad *et al*, 2006; Conrad *et al*, 2009; Hastings *et al*, 2009; McCarroll and Altshuler, 2007; McCarroll *et al*, 2008; Redon *et al*, 2006). These studies indicate that structural variants accounts for approximately 20% of genetic variation in humans, hence underlining their importance in future studies of human disease.

Importantly, altered expression of a gene does not necessarily occur because of genetic variants in that specific gene. Susceptibility genes may encode proteins that influence transcriptional pathways, which in turn may lead to compensatory or secondary changes. Finally, the relationship between genotype and disorder can be complicated by epigenetic factors, which are heritable factors that do not cause sequence variation, (*e.g.* alterations in DNA methylation and chromatin structure) (Jaenisch and Bird, 2003; Robertson and Wolffe, 2000).

Paper I, II and III address synonymous SNPs in transcription factor genes, Paper IV addresses a non-synonymous SNP in BDNF, and Paper V addresses a repeat polymorphism in the SLC6A3 gene.

Crossover and recombination

During meiosis cells divide into four gametes, each containing a haploid genome. These gametes may fuse with another gamete in sexually reproducing animals. In the course of this gamete formation, the gamete receives a combination of the two homologous chromosomes. Crossover connections during meiotic division create a possibility for the paternal chromosomes to exchange genetic material at crossover sites called chiasmata. DNA segments are exchanged at these sites at corresponding positions along pairs of homologous chromosomes by symmetrical breakage and crosswise rejoining. Crossovers are more likely to occur in some parts of the genome, called hotspots, where it is believed that conservation is less important (Kauppi *et al*, 2004). The longer the distance between two genes, the higher the probability of an odd number of crossovers, which causes so-called recombination. Recombination has the ability to split alleles that are located together on a common parental chromosome and to position alleles that originally came from different grandparents on the same chromosome. This creation of new haplotypes increases genetic variability and consequently exerts an important evolutionary influence (Dawn Teare and Barrett, 2005).

Another form of recombination is gene conversion, which involves a one-way transfer of DNA from a "donor" sequence to a highly homologous "acceptor" sequence (Chen *et al*, 2007). Gene conversion occurs in both meiotic and somatic cell division in humans, and can be defined as the transfer of information between alleles or loci without crossover, *e.g.* the meiotic products of an individual carrying Aa at a locus may be AAAa or aaaA instead of AAaa, *i.e.*, the A allele has been converted into the a allele or vice versa (Hellenthal and Stephens, 2006).

Linkage and linkage disequilibrium

Linkage and linkage disequilibrium (LD) are two important elements in genetic epidemiology. Both concepts measure a correlation, or co-segregation, between genetic markers. Whereas linkage measures co-segregation of loci in a pedigree, LD measures co-segregation of alleles in a population. When a new SNP mutation occurs close to an older one, both SNP alleles are often transmitted together. One locus is in linkage with another locus on the same chromosome if they are transmitted together from parent to offspring more often than should be expected in the case of independent inheritance; that is, if recombination occurs between them with a probability of less than 50%. Alleles at two or more loci are in LD if they are transmitted together in the same haplotype more often than expected across the total population. Each time that recombination takes place between the loci in the total population, LD between these loci becomes weaker, and is only preserved if the two loci are located very close to each other. If two loci are in strong LD, little recombination has taken place between them in the past. Two loci in LD will always be linked, but the reverse is hence not necessarily true (Dawn Teare *et al*, 2005).

LD is not a quantitative measurement and there is no natural scale for measuring it. There are several LD measures describing the statistical association between alleles at different loci, the most common being D' and r^2, both of which are two-locus measures but may be used for different purposes (Mueller, 2004). Central for both these measurements is the linkage disequilibrium coefficient D. If p equals the allele frequency at two loci (1 and 2), each containing two alleles, A and a at locus 1 and B and b at locus 2, the frequency of A and B occurring together on the same chromosome is p_{AB} and the covariance between the two loci is $D = p_{AB} - p_A p_B$, where $p_A p_B$ is the expected value of p_{AB} when allelic association is missing. The linkage disequilibrium coefficient D is constrained in the value it may take so in order to compare LD between loci with differing allele frequencies, D' and r^2 is used (Mueller, 2004). D' is determined as the ratio between D and D_{max}, always ranging between 0 and 1 (1 indicating high LD). The squared coefficient of determination r^2 is determined as D^2 divided by the product of all allele frequencies: $r^2 = D^2/\{p_A p_B (1-p_A)(1-p_B)\}$. r^2 is similar to D' in that it ranges from 0 to 1 (where 1 indicates high LD). However r^2 scales D by the standard deviations of the allele frequencies at the two loci, in contrast to D' that scales D by its maximum value. One weakness of using D' is that it tends to be overblown; D' may equal 1 while r^2 at the same time is much lower. Only if r^2 equals 1 do two alleles always occur together on a haplotype, resulting in a maximum of two possible haplotypes. Consequently the identity of an allele at locus 1 can be determined by information regarding the allele at locus 2. D' is more useful to assess probability for evolutionary recombination in a population,

whereas r^2 is more helpful in association studies (Balding, 2006; Devlin and Risch, 1995; Mueller, 2004).

The alleles of SNPs located close to each other are often correlated with one another. A series of alleles at linked loci on a single chromosome is called a haplotype (Daly *et al*, 2001). The LD between SNPs varies from place to place in the genome and between different populations. Instead of genotyping all SNPs in a non-recombining haplotype block it is possible to use a number of haplotype tag SNPs (tSNPs) as markers for disease mapping (Johnson *et al*, 2001). These tSNPs capture the genetic information in each haplotype block, making it possible to genotype fewer SNPs, which saves both money and time. However, LD between SNPs varies from place to place in the genome and between different populations.

The HapMap project was launched in 2001 with the aim to map haplotype block structure in different ethnic populations and subsequently define tSNPs for each block (Barrett *et al*, 2005). Data from the HapMap project suggest that a vast majority of SNPs with a minor allele frequency (MAF) of at least 5% could be reduced to only 550.000 LD blocks for individuals of European descent ($r^2 > 0.8$). Subsequently, information on more than 80% of SNPs across the genome can be obtained by genotyping tSNPs from each LD block (2003). It should be noted that the definition of haplotype blocks depend on tSNP density and that, for certain regions or populations, high frequency tSNPs or apparent LD block boundaries may not exist. Furthermore, susceptibility SNPs that are located in a recombinational hot spot are impossible to detect using haplotype-tSNPs (Kauppi *et al*, 2004).

CONCEPTS OF GENETIC STUDIES

Traits, penetrance and heritability

Mendelian traits are named after Gregor Mendel (1822-1884) who studied inheritance of certain traits in plants and found that these follow particular laws. Mendel crossed white and purple pea flowers and discovered that the offspring was not a hybrid but rather a 3:1 ratio of purple and white flowers, respectively. He hypothesized that genes can be paired in three ways for each trait, AA, Aa and aa, where A represents a dominant trait, in this case the colour purple. The genotype of a purple flower can thus be either AA or Aa, while white flowers (white being the recessive trait) can only be carriers of the aa genotype. If an AA genotype carrier is crossed with a carrier of the aa genotype, all offspring will carry the Aa genotype and be purple because that is the dominant trait. If Aa genotype carriers are crossed with white flowers, on the other hand, half of the offspring will be white aa carriers and the other half will carry the Aa genotype and be purple.

Mendel summarized his findings in the law of segregation and the law of independent assortment. The law of segregation states that the paternal and maternal chromosomes get separated during meiosis and alleles are segregated into two gametes. The law of independent assortment states that alleles assort independently of one another during gamete formation. A Mendelian trait is thus one that is controlled by a single locus and shows a simple Mendelian inheritance pattern. In such cases, a mutation in a single gene can cause a disease that is inherited according to Mendel's laws. Under Mendelian

inheritance, only the fitness of the individual is important; the sole determinant of whether an allele will spread when it enters a population is whether the fitness of heterozygotes is greater than that of wild-type homozygotes (Hurst, 2009).

Complex traits do not follow Mendelian mode of inheritance. In complex traits under non-Mendelian inheritance, the faith of a new allele is determined both by the rate of transmission and the fitness of the organism. Complex disorders may depend on several susceptibility gene variants, and on combinations of genes interacting with environmental factors (Hurst, 2009). Finding solid genetic risk factors for psychiatric and other complex disorders have proven difficult. There are a number of possible explanations to this. Complex traits are the result of variation in several different genes (*i.e.* locus heterogeneity), with individual risk alleles usually explaining only 1-5% of the variation in a studied trait (Burmeister *et al*, 2008). Complex disorders are often based on a diagnosis rather than actual pathophysiological changes. It is hence possible that certain complex disorders comprise several separate etiologies caused by different susceptibility genes, which vary from patient to patient. Interaction between genes and between genes and environmental factors add to a situation where a specific allele increases the risk of developing a disease in one person but not in another (Cordell, 2009). In addition, some risk alleles display incomplete penetrance, *i.e.* a particular phenotype is not always expressed in a person with a particular genotype.

Linkage studies

Genetic linkage analysis can be used to identify genomic regions that include genes predisposing to disease. In parametric (model-based) linkage analyses, the co-segregation of linkage loci in pedigrees is analysed (Dawn Teare *et al*, 2005). Loci situated close to each other on the same chromosome segregate together more often than do loci on different chromosomes, which segregate together by chance only. As discussed above, recombination at meiosis is more likely the longer the distance between two loci on a chromosome. The recombination fraction measures the recombination between loci on a chromosome in offspring. When two loci are unlinked the recombination fraction is said to be 0.5. Linkage analysis measures deviation from this number and can be attained by genotyping linkage markers and studying their segregation in pedigrees. If one or more markers show signs of co-segregation, these are said to show linkage to the disease and are hopefully associated with a gene that may be responsible for the disease. Linkage markers are usually microsatellites (short regions of tandem repeats) or SNPs (1992). In linkage studies, DNA is collected from large extended families (or pedigrees) and several hundred or thousand (depending on resolution) evenly distributed DNA markers are analysed to see whether these markers co-segregate with disease in the pedigree.

Parametric linkage analyses have been successful in finding disease loci for disorders with a simple Mendelian inheritance. However, psychiatric and neurodegenerative disorders tend to be very complex in nature and to involve several genes with no clear mode of inheritance. Non-parametric (model-free) linkage analysis does not require specification of a disease model. The underlying principle is that, between affected relatives excess sharing of haplotypes that are identical by descent (IBD) in the region of a disease-causing gene is expected, and does not depend on the mode of inheritance. However, linkage analyses of complex disorders are usually only able to identify large regions that

often contain hundreds of genes, and are thus of limited usefulness in studies of complex traits (Dawn Teare *et al*, 2005; Prathikanti and Weinberger, 2005).

Candidate genes

A candidate gene is a gene that is believed to play a causal role in a particular disease (Botstein and Risch, 2003; Cordell and Clayton, 2005). If the pathophysiology of the disease is known, defining candidate genes and determining which gene variants that predict who becomes ill may be quite straightforward. For most psychiatric and neurodegenerative disorders our definitive knowledge regarding pathophysiological mechanisms is sparse. However, for many conditions there are usually reasonable theories implicating *e.g.* certain neurotransmitters in the underlying biological aberrations, prompting researchers to study the possible importance of genes regulating the transmitter in question as putative susceptibility genes for the investigated disorder. For example, studies of disorders that have a pathophysiology tentatively involving monoamine transmitters have to a great extent focused on genes linked to monoamine neurotransmission.

Candidate genes may also arise from positional linkage studies. As discussed above, such studies may identify a certain area of a chromosome that is linked with a disorder; genes located in this specific area are subsequently candidates and may be the subject of association studies in order to determine whether they play a role in the etiology of the disorder being studied.

Association studies

In association studies the relationship (or association) between a specific allele and a phenotype (*e.g.* a quantitative trait or a disease) is studied. Association studies may thus, for example measure whether an allele is more frequent in a patient population relative to a control population.

After having decided which genes to study, one needs to decide which candidate polymorphisms in these genes to investigate. This decision is not an obvious one, since it is likely that many causal SNPs involved in common complex disorders will be non-coding, causing variation in gene regulation, expression or splicing. As discussed above, SNPs are inherited from parent to offspring in chromosome blocks containing many SNPs. Due to recombination at meiosis, the size of the DNA block containing the founder SNP shrinks over the years. Since all polymorphisms within a block are in linkage disequilibrium, and thus inherited together, all of them can function as markers for a disease causing SNP within the block. Thus, it is possible that an associated SNP has no causal role but is associated with a nearby causal polymorphism, copy-number variation or deletion (Hinds *et al*, 2006; Locke *et al*, 2006). As discussed above, the use of haplotype tSNPs, which capture the genetic variation of the full haplotype, may increase the power of an association study. Another option is to select polymorphisms of known functional importance, *e.g.* nonsynonymous SNPs that are likely to affect the function of the protein (Hirschhorn and Daly, 2005).

In addition to the investigation of candidate polymorphisms or tSNPs, association studies have also been used in fine mapping genetic loci initially detected by linkage

analysis. However, with the advance of genotyping methods it is now possible to scan a large numbers of SNPs at rapidly falling costs. This can be used to investigate associations in thousands of loci at the same time. One may, for example, study all SNPs in a gene of interest, or even 500.000 or 1.000.000 SNPs throughout the entire genome, making *a priori* selection of candidate genes redundant.

However, there are drawbacks to this approach. Statistical analysis of several thousands of genotypes thus requires harsh correction for multiple testing, requiring effect sizes to be very large in order for p-values to be considered significant (Ziegler *et al*, 2008). This obviously is a problem when studying complex disorders that may be caused by many vulnerability polymorphisms, each exerting a modest effect, which interact to cause a certain disorder.

Replication in an independent population is usually the best way of confirming that an observed association is "true" rather than accidental. However, there are many reasons why a "true" association might not be replicated, other than that should be "untrue".

First, allelic heterogeneity between different ethnic groups might result in different polymorphisms within the same gene to contribute to disease risk. Therefore, comparisons should preferably be made within ethnically homogeneous subpopulations (Cordell *et al*, 2005).

Second, it is possible that the association is modified by other factors - genetic or environmental - that differ between studied groups (Wang *et al*, 2005).

Third, the disease-causing allele may be in LD with separate markers in different groups (Hinds *et al*, 2006; Locke *et al*, 2006).

Fourth, selection of cases may differ between studies due to the use of diverse diagnostic methods; as mentioned above, complex disorders often display clinical heterogeneity. It is therefore common to try to reduce phenotypic heterogeneity by studying traits that are less heterogeneous than most psychiatric diagnoses, such as a specific symptom of a disorder (*e.g.* inattention and hyperactivity-impulsivity, respectively, in ADHD diagnosis), subpopulations (*e.g.* gender or age groups) or intermediate phenotypes (also called endophenotypes). An intermediate phenotype is a heritable phenotype that is associated with a disorder but can be measured independently of disease status (*e.g.* amygdala activity during emotional stimuli in SAD patients). Intermediate phenotypes are thought to be closer to the pathogenic genotype than the clinical phenotype itself (Meyer-Lindenberg and Weinberger, 2006; Prathikanti *et al*, 2005).

In addition, other issues that might complicate replications of genetic associations include inadequate sample sizes, differences in statistical methods and the so-called winner's curse, which may lead to an overestimation of effect sizes, thereby handicapping replication (Zollner and Pritchard, 2007).

All papers included in this thesis are association studies. Paper I, II and III look at dichotomous traits (case/control), whereas Paper IV and V investigate continuous outcome measures. Paper IV illustrates the advantage of studying diagnostic subgroups, and Paper V the endophenotype approach.

DOPAMINE

Dopamine synthesis, transmission and receptors

Neurons in the CNS communicate mainly by neurotransmitters, which in turn can be divided into three main categories: amino acids, peptides and amine transmitters. The latter group includes the catecholamine dopamine (Carlsson *et al*, 1958; Hokfelt *et al*, 1987; Moore, 1993).

Like other catecholamines, the dopamine molecule structure has a core consisting of a benzene ring with two adjacent hydroxyl groups (catechol) and a single amine group. The precursor for the synthesis of dopamine is the aromatic amino acid tyrosine, which is transformed into dopamine in two steps. The first reaction is catalysed by the rate-limiting enzyme tyrosine hydroxylase (TH), which transforms tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA). A second step catalysed by the enzyme aromatic L-amino acid decarboxylase (AADC) leads to decarboxylation of L-DOPA to dopamine (Vallone *et al*, 2000).

Pacemaker-like membrane currents in dopamine neurons drive the spontaneous baseline activity (tonic firing) seen in dopaminergic neurons, which is the subject of strong GABAergic inhibition (Dewey *et al*, 1992). Phasic firing, on the other hand, is dependent on glutamatergic excitatory input, and displays a burst spike firing pattern, which triggers high amplitude synaptic dopamine release.

Following release dopamine is inactivated by reuptake transportation into pre-synaptic terminals via the dopamine transporter (DAT) (Goto *et al*, 2007; Grace and Bunney, 1984a, b). DAT levels are high in subcortical structures such as the striatum and amygdala, where it is located within the synapse, whereas levels are low in the cortex, where DAT localization is more distant from the site of release (Slifstein *et al*, 2008), as seen in studies on both rodents and monkeys (Lewis *et al*, 2001; Sesack *et al*, 1998).

The action of dopamine released into the synaptic cleft can also be terminated by diffusion out of the synapse, or via inactivation by catechol-O-methyltransferase (COMT) (Kaakkola and Wurtman, 1992) and monoamine oxidase (MAO) (Brannan *et al*, 1995; Di Monte *et al*, 1996), respectively. COMT is primarily located in non-dopaminergic cells and inactivates dopamine and other catecholamines by methylation. MAO is an enzyme situated intraneuronally and elsewhere that inactivates catecholamines and other amines by deamination (Goldstein and Lieberman, 1992). Whereas reuptake presumably dominates removal of dopamine in the striatum, COMT is likely to have a more important role in the regulation of dopamine transmission in the cortex. In line with this, fMRI studies have shown that a DAT VNTR polymorphism has an effect on mesencephalic, but not on prefrontal, activity during episodic memory processing, whereas the COMT Val158Met genotype predicts prefrontal but not mesencephalic activity (Forbes *et al*, 2009; Schott *et al*, 2006).

Dopamine exerts its action by binding to one of five different dopamine receptors. These receptors belong to the family of seven transmembrane domain G protein-coupled receptors, and are divided into two subfamilies based on pharmacological and

biochemical properties. The D_1 subfamily includes the D_1 and D_5 receptors, while the D_2 subfamily includes D_2, D_3 and D_4 receptors. Most dopamine agonists cannot strictly differentiate between members of the same subfamily (Girault and Greengard, 2004; Vallone *et al*, 2000).

The D_1 subfamily stimulates, whereas the D_2 family inhibits, adenylyl cyclase and its second messenger, cyclic adenosine triphosphate (cAMP). The D_2 receptor exists in two isoforms generated by alternative splicing of the same gene, one version being 29 amino acids shorter. The short and long isoforms are believed to be functionally different. The short version may function as an autoreceptor, regulating dopamine synthesis and release, whereas the long version, like the D_1 receptor, is located postsynaptically (Lindgren *et al*, 2003; Usiello *et al*, 2000; Wang *et al*, 2000).

Dopamine cell groups in the CNS

The different dopaminergic neuronal cell groups in the CNS are involved in controlling or modulating various parts of the brain. This thesis focuses on genes of importance for the development of mesodiencephalic dopaminergic (mdDA) neurons, which are made up of anatomically and functionally heterogeneous populations that are separate from other dopaminergic subgroups located elsewhere in the CNS. Below is a brief summary of the dopaminergic structures that can be identified in the murine brain.

Early studies of neuronal pathways in the CNS identified catecholamine cell groups, which were named A1 to A17. The A11-A15 groups of dopaminergic neurons are found in the diencephalon. The posterior hypothalamus cell group (A11) and the zona incerta cell group (A13) in the ventral thalamus are the largest dopaminergic cell groups in this area. The A11 group sends major projections to the spinal cord and lower brain stem, the function of which is poorly understood (Bjorklund and Skagerberg, 1979). The A13 group has more diffuse projections to the amygdala as well as different areas of the hypothalamus. The A12 (arcuate nucleus) and A14 (para- and periventricular hypothalamic nucleus) groups project to the median eminence of the hypothalamus and the pituitary gland, providing the projections involved in neuroendocrine regulation mainly of prolactin release (Ben-Jonathan and Hnasko, 2001). The connectivity of the A15 group (lateral and ventral hypothalamus) is somewhat unclear. However, dopamine input to magnocellular neurons in the hypothalamic supraoptic nucleus is derived from the population of neurons located in the A14 and A15 cell groups (van Vulpen *et al*, 1999). The A16 and A17 groups consist of a small dopamine population in the telencephalon. The A16 group of the olfactory bulb makes locally restricted connections as periglomerular interneurons and the A17 group comprises retinal amacrine interneurons (Prakash and Wurst, 2006b).

Group A8-A10 are located in the mesencephalon. Evidence from molecular and electrophysiological studies suggests that mesencephalic dopaminergic neurons can be divided into three different subgroups; the retrorubral field (RRF), the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). These nuclei have historically been designated A8, A9 and A10 respectively. Three major pathways originate from the mdDA neurons: the mesolimbic pathway connects the ventral tegmental area (VTA) with the ventral striatum (nucleus accumbens, amygdala and olfactory tubercle), the mesocortical connects the VTA with the frontal cortex, and the nigrostriatal pathway

projects from the substantia nigra to the dorsal striatum (caudate nucleus and putamen). However, the exact projection pattern of the dopamine neurons originating in the mesencephalon is a matter of debate. A recent study that used retrograde tracing technique in mesocorticolimbic dopaminergic neurons from mice showed that dopamine projections in the medial prefrontal cortex, the basolateral amygdala and the core and medial shell of the nucleus accumbens originate in the medial posterior part of the VTA, whereas dopamine projections to the lateral shell of the nucleus accumbens originate in the more lateral portions of the VTA and the medial part of the SNc (Lammel *et al*, 2008). Furthermore, dopamine projections from the medial posterior VTA had low DAT mRNA and protein levels relative to their levels of TH and vesicular monoamine transporter (Vmat2), whereas dopaminergic neurons originating in the lateral VTA to the nucleus accumbens lateral shell displayed high DAT expression (Lammel *et al*, 2008). The mdDA neurons are involved in reward-related behaviour (Schultz, 2001), as well as stimulus-incentive learning (A10 neurons in particular) (Liu *et al*, 2008), controlling voluntary movements and regulating emotion-related behaviour and have been linked to various disorders, such as schizophrenia and Parkinson's disease.

TRANSCRIPTIONAL CONTROL OF DOPAMINERGIC NEURON DEVELOPMENT

Early embryogenesis

The development, properties and fate of mdDA neurons are ultimately controlled at the transcriptional level. During embryogenesis, three germ layers are formed: the endoderm, mesoderm and ectoderm. During gastrulation, neural progenitors are formed from the ectodermal layer after induction by signals from the dorsal mesoderm (Moreau and Leclerc, 2004). After this induction, the neuroectoderm grows thicker and closes-up to form the neural tube (Greene and Copp, 2009) at which time the forebrain, midbrain, hindbrain and spinal cord start to form along the anterior-posterior axis with the help of signal gradients and transcription factor domains (Lee and Pfaff, 2001; Lumsden and Krumlauf, 1996).

Development of mdDA neurons involves the activation of transcriptional cascades that have only been partially identified as of yet. In mice, the transcription factor Gbx2 is expressed in all three germ layers, but later restricted to the anterior hindbrain (Millet *et al*, 1999). Another transcription factor, Otx2 is first expressed in the epiblast, and later (at E7 in mouse) limited to the anterior neuroectoderm that will give rise to the forebrain and midbrain (Broccoli *et al*, 1999; Simeone *et al*, 2002). In $Gbx2^{-/-}$ knockout mice, the Otx2 expression pattern is moved posteriorly, leading to a faulty posterior expansion of anterior brain regions (Martinez-Barbera *et al*, 2001; Millet *et al*, 1999). Mice in which Otx2 has been conditionally deleted at E9.5 display a severe decrease in mdDA neurons (Puelles *et al*, 2004). $Otx2^{-/-}$ knockout mice display even greater abnormalities, failing to develop forebrain and midbrain (Acampora *et al*, 1995). The additional deletion of the homeodomain protein Nkx2.2 in conditional Otx2 knockout mice reverses the mdDA neuronal deficiency, thus suggesting that Nkx2.2 is a negative regulator of mdDA development, and that Otx2 normally represses Nkx2.2 (Prakash *et al*, 2006a).

The transcription factors En1/2, Foxa1/2 and Pax2/5 are activated shortly after induction of Otx2 and Gbx2, around E8 in mice (Alavian *et al*, 2008; Ferri *et al*, 2007). En1/2, Foxa1/2 and Pax2/5 participate in the regional specification of the midbrain and hindbrain and are essential for the development of mdDA neurons, as demonstrated by studies of knockout mutant mice (Liu and Joyner, 2001; McMahon and Bradley, 1990; Schwarz *et al*, 1997; Sgado *et al*, 2008).

The secreted glycoproteins Wnt1 and Wnt5a regulate cell proliferation, fate decisions, and differentiation (Castelo-Branco *et al*, 2003). Studies suggest that Wnt1 and Wnt5a work in concert with other signals such as Shh and Fgf8 to promote mdDA neuronal development by establishing the dopaminergic progenitor domain in the mammalian ventral midbrain (Ye *et al*, 1998). Wnt1 has been suggested to be involved in the regulation of transcription factors Otx2 and Nkx2.2 (Prakash *et al*, 2006a; Prakash and Wurst, 2007), and for induction of the En1/2 genes (Danielian and McMahon, 1996). Wnt1 increased the proliferation of Nurr1 (orphan nuclear hormone receptor Nr4a2) expressing precursors, whereas Wnt5a increased the proportion expressing Nurr1 and Pitx3, which are specifically expressed in mdDA neurons (Figure 1) (Castelo-Branco *et al*, 2003).

Formation of the isthmus and mdDA neuronal field

During development of the brain, neurons and other cells emerge at specific locations, partly controlled by local organizing centres that release inductive factors (Ye *et al*, 2001). The midbrain-hindbrain (mesencephalon-rhombencephalon) organizing centre, called isthmus, is established by mutual repression between transcription factors Otx2 and Gbx2 (Millet *et al*, 1999). After formation of the isthmus, signals from the floor plate and roof plate divide the mesencephalon and diencephalon into a dorsal and a ventral part, each governed by a unique transcriptional cascade (Smits *et al*, 2006). Each organizing centre plays a unique role; the isthmus is essential for the location and size of mdDA neurons (Nakamura *et al*, 2008). Cells at this organizing centre produce Fgf8, which interacts with the glycoprotein Shh secreted by the floor plate, which runs throughout almost the entire length of the neural tube, to designate the specific place where mdDA neurons are formed in the ventral part of the mesencephalon and diencephalon (Hynes *et al*, 1995; Ye *et al*, 1998). Whereas the early onset of Fgf8 expression appears to be independent of En1 and En2 (Liu *et al*, 2001), these transcription factors influence the size of Fgf8 expression in the isthmus, as demonstrated by animal studies (Shamim *et al*, 1999).

In addition to being expressed during embryo regionalization at E8 in mice, En 1 and En2 are also involved in later (*i.e.* E11-E12) specification of neuronal phenotype (Simon *et al*, 2004). Studies of *En1/2*$^{-/-}$ double knockout mice have hence demonstrated that these genes are required for survival of mdDA neurons (Wurst *et al*, 1994). Similarly, inactivation of these genes by RNA interference induces apoptosis (Simon *et al*, 2004).

Tgfβ is a factor that is required for survival of mdDA neurons; *in vitro* studies suggest that Tgfβ acts in cooperation with Shh and Fgf8 to induce TH expressing cells, but it does not appear to affect proliferation (Roussa *et al*, 2004). Tgfβ has been shown to reduce apoptosis *in vitro* and neutralization of this gene results in a reduced number of mdDA neurons (Farkas *et al*, 2003). Furthermore, Tgfβ induces glial cell line-derived

neurotrophic factor (GDNF), suggesting that the role of Tgfβ in mdDA neuron development and survival might rely on neurotrophic support (Peterziel *et al*, 2002).

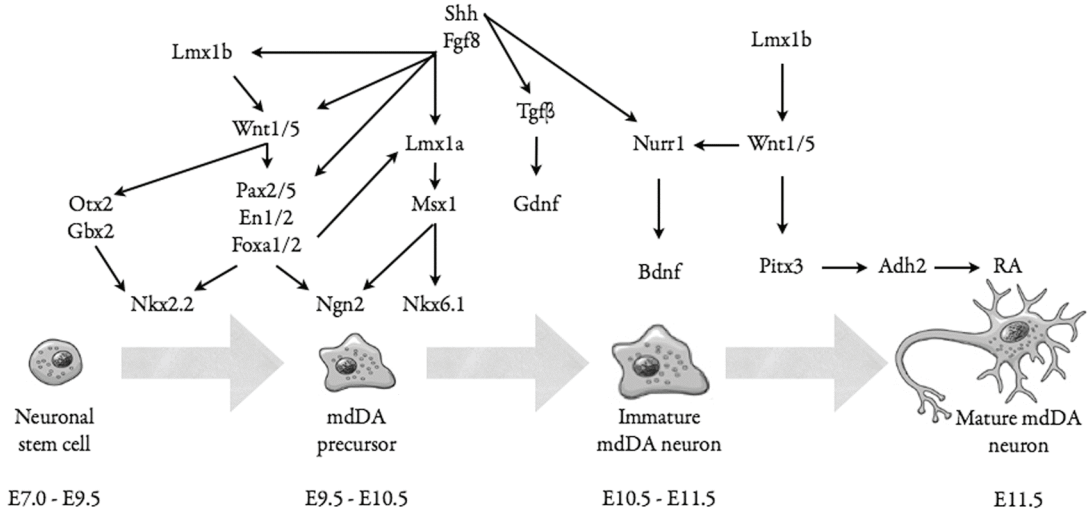

Figure 1. The development of mdDA neurons requires a complex combination of diffusible signals and transcription factors in order to control both the acquirement and maintenance of a correct phenotype. Stem cells are first patterned to a ventral cell fate, after which they are further specified towards an mdDA neuronal fate by a transcriptional cascade comprising several stages.

Differentiation of mdDA neurons requires Lmx1a

A transcriptional cascade involving several steps of specification controls the development of mature mdDA neurons (Alavian *et al*, 2008). After formation of the isthmus and the induction of Shh and Fgf8, around E8.5 in mouse, precursor cells destined to become mdDA neurons begin to produce Lmx1a (Failli *et al*, 2002). Lmx1a is of great importance for mdDA neuronal development, since it alone is both sufficient (in conjunction with Shh) and required to trigger dopaminergic cell differentiation (Andersson *et al*, 2006b; Thameem *et al*, 2002). Although Lmx1a is essential for the development of mdDA neurons, it is also expressed in other areas of the brain, including the hippocampus, cerebellum and the dorsal spinal cord (Failli *et al*, 2002).

Lmx1a induces the expression of Msx1, which in turn inhibits negative regulators of neurogenesis, including Nkx6.1 (Figure 1) (Andersson *et al*, 2006b). Consequently, Lmx1a and Msx1 function as determinants of mdDA neuron cell fate (Andersson *et al*, 2006b), initiating a transcriptional cascade that eventually induces the proneural protein Ngn2 and neuronal differentiation. In addition to Msx1, Otx2 also regulates Ngn2, as expression of the latter is absent in conditional Otx2 mutant knockout mice (Vernay *et al*, 2005). *Ngn2*$^{-/-}$ knockout mice have a reduced number of postmitotic mdDA neuronal markers, such as Nurr1 and TH (the rate-limiting enzyme in dopamine synthesis) (Andersson *et al*, 2006a), and Ngn2 is thus required for normal generation of mdDA neurons. Over-expression of Ngn2 *in vitro* results in increased neuronal differentiation and induces the expression of the mdDA neuronal marker Vmat2, but not Nurr1, indicating that Ngn2 is not sufficient for mdDA neuronal differentiation (Andersson *et al*, 2007; Roybon *et al*, 2008).

The spontaneously generated mutant *dreher* mouse (Sekiguchi *et al*, 1994; Sekiguchi *et al*, 1992) has been identified to carry a mutation in the *Lmx1a* gene (Millonig *et al*, 2000) and exhibits neurogenesis defects in mdDA neurons (Ono *et al*, 2007). Silencing the expression of *Lmx1a* using RNA interference in chick embryos also results in loss of mdDA neurons (Andersson *et al*, 2006b). Furthermore, forced expression of Lmx1a can under permissive conditions promote generation of mdDA neurons in mouse and human embryonic stem cells (Friling *et al*, 2009). After induction of Lmx1a expression, cells destined to become mdDA neurons gradually mature and become postmitotic.

Development of postmitotic mdDA neurons involves Lmx1b and Pitx3

Early phenotypic markers of postmitotic mdDA neurons, such as TH, are induced around E11.5 in mice (Abeliovich and Hammond, 2007). Transcription factors Nurr1, Lmx1b and Pitx3 are activated around this time. These genes are essential for differentiation and survival of mdDA neurons (Nunes *et al*, 2003; Smidt *et al*, 2000; Wallen and Perlmann, 2003).

The transcription factor Nurr1 is expressed immediately after mdDA neurons become postmitotic, briefly prior to TH induction at around E10.5 in mice (Wallen *et al*, 2003), and then stays expressed throughout adulthood (Backman *et al*, 1999). Nurr1$^{-/-}$ knockout mice fail to express TH in the SNc and VTA, but other markers such as Pitx3 and Lmx1b are unaltered (Castillo *et al*, 1998; Zetterstrom *et al*, 1997). Consequently, Nurr1 deficient mice do not fail to generate mdDA neurons, but because Nurr1 is responsible for the activation of TH, Vmat2, and DAT, these neurons lack proper dopaminergic phenotype (Smits *et al*, 2003). Later in life, Nurr1 has been suggested to play a role in survival and migration of mdDA neurons (Wallen *et al*, 1999), but this finding is disputed (Witta *et al*, 2000). Genetic studies in humans however have associated mutations in Nurr1 to PD (Jankovic *et al*, 2005), whereas studies in mice have linked Nurr1 deficiency to progressive loss of mdDA neurons in SNc and increased sensitivity to the dopaminergic neurotoxin MPTP (Jiang *et al*, 2005; Le *et al*, 1999), suggesting that Nurr1 is indeed of importance for survival of some mdDA neurons. Interestingly, the neurotrophin BDNF, which is also involved in the maturation of mdDA neurons, is a Nurr1 target gene; hence it is possible that some of the effects of Nurr1 on the development of mdDA neurons are mediated via regulation of BDNF expression (Volpicelli *et al*, 2007). Transcription factors Foxa1/2, first expressed at E8.0, are required for the expression of Nurr1 as well as Ngn2 (Ferri *et al*, 2007). Studies of Foxa1/2 function in conditional mutant mice suggest that these transcription factors also regulate Lmx1a and Lmx1b expression and inhibit Nkx2.2 expression, in mdDA progenitors (Lin *et al*, 2009).

Lmx1b is first expressed in midbrain at E7.5 and partly co-expressed with Lmx1a and Msx1 in mdDA neuronal progenitor cells (Andersson *et al*, 2006b), but also in other cells outside the midbrain (Smidt *et al*, 2000). Expression of Lmx1b is down-regulated at around E11 only to reappear in postmitotic neurons at E16. Expression of Lmx1b at this later stage is co-localized with Pitx3 and TH and continues throughout adulthood (Smidt *et al*, 2000). Lmx1b is structurally related to Lmx1a, but functions differently, not being able to induce mdDA cell fate. Experiments on chick embryos have shown that *Lmx1b* acts as an effector of the growth factor *Fgf8* in the regulation of *Wnt1* in developing mdDA neurons. Wnt1 expression is localized to the Lmx1b expression

domain. (Adams *et al*, 2000). Lmx1b$^{-/-}$ knockout mice display TH-positive neurons, but fail to express Pitx3, and mdDA neurons are subsequently lost at the time when the mice are born (Smidt *et al*, 2000).

Lmx1b may regulate an independent pathway necessary for expression of *Pitx3* (Figure 1) (Smidt *et al*, 2000). *Pitx3* is expressed exclusively in mdDA neurons shortly after induction of Nurr1 at E11.5 (Smidt *et al*, 1997), and expression continues into adulthood. Pitx3 appears to be required for the regulation of TH expression in mdDA neurons as well as for the generation and maintenance of these cells (Maxwell *et al*, 2005).

It has been suggested that Nurr1 and Pitx3 interact in regulating the dopaminergic pathway gene battery. A study of downstream target genes of Pitx3 revealed that expression of VMAT2 and DAT were greatly reduced in mdDA neurons of Pitx3-deficient mice (Hwang *et al*, 2009). Pitx3 has been suggested to directly activate transcription of VMAT2 and DAT, and thereby contributing to function and/or survival of mdDA neurons. Since both VMAT2 and DAT are also known to be regulated by Nurr1 (Smits *et al*, 2003), it is possible that Pitx3 and Nurr1 cooperate in regulating mDA specification and maintenance, partly through an overlapping downstream pathway (Hwang *et al*, 2009).

In the absence of Pitx3, the Nurr1 transcriptional complex is kept in a repressed state by co-repressors, including SMRT and PSF, through recruitment of histone deacetylase (HDAC), which keep the target promoters de-acetylated and thus repressed. Recruitment of Pitx3 induces the release of SMRT from the transcriptional complex, and hence initiates target gene transcription (Jacobs *et al*, 2009a; Jacobs *et al*, 2009b; Martinat *et al*, 2006).

Moreover, Pitx3 is able to upregulate the expression of BDNF, which also has effects on mdDA neuron proliferation, survival and differentiation (Yang *et al*, 2008). BDNF has been shown to promote the survival of human fetal mdDA neurons *in vitro* (Studer *et al*, 1996). Conditional knockout elimination of BDNF in mice brain throughout postnatal development disrupts the organization of SNc dopaminergic neurons (Oo *et al*, 2009).

A naturally occurring blind mutant mouse strain, first discovered in 1968 and called aphakia, lacks part of the Pitx3 gene, making them Pitx3 deficient (Rieger *et al*, 2001; Smidt *et al*, 1997). Aphakia mice initially express normal levels of TH but later develop a deficit of midbrain dopaminergic precursor cells that would later have matured into SNc neurons (Hwang *et al*, 2003; Semina *et al*, 2000; Smidt *et al*, 2004a; van den Munckhof *et al*, 2003). Interestingly, by birth, aphakia mice display a specific absence of dopaminergic neurons in the SNc as well as a loss of axonal projections to the dorsal striatum (Hwang *et al*, 2003; Semina *et al*, 2000; Smidt *et al*, 2004a; van den Munckhof *et al*, 2003). In contrast, dopaminergic neurons in the VTA are relatively unaffected by the lack of Pitx3 in aphakia mice.

Normally, Pitx3 is expressed in both VTA and SNc, but precedes TH in SNc, whereas it is concurrent with TH in the VTA (Maxwell *et al*, 2005; Messmer *et al*, 2007; Smidt *et al*, 1997). Studies suggest that the dependence of the SNc on Pitx3 is caused by SNc-specific activation of aldehyde dehydrogenase 2 (Ahd2) (Smidt and Burbach, 2009).

Pitx3 thus has been shown to act on the promoter of Ahd2 (Chung *et al*, 2005; Jacobs *et al*, 2007), which is expressed by dopaminergic neurons of the SNc and catalyses the formation of retinoic acid (RA). RA is a small signal molecule that has a crucial role in neuronal patterning, differentiation and survival of mdDA neurons (Jacobs *et al*, 2007; McCaffery and Drager, 1994; Smidt *et al*, 2009). *In vitro* studies have shown that overexpression of Pitx3 increases the generation of dopaminergic neurons expressing Ahd2 (Chung *et al*, 2005; Martinat *et al*, 2006); moreover, restoration of RA signalling in the embryonic mdDA area counteracts the developmental defects caused by Pitx3 deficiency (Jacobs *et al*, 2007). Furthermore, *in vitro* studies have shown that *Pitx3*-deficient embryonic stem cells generate 50% fewer mature dopaminergic neurons, and that dopamine release was dysfunctional in these neurons (Papanikolaou *et al*, 2009). The reduced number of generated cells was partially restored by the addition of RA, adding support for the notion that the effects of Pitx3 on mdDA neuron specification are mediated, at least in part, via the retinoic acid pathway (Papanikolaou *et al*, 2009).

Several of the transcription factors described above are known to play an important role in the maintenance and survival of mdDA neuron, as are numerous neurotrophic factors, including GDNF, conserved dopamine neurotrophic factor (CDNF), NRG1, Tgfα/β, Fgf2 and BDNF (Hyman *et al*, 1991; Lindholm *et al*, 2007; Peterziel *et al*, 2002; Roussa *et al*, 2004; Timmer *et al*, 2004; Yang *et al*, 2008; Yurek *et al*, 2004).

PARKINSON'S DISEASE

Background, symptomatology and treatment

In his monograph published in 1817 entitled *An Essay on the Shaking Palsy*, James Parkinson described a disorder where patients display "involuntary tremulous motion with lessened muscular power, in parts not in action even when supported, with a propensity to bend the trunk forward and to pass from a walking to a running pace". In acknowledgement of his work, the famous neurologist Jean Martin Charcot later proposed that this primarily sporadic neurodegenerative disorder should be called Parkinson's disease (PD) (Kempster *et al*, 2007).

The incidence rate of PD rises considerably with age, with a lifetime risk of developing the disease of 1.5%, and with a median onset age of 60 years. Because of an aging population in the western world, PD is becoming more prominent and PD is now the second most frequent neurodegenerative disorder after Alzheimer's disease (de Rijk *et al*, 1995; Lesage and Brice, 2009).

The term parkinsonism refers to the cardinal symptoms of PD, which include akinesia (inability to initiate movement), resting tremor (4–6 Hz), muscular rigidity and bradykinesia (slowness of voluntary movement with progressive reduction in speed and amplitude or repetitive actions). The onset of PD is gradual and the earliest symptoms might go unnoticed for some time. The symptoms mentioned above are typical but not unique to PD, and are often accompanied by other symptoms, including depression, dementia, and hyposmia (inability to detect odor). Other supportive criteria for PD include a unilateral onset with a progressive course and, initially, an excellent response to L-DOPA treatment (Lees *et al*, 2009).

L-DOPA is a precursor to dopamine, which in contrast to dopamine, is transported across the blood-brain-barrier. L-DOPA has been the standard treatment for PD since its release in 1968. It was first considered to be extremely effective, but a progressive loss of therapeutic response and its effectiveness was found to be limited to approximately ten years. PD patients on chronic L-DOPA therapy may also develop "on-off" effects where a good response to L-DOPA alternates with periods of akinesia and rigidity (Nutt *et al*, 1992). L-DOPA is usually used in combination with a peripheral decarboxylase inhibitor to lessen peripheral side effects like nausea and hypotension. COMT inhibitors are a relatively recent addition to the group of drugs used to treat PD. They are mainly used to treat "wearing off" effects of L-DOPA prior to the next dose and probably act primarily by reducing the conversion of L-DOPA to the inactive metabolite 3-O-methyldopa in the gastrointestinal tract (Hauser, 2009). Other drugs used to treat PD include MAO-B inhibitors and dopamine receptor agonists.

All these treatment options only relieve symptoms but cannot slow the progression of the disease itself. Although L-DOPA remains the most effective medication, dopamine receptor agonists in combination with MAO-B inhibitors may provide symptom relief for a number of years before L-DOPA treatment is necessary (Rezak, 2007). Additionally, two older classes of drugs can also be used to treat some PD symptoms. Anticholinergic drugs may provide control of some symptoms when used as monotherapy, but have severe side effects that make them inappropriate for the treatment of the elderly. Amantadine enhances dopamine transmission, and has also anti-glutamatergic activity, and is used as an antidyskinetic drug in late-stage PD (Lees, 2005).

Pathophysiology and etiology of Parkinson's disease

Sporadic PD is above all associated with a progressive loss of dopaminergic neurons in the SNc. Symptoms first appear when 50-70% of dopaminergic neurons in the SNc have disappeared (Lesage *et al*, 2009). The degeneration of dopaminergic neurons in the SNc however can probably not explain all of the symptoms related to the disorder (*e.g.* depression, anxiety, cognitive decline, and dementia).

Although significant genetic and pathological clues have been found, the etiology of sporadic PD cases remains almost as elusive as when the disorder was described in 1817 (Lees *et al*, 2009). In addition to SNc degeneration, PD is also associated with a build up of aggregates of α-synuclein proteins, known as Lewy bodies, in the central and peripheral nervous systems (Braak and Del Tredici, 2009), as well as with increased microglial activation (Long-Smith *et al*, 2009). Sporadic PD was for many years thought to be non-heritable, but this is most likely not entirely true (Sveinbjornsdottir *et al*, 2000). Possible genetic factors are further discussed below.

Besides genetic variation, several environmental factors have been identified that either decrease or increase the risk of developing PD. Smokers are less likely to develop the disease (Allam *et al*, 2004). This is possibly attributable to the nicotine in tobacco, as previous studies have shown a protective effect of nicotine against toxic insults. Moreover, nicotine attenuates L-DOPA induced dyskinesias, a common and debilitating side effect affecting a majority of PD patients on L-DOPA (Quik *et al*, 2008).

Caffeine, an adenosine A2 receptor antagonist, also appears to be protective against PD (Schwarzschild *et al*, 2003). Both nicotine and caffeine increase striatal dopamine levels, but it has also been suggested that the association between refraining from caffeine and nicotine consumption on the one hand, and enhanced risk of PD on the other, is not a causal relationship, but a result of certain personality traits often being associated with PD (Lees *et al*, 2009).

Environmental factors that, on the other hand, may contribute to an increased risk of developing PD, include obesity, living in a rural environment, drinking well water, head injury, lack of exercise and exposure to insecticide and herbicide. As is the case with many other neurodegenerative disorders, aging is also an important risk factor in PD (Elbaz and Tranchant, 2007). Certain toxins (*e.g.* MPTP) can produce a clinical picture that is similar, but not identical, to that of PD (Lees *et al*, 2009).

Heredity and genetic factors in Parkinson's disease

Although PD long appeared to be a sporadic non-genetic disorder, several disease-related genes have now been identified. Whereas some of these cause a highly hereditary form of the disorder in certain families, others contribute to an increased susceptibility to PD. Less than 10% of PD patients have a familial form of PD with an autosomal recessive or autosomal dominant mode of inheritance. These forms are due to mutations *e.g.* in genes associated with protein quality control, kinase activity and mitochondrial function (Schulz, 2008).

It is not known why Lewy bodies occur in patients with sporadic PD. However, in some cases of familial PD, aggregation of α-synuclein proteins is caused by mutations in the α-synuclein gene (SNCA/PARK1/PARK4) (Polymeropoulos *et al*, 1997). Misfolded proteins may aggregate in the brain and must therefore be refolded by chaperone proteins, or degraded by the ubiquitin proteasome system. Carriers of loss of function mutations in the E3 ubiquitin ligase gene (Parkin/PARK2) develop PD at a young age (Kitada *et al*, 1998; Shimura *et al*, 2000).

In sporadic PD, protein aggregation may be caused by environmental risk factors (mentioned above), or by a general decline in function of the ubiquitin proteasome system (McNaught *et al*, 2003). It is a matter of debate whether or not the build up of Lewy bodies results in toxicity. It is possible that they are a protective measure by the cell, a view that gains support from the fact that almost all surviving dopaminergic neurons at the end stages of PD disease contain Lewy bodies (Goldberg and Lansbury, 2000).

Several mutations in genes resulting in aberrant kinase activity have been identified to cause familial forms of PD (Shen, 2004). Mutations in leucine-rich repeat kinase 2 (PARK8/LRRK2) is the most common cause of familial PD in some parts of Europe (Paisan-Ruiz *et al*, 2004), and mutations in this gene have also been associated with sporadic PD (Paisan-Ruiz *et al*, 2008).

Numerous studies have linked mitochondrial dysfunction to PD. Familial forms of PD have thus been linked to mutations in PTEN induced putative kinase 1 (PINK1/PARK6) and DJ-1 (PARK7), which, in common with the SNCA and LRRK2

genes mentioned earlier, are either mitochondrial proteins or associated with mitochondria in some way. Moreover, these genes are involved in pathways of oxidative stress and free radical damage (Bonifati *et al*, 2003; Gegg *et al*, 2009; Schapira, 2008; Valente *et al*, 2004).

Importantly, more than 90% of PD cases are sporadic and most likely the result of complex interactions between multiple genes and environmental factors. However, some studies have found that genes responsible for familial PD are also associated with sporadic forms of the disease, either by increasing susceptibility or by affecting penetrance, age at onset, severity or progression of the disorder (Lesage *et al*, 2009).

Genome wide association studies of sporadic PD patients have been inconclusive (Evangelou *et al*, 2009; Fung *et al*, 2006), but appear to lend support the role of SNCA in PD (Pankratz *et al*, 2009). Genome wide association studies have also implicated several variants in axon guidance pathway genes to increase PD risk as well as to influence age of disease onset (Lesnick *et al*, 2007; Srinivasan *et al*, 2009); however, the importance of axon guidance pathway genes in PD has later been questioned (Li *et al*, 2008).

Paper I and II investigate the influence of polymorphisms in genes of importance for the development and survival of dopaminergic neurons in PD patients.

SCHIZOPHRENIA

Background, symptomatology and treatment

In the beginning of the 20th century, Emil Kraeplin coined the term dementia praecox to describe a disease of the brain that was separated from affective disorders. Eugene Bleuler later renamed it schizophrenia, from the greek words for split (schizo) and mind (phreno), reflecting his image of the disease at the time. Bleuler depicted schizophrenia as a group of psychoses characterized by alterations in the way patients were thinking, feeling and relating to the world. According to Bleuler, schizophrenia was characterized by four fundamental symptoms: inappropriate affect, autism, ambivalence and loosening of associations (Ban, 2004). Since the time Kraeplin first came up with the concept of dementia praecox, the diagnostic criteria of the disorder has been the subject of intense debate. It was thus argued early on that symptoms like paranoia, hebephrenia (disorganized behaviour) and catatonia (motor disturbances) were so different that they could not possibly be caused by the same disorder (Healy, 2002).

Schizophrenia has a median lifetime risk of approximately 0.7%, with slightly higher incidence rates in men (as compared to women), immigrants, people of lower socio-economic class and those exposed to urban environments (Dohrenwend *et al*, 1992; McGrath *et al*, 2008). It has been described as one of the worst diseases affecting mankind. Because of the commonness of associated deficits, and the often life-long suffering caused by schizophrenia, it is considered one of the ten leading causes of disease-related disability in the world (Murray *et al*, 1996).

Schizophrenia normally makes it first appearance in early adulthood, and while the prognosis varies somewhat from person to person, it is generally poor. A majority of

patients face a lifelong struggle with a debilitating illness for which there is no cure, or even very effective medication (Saha *et al*, 2005). This is reflected by a high suicide rate in schizophrenic patients, with as many as 10% ending their life prematurely (Tandon, 2005).

Since there is no biological marker for schizophrenia as of date, diagnosis of the disorder is based on symptom assessment. In spite of the heterogeneous nature of its symptoms, schizophrenia is regarded as one diagnosis in the 10th edition of the International Classification of Diseases (ICD-10) of the World Health Organization (World Health Organization., 1992) as well as in the 4th edition of the Diagnostic and Statistical Manual (DSM-IV) of the American Psychiatric Association (DSM-IV., 1994).

These manuals, which function as diagnostic instruments for clinicians, both divide schizophrenia symptoms into two categories, namely negative and positive. Negative symptoms are described as a loss of normal function and include lack of motivation and interest, blunted affective range, and symptoms of cognitive impairments (sometimes described as a separate category of symptoms) in attention, working memory, and a variety of executive functions.

Positive symptoms, on the other hand, are episodic in nature and associated with psychosis, including hallucinations, delusions and disorganized behaviour (DSM-IV., 1994). While positive symptoms are associated with an acute psychotic state, negative symptoms and cognitive impairments are linked to the chronic shape of the disorder and contribute more to the long-term disability in patients (Gray and Roth, 2007). However, diagnosis of the disorder is complicated by the fact that symptomatology varies significantly from patient to patient.

Although the introduction of the first antipsychotic drugs more than 50 years ago have profoundly affected the treatment of schizophrenia, big challenges remain in the long-term treatment of this disorder. Three basic classes of drugs are used in treatment of the disorder: conventional (typical) antipsychotic drugs, atypical antipsychotic drugs and partial dopamine D_2 receptor agonists (Lieberman, 2004). Despite having varying mechanisms, all three groups act primarily on dopamine systems (Miyamoto *et al*, 2005).

While atypical antipsychotic drugs and partial D_2 receptor agonists are generally believed to offer some advantages over typical antipsychotics, all three groups are mainly effective in treating positive symptoms, and have a limited efficacy on negative symptoms and cognitive deficits (Goldberg *et al*, 2007; Keefe *et al*, 2007). It has therefore been proposed that future drugs should target specific symptoms rather than try to be effective monotherapies for a very complex disorder (Hyman and Fenton, 2003).

Pathophysiology and etiology of schizophrenia

Despite numerous studies on the disorder during the last century, the pathophysiology of schizophrenia remains relatively obscure. Most of the reported neuropathological changes are subtle. Loss of interneurons in select brain regions has been reported (Benes *et al*, 1991), and a reduction in markers of axon terminals and interneuronal neuropil in the prefrontal cortex has been suggested to be an important feature of the pathology (Selemon and Goldman-Rakic, 1999). One of the first computed tomography studies of

the disorder reported increased ventricular volume in a small group of schizophrenic patients (Johnstone *et al*, 1976). Enlargement of the lateral and third ventricles, accompanied by a loss of brain tissue averaging 3%, have been confirmed in numerous imaging studies as well as in meta-analyses (Lawrie and Abukmeil, 1998; Van Horn and McManus, 1992). Imaging studies also indicate regional pathological changes in the temporal lobe, hippocampus, parahippocampal gyrus and amygdala (Gur *et al*, 2000; Lawrie *et al*, 1998; Shenton *et al*, 2001).

Although there is a significant overlap between patients and controls for each pathophysiological parameter, twin and family studies add support to these findings. In monozygotic twins who are discordant for schizophrenia, the affected twins thus had significantly larger ventricles than both control twins and their own co-twins (Reveley *et al*, 1982). Moreover, family studies suggest that family members suffering from schizophrenia display significant ventricular enlargement compared to unaffected members (Sharma *et al*, 1998). Furthermore, adolescents who, by virtue of their family history, are at high risk of developing schizophrenia, also display enlarged ventricles and smaller medial temporal lobes (Cannon *et al*, 1993; Lawrie *et al*, 1999), suggesting that the pathophysiological changes precedes onset of symptoms, supporting a neurodevelopmental model of schizophrenia.

The neurodevelopmental hypothesis of schizophrenia has become one of the most prevailing theories about the disorder since it was first stated over 20 years ago (Murray and Lewis, 1987; Weinberger, 1987). It suggests that the pathology of schizophrenia begins in the early stages of intrauterine life. The absence of *gliosis* in schizophrenia is taken to mean that the disorder is not neurodegenerative in nature, and hence that neurodevelopmental changes must have taken place before the third trimester (Lewis and Levitt, 2002). Other indirect evidence of the neurodevelopmental hypothesis includes manifestation of neuromotor, behavioural and intellectual impairment in children destined to develop schizophrenia (Jones, 1997). Interestingly, environmental factors that increase the risk of schizophrenia include obstetric complications and prenatal maternal malnutrition and infections (Geddes and Lawrie, 1995; Lewis *et al*, 2002). The fact that there is a delay between these environmental events and manifestation of schizophrenia symptoms supports the idea that schizophrenia may be a disorder of neural development. Furthermore, experimental neonatal lesions also have delayed effects (Lipska and Weinberger, 1995; Saunders *et al*, 1998).

The dopamine hypothesis of schizophrenia postulates that dopamine hyperactivity plays a key role in the disorder. It has been one of the most enduring ideas about the disorder since Carlson and co-workers first suggested it over fifty years ago (Carlsson, 1988). The idea emerged from studies of antipsychotic drugs that were shown to block dopamine D_2 receptors, and was also based on the ability of dopamine agonists to induce symptoms that mimic psychosis. Amphetamine and other drugs that increase striatal dopamine levels may induce psychosis in non-schizophrenic individuals (Guillin *et al*, 2007), and cause symptoms to worsen in schizophrenic patients (Lieberman *et al*, 1987). Moreover, among the side effect of L-DOPA therapy in PD patients are psychosis and paranoid delusions (Breier *et al*, 2002). In addition, several neuroimaging studies suggest that psychotic symptoms in schizophrenic patients are associated with exaggerated dopaminergic transmission (Breier *et al*, 1997; Laruelle *et al*, 1996).

The hypothesis was later modified; for example it was suggested that the striatal hyperdopaminergia was associated with frontal hypodopaminergia (Davis *et al*, 1991). There were several reasons for this revision. The enduring negative symptoms of schizophrenia were resistant to D_2 receptor antagonists (Laruelle *et al*, 2003). Moreover, atypical antipsychotic drugs like clozapine were effective in spite of having relatively low affinity for the D_2 receptor (Howes and Kapur, 2009). Furthermore, imaging and post-mortem studies suggesting increased D_2 receptor binding in schizophrenic brains, were disputed since it was unclear whether this was caused by prior use of antipsychotic medications or not (Zakzanis and Hansen, 1998).

Studies in animals support an association between hypo- and hyperdopaminergia; experimental lesions of dopamine neurons in the prefrontal cortex thus gave rise to augmented levels of dopamine and D_2 receptor density in the striatum (Pycock *et al*, 1980), while dopamine agonist exposure in the prefrontal area was shown to reduce dopamine concentration in the striatum (Scatton *et al*, 1982). Davis suggested that negative symptoms were caused by frontal hypodopaminergia while positive symptoms were the result of striatal hyperdopaminergia (Davis *et al*, 1991). Whereas Davis did not take the neurodevelopmental aspects into account, it has been suggested that dysfunctional development of the mesolimbic and mesocortical dopamine pathways could lead to an imbalance in the dopamine system, later interacting with normal developmental events to generate schizophrenia (Murray *et al*, 1987; Sesack and Carr, 2002). Furthermore, it has been suggested that negative symptoms and cognitive impairment in particular may be related to lower prefrontal D_1 receptor activity (Weinberger, 1987). PET studies have shown a reduced D_1 receptor binding in the PFC of schizophrenic patients (Hirvonen *et al*, 2006; Okubo *et al*, 1997). Interestingly, increased D_1 receptor availability, tentatively being a compensatory effect of reduced activation, has been associated with impaired working memory in schizophrenic patients (Abi-Dargham *et al*, 2002). It has also been suggested that tonic dopamine activity may actually be lowered, whereas the phasic activity may be increased, in schizophrenic patients (Grace, 1991).

Several other transmitter substances have been implicated in schizophrenia, including glutamate, GABA and NO (Lewis *et al*, 2005; Volk *et al*, 2002). PCP is a non-competitive NMDA receptor antagonist developed in the 1950s as an anaesthetic. It was soon discovered that this substance could induce symptoms in humans that closely resemble those of schizophrenia (Luby *et al*, 1959). This laid the foundation for the glutamate hypothesis of schizophrenia (Kim *et al*, 1980), according to which the disorder is associated with lowered NMDA receptor-mediated glutamate neurotransmission (Moghaddam, 2003; Olney and Farber, 1995). This gains some support from post-mortem studies, reporting reduced expression of NMDA receptors in various brain regions of patients with schizophrenia (Harrison *et al*, 2003). Interestingly, dopamine plays a role in the regulation of glutamate neurotransmission, and mdDA neurons are in turn modulated by glutamatergic projections (Carlsson *et al*, 1999; Laruelle *et al*, 2003).

Heredity and genetic factors in schizophrenia

That mental illness is more common in certain families has been documented since the 18th century. The first theory about heredity in mental illness was put forward by Morel who postulated that insanity was the result of a biological defect. Morel's theory of

"degeneration" concluded that the severity of mental disorders increased in lineal descents (Ban, 2004). Kraeplin noted that as many as 70% of his patients with dementia praecox had a family history of psychosis. Even though most cases are seemingly sporadic, the risk of developing the disorder increases as the degree of genetic affinity with the affected family member increases (Kendler *et al*, 1993). Adoption studies have found that the risk of developing schizophrenia is associated with the presence of the disorder in the biological parents but not in the adoptive parents (Heston, 1966). Moreover, family studies have found that relatives of schizophrenic patients are more likely to develop the disease, than people in the general population, with higher risk for first-degree relatives like siblings and parents (Li *et al*, 2009). In line with this, twin studies have consistently found that monozygotic twins, sharing 100% of their genes, are more likely to be concordant for the disease than dizygotic twins, who like ordinary siblings share 50% of their genes. A dizygotic twin of a schizophrenic patient has a 10–15% risk of developing the disease, whereas in a monozygotic twin the risk of schizophrenia is 40–50% (Farmer *et al*, 1987; Sullivan *et al*, 2003). With a heritability of 80% (Sullivan *et al*, 2003), the genetic basis for schizophrenia is clearly established. It is plausible that the genetic contribution to schizophrenia is complex in nature and involves multiple interacting genes (Faraone and Tsuang, 1985). Numerous publications have reported several susceptibility loci for schizophrenia in different chromosomes, but only a few have reached acceptable levels of statistical significance, and more often than not, these observations have not been replicated in subsequent linkage studies. Subsequent meta-analyses of linkage studies concluded that multiple regions of the genome were likely to contain risk factors for the disorder (Badner and Gershon, 2002; Lewis *et al*, 2003), and this notion also gains support from family studies (Harrison and Weinberger, 2005). Some risk-increasing gene variants may interact with other genes or with environmental factors (Riley and Kendler, 2006).

Variants of several genes that have been suggested to be associated with schizophrenia on the basis of association studies, including COMT, DAO, DRD1, DRD2, DRD4, NRG1, AKT1, BDNF, DISC1 and DTNBP1 (Alaerts *et al*, 2009; Allen *et al*, 2008; Bellon, 2007; Harrison *et al*, 2005; Tunbridge *et al*, 2006; Zintzaras, 2007) are known to participate in dopaminerigic neurotransmission and neurodevelopment. Of these genes, only COMT and NRG1 map to loci identified in the two linkage meta-analyses mentioned earlier (Lewis *et al*, 2003)}(Badner *et al*, 2002).

The Val158Met polymorphism in the COMT gene has been associated with an increased risk for developing schizophrenia, and with prefrontal function in schizophrenia in particular (Takizawa *et al*, 2009). Studies in cellular and animal models suggest that NRG1, DISC1 and AKT1 play an important part in neurite formation (Bellon, 2007).

Subsequent genome-wide association studies (GWASs) have implicated SNPs in other genes, including FGF2, myosin 18B (MYO18B), coiled-coil domain containing 60 gene (CCDC60), retinol binding protein 1 (RBP1), zinc finger protein 804A (ZNF804A), reelin (RELN), neurogranin gene (NRGN), transcription factor 4 (TCF4) and as many as 450 SNPs on chromosome 6p spanning the major histocompatibility complex (MHC) in the etiology of schizophrenia (Kirov *et al*, 2009b; O'Donovan *et al*, 2008; O'Donovan *et al*, 2009; Purcell *et al*, 2009; Shifman *et al*, 2008; Stefansson *et al*, 2009). In addition, several studies suggest the involvement of copy-number variations and deletions in

several chromosomal areas, including the neurexin 1 gene (NRXN1) (Ingason *et al*, 2009; Kirov *et al*, 2009a; Need *et al*, 2009; Rujescu *et al*, 2009; Stefansson *et al*, 2008). However, given the inconsistent results from different association studies, it is likely that polymorphisms in these genes only account for small effects in schizophrenia etiology (Sanders *et al*, 2008). Furthermore, contradictory results from studies trying to replicate genetic findings, prevent any risk gene to be singled out at this time (Jonsson *et al*, 2009; Tandon *et al*, 2008). It is possible that more complex genetic analyses and/or studies of intermediate phenotypes are necessary in order to identify the genes responsible for the high heritability of this disorder.

Paper III examines if polymorphisms in PITX3, LMX1A and LMX1B increase the risk of schizophrenia.

ATTENTION DEFICIT/HYPERACTIVITY DISORDER

Background, symptomatology and treatment

Attention-deficit/hyperactivity disorder (ADHD) is one of the most common neurodevelopmental disorders, affecting approximately 5% of the school-aged population (Faraone *et al*, 2003). The criteria given by DSM-IV include nine symptoms in each of two domains (inattention and hyperactivity-impulsivity), and three different subtypes are defined: predominantly inattentive, predominantly hyperactive impulsive, and combined. Displaying six out of nine symptoms in either domain, or in both domains in the case of the combined type, is necessary to be diagnosed with the disorder (DSM-IV., 1994).

Children with mainly hyperactive/impulsive symptoms display high distractibility and reduced persistence, often have memory retrieval problems, and often exhibit aggressive and/or oppositional behaviour leading to an increased risk of adolescent delinquency and substance abuse. Predominantly inattentive children, on the other hand, display poorly focused attention and impaired information processing.

ADHD usually onsets in early childhood and is more prevalent in males than females (Biederman and Faraone, 2002a). As shown by clinical and epidemiological studies, around 50% of all children with ADHD display psychiatric and cognitive comorbidity during their lifetime, including increased risk of conduct disorder, mood disorders and anxiety disorders. Girls are at much lower risk to manifest conduct disorder, whereas the risk of developing mood or anxiety disorders are not dependent on sex. This gender difference implies that girls with ADHD might be under-identified and under-treated (Biederman *et al*, 2002b).

Although ADHD is a chronic disorder, research indicates that the symptoms usually tend to decline over time (Faraone *et al*, 2006a); however, a significant number of youths follow a persistent high ADHD trajectory (Biederman *et al*, 1996; van Lier *et al*, 2007). A meta-analysis thus suggests the prevalence of adult ADHD to be approximately 2.5%(Simon *et al*, 2009). ADHD persisting beyond childhood is associated with an increased risk for a wide range of emotional, educational, and social adjustment problems later in life (Biederman *et al*, 1998). Predictors of illness persistence include family history of ADHD, psychiatric co-morbidity and adversity (Biederman, 2005).

Amphetamine, methylphenidate and other stimulant medications commonly used to treat ADHD facilitate catecholamine transmission by blocking dopamine and noradrenergic transporters (Arnsten and Li, 2005). In animal studies, these drugs usually induce locomotor hyperactivity. In contrast, ADHD patients given stimulant medications, display reduced locomotor activity and improved attention. These effects are not as paradoxical as one might think; human control subjects thus respond in a similar way, and rats given low doses of central stimulants, resulting in blood levels similar to those in treated ADHD patients, display decreased locomotor activity (Kuczenski and Segal, 2002; Rapoport and Inoff-Germain, 2002). High doses produce a sharp increase in dopamine release, whereas lower oral doses of stimulants results in a more subtle and slow effect on dopamine release in the striatum (Volkow *et al*, 2002). Whereas drugs targeting dopaminergic and noradrenergic transporters are thus effective for ADHD, serotonergic drugs have little effect in the treatment of the disorder. A possible mechanism that has been suggested to explain the effect of stimulant drugs on ADHD symptoms propose that these increase the inhibitory effect of the prefrontal cortex (PFC) on subcortical structures (Biederman, 2005). The PFC mediates executive function (*i.e.* working memory, behavioural inhibition, attention regulation, planning, and organization). Animal studies indicate that norepinephrine and dopamine, via D_1 receptors, are essential for PFC function (Arnsten *et al*, 2005). Moreover, imaging studies suggest that patients with ADHD have reduced PFC function. Stimulant medications, like methylphenidate, improve performance of PFC tasks in both ADHD patients and healthy controls (Aron *et al*, 2003; Mehta *et al*, 2000).

Pathophysiology and etiology of ADHD

The broad diagnosis criteria for ADHD in the DSM-IV make it a relatively heterogeneous disorder, which may render studies aiming to clarify the underlying pathophysiology difficult. Moreover, inattention and hyperactivity are not specific symptoms that are found exclusively in ADHD. While it is hence possible that the ADHD diagnosis encompass multiple disorders of different etiology, there may also be a common foundation that is being manifested in different forms. This notion gains support from the fact that stimulant drugs are often very effective in treating all symptoms of the disorder.

Several environmental factors have been suggested to contribute to the etiology of ADHD, including cigarette and alcohol exposure, lead contamination (Gittelman and Eskenazi, 1983), and food additives (Swanson and Kinsbourne, 1980). Some of these factors, including food additives and lead contamination, are unlikely to account for any major number of ADHD cases (Conners *et al*, 1980; Williams and Ross, 2007). However, studies have demonstrated that lead exposure can cause hyperactivity, distractibility, restlessness, and lower intellectual functioning (Williams *et al*, 2007). In contrast, complications during pregnancy or birth (*i.e.* low birth weight) appear to increase the risk of ADHD. Similarly, maternal smoking during pregnancy has also been suggested to be a risk factor for ADHD. This claim gains support from animal studies that have associated chronic exposure to maternal nicotine intake during pregnancy with hyperactivity in the offspring, and is intriguing given that nicotinic receptors modulate dopamine activity, and dopaminergic dysfunction is believed to be involved in the pathophysiology of ADHD (Biederman *et al*, 2009; Drenan *et al*, 2008; Mick *et al*, 2002).

Numerous imaging studies have been conducted on ADHD patients; most have focused on boys between the ages of 4 and 18. The few studies that have assessed girls are largely consistent with the results on boys (Castellanos *et al*, 2002; Giedd *et al*, 2001; Seidman *et al*, 2005). As mentioned above, several studies have shown that ADHD patients have an altered dopamine signalling, including a reduction in dopamine synaptic markers in the midbrain, PFC, and other brain areas (Arnsten *et al*, 2005; Jucaite *et al*, 2005; Volkow *et al*, 2009; Volkow *et al*, 2007).

Neuroimaging studies have found an overall decrease of around 3% to 5% in total brain volume, especially in the right hemisphere, in children with ADHD (Castellanos *et al*, 1996; Castellanos *et al*, 2002; Giedd *et al*, 2001; Seidman *et al*, 2005); however, this finding has not been replicated in studies of adult ADHD patients (Hesslinger *et al*, 2002). Other studies have found smaller volumes of gray and white matter (Castellanos *et al*, 2002; Seidman *et al*, 2005).

Several studies have also found changes in specific regions of the brain in children with ADHD. A number of MRI studies on the PFC have thus reported smaller volumes in ADHD patients when compared with healthy controls (Castellanos *et al*, 1996; Hesslinger *et al*, 2002; Hynd *et al*, 1990). Reduction of the PFC has been shown to account for as much as 48% of the reduction in total brain volume in ADHD patients (Krain and Castellanos, 2006). Studies of animal models, as well as observations of patients with cortical lesions have demonstrated that the PFC is important for inhibiting distraction, sustaining attention and dividing attention, and that these functions are modulated by dopamine through actions at the D_1 and D_2 families of receptors (Brennan and Arnsten, 2008).

The PFC has connections with several other regions, including the striatum and cerebellum. The caudate nucleus and globus pallidus of the striatum, which contain a high number of dopamine synapses, are decreased in size in ADHD patients when compared with controls (Castellanos *et al*, 1996; Dougherty *et al*, 1999; Hynd *et al*, 1993; Seidman *et al*, 2005). Striatum is vulnerable to perinatal hypoxic complications and dysfunction in this region of the brain has been suggested to play an important role in the etiology of ADHD (Lou, 1996). This idea gains support from studies in animals, which develop hyperactivity and poor performance on working memory and response inhibition tasks after striatal lesions. In addition, the drugs commonly used to treat ADHD are known to affect the striatum (Volkow *et al*, 2002).

Interestingly, the smaller volume of the caudate nucleus seen in children with ADHD has been reported to diminish from age 16 and forward, suggesting that anatomical changes may differ between children and adults with the disorder (Castellanos *et al*, 2002). Some studies have also found changes in the normal symmetry of the caudate nucleus, with greater left than right volume, in children with ADHD (Krain *et al*, 2006). However, results are inconclusive, and may be dependent on alterations of the head and body of the caudate nucleus (Tremols *et al*, 2008).

Other brain regions of interest for ADHD include the corpus callosum and cerebellum. The corpus callosum is made up of myelinated axons connecting the two cerebral hemispheres. Size differences observed in ADHD patients may reflect differences in the number or size of axons that connect the two hemispheres and consequently the

communication between these (Castellanos *et al*, 1996; Giedd *et al*, 2001; Hynd *et al*, 1991; Seidman *et al*, 2005). The cerebellum is usually thought of as a region that is mostly involved in motor control; however, research suggests that it is involved in a number of cognitive and affective processes. Specifically, studies of this region in ADHD children have shown structural abnormalities including volume reduction in the vermis (Tripp and Wickens, 2009). Interestingly, specific markers of dopamine axons have been found in certain lobules of the cerebellar vermis, suggesting that dopamine may play a role in certain cerebellar functions (Melchitzky and Lewis, 2000).

Heredity and genetic factors in ADHD

Based on family, twin, adoption, and segregation analysis studies, ADHD is a highly heritable disorder with a clear genetic etiology. Twin studies have been highly consistent in showing strong genetic influences on ADHD; with heritability estimates in the region of 60-90%. Family studies have shown that parents and siblings of children with ADHD have a two- to eightfold increase in the risk of developing the disorder. Moreover, adoption studies support the notion that genetic factors play an important role in its etiology (Biederman, 2005; Biederman *et al*, 2002a; Faraone, 2004; Thapar *et al*, 2007).

Most efforts to identify the genes responsible for the high heritability of ADHD have focused on dopamine-related genes, including DRD4, DRD5, DAT1/SLC6A3, COMT, DBH, MAOA and TPH2 (Kebir *et al*, 2009; Thapar *et al*, 2007). In addition, BDNF is important for the development of dopaminergic neurons (see above) and variation also in this gene has been associated with the disorder (Kent *et al*, 2005).

Some of these associations have been successfully replicated, though it is clear that their contribution to the overall phenotype is small, demonstrating the complex genetic architecture of the disorder (Faraone and Khan, 2006b). Due to the heterogeneity and complexity of ADHD, it has been proposed that analysis of symptom dimension subgroups would allow for increased detection of genetic effects. Moreover, there may be genes with specific effects on either the hyperactive-impulsive or the inattentive component of ADHD. Results from twin studies suggest a strong genetic overlap between the two symptom dimensions, but also the possibility of dimension-specific genes (Larsson *et al*, 2006; McLoughlin *et al*, 2007). Moreover, genetic studies indicate that some candidate genes may be associated with specific dimensions of ADHD (Neuman *et al*, 1999; Rasmussen *et al*, 2004); for example, two SNPs in the DRD4 promoter region were linked to inattentive ADHD symptoms (Lasky-Su *et al*, 2008), a microsatellite marker near the DRD5 gene was linked to the inattentive and combined subtypes of ADHD (Lowe *et al*, 2004), and a polymorphism in DAT1/SLC6A3 gene was associated with symptoms of hyperactivity-impulsivity (Lee *et al*, 2007b).

Previous studies have shown differences in both etiology (Faraone *et al*, 2000) and later adjustment problems (McGee *et al*, 2002) between children with ADHD symptoms that persists into adolescence and those that show declining ADHD symptoms. Given the differences in children and adults with ADHD, there is a possibility that certain genes may have specific effects on the persistence of ADHD. For example, the DRD4 7-repeat allele appears to influence the developmental continuity of this condition (Langley *et al*, 2009).

In Paper IV we explore the effect of the BDNF Val66Met polymorphism on both age-specific and persistent symptoms of inattention and hyperactivity-impulsivity.

SOCIAL ANXIETY DISORDER

Background, symptomatology and treatment

Social anxiety disorder (SAD) is a common psychiatric disorder with a lifetime prevalence of 12.1-13.3% (Kessler *et al*, 1994; Ruscio *et al*, 2008), affecting more women than men, and with a typical age of onset in early adolescence (Chartier *et al*, 1998). SAD is also called social phobia and was separated from specific phobias only some 40 years ago (Marks and Gelder, 1966).

SAD patients fear and avoid the scrutiny of others and are very shy when meeting new people, quiet in groups, and withdrawn in unfamiliar social settings. SAD has, in the last decade, been the focus of increased attention from the medical community. While some critics claim that SAD is simply extreme shyness, and not a disorder *per se*, the fact is that merely half of all adult SAD patients do not report excessive shyness as children (Cooper and Eke, 1999). Thus, shyness is not synonymous with SAD, which is now being recognized as a debilitating, but treatable, condition (Schneier, 2006; Stein, 1996).

Both the DSM-IV (DSM-IV., 1994) and the ICD-10 (World Health Organization., 1992) classify SAD as a phobic disorder that is characterized by a notable and persistent fear of one or more social or performance situations with exposure to unfamiliar people or possible scrutiny by others. Although SAD patients often crave the company of others, they avoid social situations for fear of being considered unlikeable, stupid, or boring, and when they do interact with others, they experience intense emotional and/or physical symptoms (*e.g.* fear, heart racing, blushing, trembling and sweating).

Individuals suffering from SAD recognise that their fear is excessive or unreasonable. The disorder interferes considerably with the normal life of the patient, including occupational/academic functioning, relationships, and social activities, and they have usually notable distress about having the phobia. DSM-IV recognises a common subtype of SAD that it refers to as generalized SAD, characterized by a fear of most social situations (DSM-IV., 1994) (in contrast to a condition where phobia is restricted to certain specific situations). Due to its neglected nature, risk for comorbidity and role as a risk factor for substance abuse, SAD is by many regarded as a major public health concern (Katzelnick *et al*, 2001; Schneier *et al*, 1992).

Numerous studies in SAD patients have demonstrated the efficacy of selective serotonin reuptake inhibitors (SSRI) (Fedoroff and Taylor, 2001; Stein *et al*, 2004; Stein and Stein, 2008), which are now considered the pharmacotherapy of choice in the treatment of SAD (Muller *et al*, 2005; Van Ameringen *et al*, 2003). Some benzodiazepine drugs and MAO inhibitors have also been shown to be of benefit in the treatment of SAD (Blanco *et al*, 2002; Davidson *et al*, 1993; Fedoroff *et al*, 2001; Gelernter *et al*, 1991). However, MAO inhibitors are of limited usefulness because of the risk of substantial adverse side effects and the need for strict dietary restrictions. The introduction of reversible MAO-A inhibitors (*i.e.* moclobemide) with fewer side effects that did not require dietary

restriction, seemed ideally suited for treating SAD, but short-term clinical trials of moclobemide were inconclusive (Stein *et al*, 2004). The use of benzodiazepines is also problematic because of adverse side effects like the development of physical dependence (Muller *et al*, 2005; Van Ameringen *et al*, 2003). Furthermore, cognitive behavioural therapy has also been suggested to be a suitable first-line intervention for SAD. Comparing pharmacotherapy and psychotherapy is difficult, but some studies suggest that pharmacotherapy may have faster effects, whereas the effects of cognitive behavioural therapy might last longer (Davidson *et al*, 2004; Gelernter *et al*, 1991).

Pathophysiology and etiology of social anxiety disorder

Numerous neuroimaging studies of SAD patients have looked at the neural response to emotional stimuli (*e.g.* emotional facial expressions) (Goldin *et al*, 2009; Phan *et al*, 2006; Stein *et al*, 2002b). Studies using fMRI have shown that patients with SAD have an exaggerated amygdala response (see next section) to the perception of anger or contempt (Stein *et al*, 2002b), fear-relevant stimuli (Birbaumer *et al*, 1998), and to learned fear responses (Schneider *et al*, 1999). Moreover, PET studies of SAD patients suggest that anxiety related to performance or anticipation of a public speaking task was associated with increased activity of the amygdala, dorsolateral prefrontal cortex and inferior temporal cortex (Tillfors *et al*, 2001b). Furthermore, in SAD patients, a decreased activity was observed in the amygdala, hippocampus and neighbouring cortical areas in response to public speaking following either cognitive behavioural therapy or administration of the SSRI citalopram (Furmark *et al*, 2002). These findings suggest that SAD may be associated with amygdala hyperactivity.

Whereas SAD patients may respond to treatment with drugs exerting pro-dopminergic effects, like MAO inhibitors (Blanco *et al*, 2002), treatment with dopamine-blocking antipsychotic drugs increases social anxiety symptoms (Stein *et al*, 2002a). Lowered plasma levels of pregnenolone sulphate, associated with increased dopamine release in the CNS, have been observed in male SAD patients (Heydari and Le Melledo, 2002). In addition, SAD patients have been reported to display a lowered density of striatal D_2 receptors (Schneier *et al*, 2000), as well as lowered dopamine reuptake site densities, compared with controls (Tiihonen *et al*, 1997). Moreover, there is a higher incidence of SAD in PD patients (Richard, 2005; Stein *et al*, 1990).

The efficacy of SSRI in treatment of SAD patients suggests an involvement of serotonin in this disorder. The amygdala is densely innervated by serotonergic neurons (Bauman and Amaral, 2005). As previously mentioned, amygdala abnormalities might normalise with SSRI drug treatment (Furmark *et al*, 2002). Serotonin-related genes have also been implicated in the disorder (see below), supporting a role for serotoninergic dysfunction in SAD.

The neuropeptides oxytocin and vasopressin are involved in social behaviour (Bartz and Hollander, 2006). As shown in behavioural studies, oxytocin increases trust (Kosfeld *et al*, 2005), indicating an involvement of the amygdala, which is linked to trust, in the regulation of social behaviour (Winston *et al*, 2002). Oxytocin has been shown to reduce amygdala activation and decrease the coupling between this brain region and brainstem regions implicated in autonomic and behavioural manifestations of fear, suggesting mechanism for the effects of oxytocin in social cognition, which could have implications

for understanding and treating disorders marked by social deficits, such as SAD (Kirsch *et al*, 2005).

Heredity and genetic factors in social anxiety disorder

Several twin studies suggest a genetic contribution to SAD in both women and men (Hudson and Rapee, 2000; Kendler *et al*, 2001; Kendler *et al*, 1992). Moreover, family studies indicate that SAD is more common in relatives of SAD patients (Tillfors *et al*, 2001a), generalized SAD being more aggregated in families than non-generalized SAD (Mannuzza *et al*, 1995). Interestingly, relatives of SAD patients score higher on measures of social anxiety, trait anxiety and harm avoidance, compared with control subjects (Stein *et al*, 2001).

As is often the problem with complex disorders, the nature of SAD may not adhere to DSM-IV diagnostic nomenclature (DSM-IV., 1994), making genetic studies of the disorder difficult. Some association studies have therefore focused on behavioural traits that are thought to be associated with the disorder (*e.g.* introversion, behavioural inhibition and harm-avoidance). Although far from consistent, findings include associations of low extraversion with polymorphisms in COMT (Hoth *et al*, 2006; Reuter and Hennig, 2005; Stein *et al*, 2005), DRD4 (Bookman *et al*, 2002; Eichhammer *et al*, 2005), BDNF (Terracciano *et al*, 2008) and the serotonin transporter (5-HTT) (Gillihan *et al*, 2007). The latter gene has also been the target of neuroimaging studies of SAD patients that have linked a repeat polymorphism in this gene, 5-HTTLPR, to amygdala activity during a stressful speaking task (Furmark *et al*, 2004), amygdala responsiveness to processing of angry faces (Furmark *et al*, 2009), and amygdala activity after sustained placebo treatment (Furmark *et al*, 2008; Munafo *et al*, 2008). In addition, the same repeat polymorphism has been linked to reduced SSRI reponse in SAD patients (Stein *et al*, 2006). Furthermore, a genome-wide linkage scan of SAD probands found evidence of linkage between SAD and a marker on chromosome 16 close to the gene encoding the norepinephrine transporter protein (SLC6A2) (Gelernter *et al*, 2004).

Paper V reports on the influence of a dopamine transporter polymorphism on amygdala activity in SAD patients and healthy controls.

THE AMYGDALA

Anatomy and connections of the amygdala

The anatomist Burdach first used the term amygdala in 1819 to describe the almond-shaped structure in the human anterior temporal lobe, hence the name amygdala (latin for almond). Like most brain regions, the amygdala consists of several distinct cell groups; the almond shaped basal nucleus is thus only one of several nuclei that make up the amygdala (Davis and Whalen, 2001). Exactly how the amygdala is to be partitioned, and how the subdivisions relate to other regions, is a matter of debate. One enduring idea is that the amygdala consists of two main parts: the cortico-medial region, and an evolutionarily newer division called the basolateral amygdala. However, the terminology is complicated by the fact that different nuclei sometimes are included in the two subregions. For the most part, the term basolateral complex is used to describe the lateral, basal, and accessory basal nuclei, whereas the cortico-medial region usually is said

to consist of the central, medial and cortical nuclei (LeDoux, 2007; Roozendaal *et al*, 2009). The nuclei are often further partitioned into subnuclei. That such fine distinctions are relevant is illustrated by the fact that cells in the superior and inferior parts of the dorsal subregion of the lateral nucleus are involved in different aspects of fear and memory (LeDoux, 2003, 2007).

Each nucleus of the amygdala has unique connections with other brain regions. The basolateral complex of the amygdala projects to many different brain regions involved in learning and memory, including the prefrontal cortex, the hippocampus, the caudate nucleus and the nucleus accumbens (Roozendaal *et al*, 2009). Because it receives information from numerous sensory systems (*i.e.* visual, olfactory, taste, somatosensory, auditory), the lateral amygdala is sometimes referred to as the gatekeeper of the amygdala. The central nucleus, on the other hand, is considered to be an important output region, sending efferents to autonomic, behavioural and hormonal regulatory centres in the hypothalamus, pons, mesencephalon, and stria terminalis (Roozendaal *et al*, 2009).

Because direct connections from the lateral nucleus to the central nucleus are few in number, sensory inputs to the lateral amygdala usually connect to the dorsal subnucleus that convey the information to other amygdala nuclei, which in turn connect with the central nucleus. In addition, the basolateral complex project to intercalated cells that subsequently connect with the central nucleus (LeDoux, 2007). The central nucleus also receives input from the basal nucleus, which is also an important output region in the amygdala, connecting with the ventral striatum and prefrontal cortex (LeDoux, 2007).

Amygdala functions

The amygdala has long been implicated in regulating affective and social behaviour (*e.g.* learned associations beetween stimuli and affective responses) (Adolphs *et al*, 1998; Jonason and Enloe, 1971). Fear is the function most commonly associated with the amygdala. The amygdala reacts to emotional stimuli, and threatening and fear-inducing stimuli in particular (Buchel *et al*, 1999; Morris *et al*, 1996; Whalen *et al*, 1998).

The function of the amygdala in relation to fear has been studied using a number of various methods, Pavlovian fear conditioning being the most common. In fear conditioning, an emotionally neutral conditioned stimulus (*e.g.* a tone) is presented together with an aversive unconditioned stimulus (*e.g.* electric footshock) (Sah *et al*, 2008). After a number of such pairings, the conditioned stimulus acquires the capacity to elicit involuntary fear responses (*i.e.* freezing or escape responses). Numerous such studies in animal models have shown that damage to the amygdala interferes with the acquisition and expression of conditioned fear (Davis, 1997). Similarly, human subjects with amygdala damage display impaired conditioned response acquisition relative to control subjects (Bechara *et al*, 1995; LaBar *et al*, 1995). Electric stimulation of the amygdala on the other hand, increases fear-induced attention and behaviour (Kapp *et al*, 1994).

Fear conditioning increases amygdala functional activity, as assessed by fMRI (LaBar *et al*, 1998). Interestingly, amygdala reactivity (*i.e.* activity during processing of emotional faces, as compared to during the processing of neutral faces) is augmented in patients

suffering from depression, general anxiety disorder, social anxiety disorder, and specific phobias (Etkin and Wager, 2007; Evans *et al*, 2008; Monk *et al*, 2008; Siegle *et al*, 2007).

In addition to its role in processing of emotionally charged stimuli, the amygdala is also involved in the modulation of other cognitive functions, such as attention, perception and memory. Emotionally charged events are better remembered than neutral events (Hamann *et al*, 1999), and it is believed that the amygdala modulates the emotional significance of external stimuli, and that the amygdala is of importance for remembering emotionally arousing events (Cahill *et al*, 1996; Hamann *et al*, 1999).

Influence of dopamine on amygdala function

The amygdala is innervated by dopaminergic neurons, and *in vivo* studies indicate that dopamine modulates amygdala neuronal activity. Dopamine projections from the mesencephalon to the amygdala are thought to modulate associative learning processes, especially in regard to behavioural responses to rewarding or aversive stimuli (Everitt *et al*, 1999; Koob, 1999).

Several neuroimaging studies in humans suggest that dopamine exerts a stimulatory influence on the amygdala. Amphetamine has been shown to potentiate amygdala response during processing of angry and fearful facial expressions (Hariri *et al*, 2002). Amygdala activation is absent, but partially restored after dopamine repletion, in PD patients (Tessitore *et al*, 2002). Furthermore, dopamine release in the human amygdala has been shown to be positively correlated with amygdala activity during processing of aversive stimuli (Kienast *et al*, 2008).

It appears that dopamine has direct effects on basolateral amygdala projection neurons and indirect effects through modulation of interneurons (Kroner *et al*, 2005). Dopamine levels in the basolateral amygdala are increased in the presence of affective stimuli and studies suggest that dopamine transmission in the amygdala contributes to the acquisition and expression of fear conditioning (Guarraci *et al*, 1999). Dopamine also potentiates sensory input to the amygdala, and administration of dopamine agonists facilitate, whereas dopamine antagonists diminish, behaviours that are amygdala-dependent (*i.e.* the retrieval of conditioned fear response) (Greba and Kokkinidis, 2000; Nader and LeDoux, 1999). Moreover, during affective learning, dopamine neurons innervating the amygdala display increases in input resistance and enhanced responses to conditioned stimuli, which are blocked by the dopamine antagonist haloperidol (Rosenkranz and Grace, 2002). An influence of dopamine on amygdala function is thus well established (Grace and Rosenkranz, 2002; Kroner *et al*, 2005; Salgado-Pineda *et al*, 2005) and also gains support from several functional neuroimaging studies in humans.

Noteworthy, the strong amygdala activation seen in healthy individuals during processing of angry and fearful faces is potentiated by the dopamine releaser amphetamine (Hariri *et al*, 2002). Moreover, dopamine storage in the human amygdala, measured with 6-[^{18}F]fluoro-L-DOPA PET, has been found to be positively correlated with changes in amygdala blood oxygen levels during presentation of negative emotional visual stimuli, which in turn, was related to trait anxiety (Kienast *et al*, 2008).

Paper V reports on the influence of a dopamine transporter polymorphism on amygdala activity in SAD patients and healthy controls.

AIMS OF THIS THESIS

With some intriguing exceptions, studies on dopamine-related genes have as yet not yielded strong support for the notion that heritable dopamine-related disorders, or other dopamine-related traits, are caused by variations in genes influencing dopamine transmission. Given the ever accumulating evidence that dopamine does indeed play a key role in the regulation of human behaviour, and in the pathophysiology of a number of neurological and psychiatric disorders, studies on the possible influence of such genes on various aspects of the human phenotype however remain highly motivated.

One important incentive for the present studies was the fact that most studies on genes influencing dopamine transmission so far have focussed on those encoding dopamine receptors, enzymes catalyzing the formation and inactivation of dopamine and the dopamine transporter; in contrast, those influencing the early differentiation and development of dopamine neurons, as well as the maintenance of these neurons, have to a great extent been neglected in association studies, in spite of the fact that developmental aspects seem to play a key role for the pathophsyiology of, *e.g.*, schizophrenia, and in spite of the fact that the number of dopaminergic neurons in the premorbide phase is probably of large importance for the debut of the symptoms of PD. One important aim of this thesis hence was to shed light on the possible influence of a number of such genes (PITX3, LMX1A, LMX1B) in these two disorders (Papers I, II, III).

BDNF is another gene involved in the formation and maintenance of dopaminergic neurons. The fourth paper was motivated by previous studies suggesting that this gene may influence the risk for another dopamine-related disorder, *i.e.* ADHD, and by the fact that these studies - the results of which have been partly conflicting - have mostly failed to take the longitudinal course and the subtype of the condition into consideration. In Paper IV the possible role of BDNF for ADHD was hence reassessed with particular emphasis on these aspects.

Paper V, finally, was inspired by a number of previous studies suggesting that a polymorphism in the serotonin transporter gene may influence amygdala reactivity, and motivated by the fact that no corresponding studies have been performed investigating the possible influence of genetic variation in the dopamine transporter gene, in spite of the fact that pharmacoloigical data reveal a strong influence of dopamine on amygdala function. The study included both controls and patients with SAD, but the main purpose was not to compare these groups, in order to reveal differences between them, but to study the relationship between genotype and amygdala reactivity.

The specific aims of the five papers have been:

I. … to investigate if three SNPs (rs2281983, rs3758549 and rs4919621) in the *PITX3* gene influence the risk of developing PD in 361 PD patients and 333 controls.

II. … to investigate if genetic variation in *LMX1A* and *LMX1B* differs between patients with PD (n=357) and control subjects (n=1446), by genotyping 33 SNPs in *LMX1A* and 11 SNPs in *LMX1B*.

III. … to investigate if five polymorphisms in PITX3, LMX1A and LMX1B, previously linked to PD, are associated with schizophrenia.

IV. … to assess, using PET and fMRI, whether a repeat polymorphism in the DAT gene (SLC6A3) influences amygdala function during processing of aversive emotional stimuli in two separate samples, one consisting of 95 SAD patients and 17 controls, and the other consisting of 78 healthy subjects.

V. … to assess longitudinal, quantitative phenotypes of hyperactivity-impulsivity and inattention in order to determine whether the Val66Met polymorphism in BDNF is associated with age-specific and/or persistent symptoms of hyperactivity-impulsivity and/or inattention in a community-based cohort of 1236 Swedish twins with ADHD symptoms data from three time points.

RESULTS AND DISCUSSION

Paper I. PITX3 polymorphism is associated with early onset Parkinson's disease.

As discussed above, PITX3 is a transcription factor that is crucial for the differentiation and maintenance of mdDA neurons. Moreover, the PITX3 gene is disrupted in a putative mouse model for PD (Rieger *et al*, 2001; Semina *et al*, 2000; Smidt *et al*, 2004b).

In Paper I, genetic variations in the PITX3 gene were investigated to determine if they influence the risk for PD or the age at onset of this disorder. To this end, two SNPs, one of which had been identified during a prior sequencing of the gene in 23 PD patients (rs2281983), and another (rs4919621), that was selected since it, according to the HapMap data base, captured (r^2 cut-off of 0.9) three other SNPs (rs733283, rs3808938 and rs3758553), were analyzed in 361 PD patients, 69 of which had early (≤50 years) onset PD (Hakansson *et al*, 2005), and in 333 controls. Following the completion of these analyses, data from another group were made public suggesting that a SNP in the putative promoter region of the gene (rs3758549) was associated with PD (Fuchs *et al*, 2007); consequently, this SNP was also genotyped.

Whereas no significant differences in genotype or allele frequencies were observed for any of the investigated SNPs when PD patients were compared with controls, the A allele of the rs4919621 SNP in intron 1 was significantly more common in PD patients with an early age of onset (≤50 years) than in controls (p=0.002), and also more frequent in PD patients with an early age of onset than in those with a late age of onset (>50 years) (p=0.001). A Kaplan-Meier analysis comprising all PD patients confirmed that the AA genotype was associated with an earlier age of onset than the other genotypes (p=0.004). The rs2281983 SNP in exon 3 was in nearly complete linkage with the rs4919621 SNP (r^2=0.96); consequently, this SNP was also found to be associated with early onset PD (p=0.004). In contrast, the rs3758549 promoter SNP was not associated with PD or age at onset.

The relatively small number of patients in our study suggests that our findings should be interpreted with caution. After our first submission of this paper, Fuchs and co-workers however published a paper on two independent German cohorts, the first consisting of 340 PD patients and 680 controls, and the second of 669 patients and an equal number of controls (Fuchs *et al*, 2007). Whereas Fuchs and co-workers did not analyze the rs4919621 SNP, the rs2281983 SNP, in LD with rs4919621, was found to be associated with PD in one of the two studied PD populations, with a weak tendency in the same direction also in the other population (Fuchs *et al*, 2007). Although these associations were with PD *per se* rather than with PD with early age at onset, they may be regarded as a semi-replication of our observation.

Moreover, since our publication two additional papers have been published that support the involvement of PITX3 in the pathogenesis of PD (Haubenberger *et al*, 2009; Le *et al*, 2009). Haubenberger and co-workers looked at six PITX3 SNPs, including the three SNPs that were investigated in our paper, in 365 Austrian PD patients and 418 controls. Whereas they could they could not replicate our finding of an association between rs4919621 and age at onset, they did find a strong association between the T allele of the promoter SNP rs3758549 and PD (p=0.0001), *i.e.* an association in opposite direction

as compared to that reported by Fuchs and co-workers (Fuchs *et al*, 2007). Haubenberger and co-workers speculate that the large difference between patients and controls with respect to the frequency of subjects being heterozygous for the rs3758549 SNP could suggest an overdominant effect model, which implies that alleles that are recessive and harmful in homozygotes are an advantage to heterozygotes (Haubenberger *et al*, 2009).

The latest study to involve PITX3 in the pathogenesis of PD is from Le and co-workers who looked at two PITX3 SNPs (rs4919621 and rs2281983) in 265 North American PD patients and 210 controls. They found the same alleles as our paper to be associated with PD, and with early onset PD in particular (Le *et al*, 2009). The involvement of PITX3 in PD onset age gains support from a linkage study suggesting the region in which PITX3 is situated to be associated with early onset PD (Li *et al*, 2002).

As discussed above, expression of PITX3 is highly restricted to mdDA neurons during development (Nunes *et al*, 2003; Semina *et al*, 1997; Smidt *et al*, 2000; Smidt *et al*, 1997; Zhao *et al*, 2004) and is crucial for the survival of these neurons after birth (Smidt *et al*, 2004b). The *aphakia* mutant mouse is characterized by several deletions in and around *Pitx3* gene (Rieger *et al*, 2001; Semina *et al*, 2000), and can hence be considered a null mutant for this gene (Smidt *et al*, 2004b). As a consequence, mdDA neurons in the *aphakia* mouse brain fail to develop (Nunes *et al*, 2003; van den Munckhof *et al*, 2003). Dopamine concentrations in *aphakia* mice are hence reduced by 90% and the animals develop DOPA-reversible PD-like motor impairment (Hwang *et al*, 2005). Furthermore, aphakia mice have an impaired ability to perform striatum-dependent cognitive tasks (*i.e.* rotarod learning, T-maze and inhibitory avoidance tasks), but not striatum-independent tasks (Ardayfio *et al*, 2008).

A particular subtype of aldehydedehydrogenase 2 (ALDH2) expressing mdDA neurons, projecting from the SNc to the striatum, are especially vulnerable to degeneration in PD. The differentiation of mouse embryonic stem cells into this particular kind of mdDA neuron is facilitated by PITX3 (Chung *et al*, 2005). Moreover, a study on microRNA feedback circuits in mdDA neurons identified microRNA miR-133b as a regulating factor in mdDA maturation, functioning in a negative feedback circuit involving PITX3, which in itself induces transcription of miR-133b (Kim *et al*, 2007).

One possible explanation for the association with early onset PD found both by us and by Le and co-workers would be that the pathophysiology of early onset PD is different from that of late onset of PD, rs4919621 being of importance for the former but not the latter condition. Given that PITX3 influences the survival of mdDA neurons, the possibility that a polymorphism in its gene may influence the risk for early degeneration of dopaminergic neurons is not unlikely.

It should be taken into consideration that approximately 50-70% of the dopaminergic neurons in the SNc must have degenerated before symptoms of PD occur (Barzilai and Melamed, 2003). It is reasonable to suggest that early onset PD patients, as compared to those displaying late onset, are characterized by a relatively low number of mdDA neurons before the degeneration starts, so that the threshold where the degeneration-induced dopamine deficiency causes symptoms is reached at a comparatively early age. Variants of a gene tentatively influencing the number of dopaminergic neurons in SNc,

such as PITX3, may consequently be associated with PD onset age without being involved in the degenerative process.

In conclusion, the results of Paper I suggest that polymorphisms in the PITX3 gene are associated with PD, and early onset PD in particular. Our findings have recently been replicated in an independent study, and there are also other recent reports suggesting the PITX3 gene to play a role in PD.

Paper II. Do polymorphisms in transcription factors LMX1A and LMX1B influence the risk for Parkinson's disease?

Lmx1a is involved in the differentiation process of mdDA neurons (see above), silencing the expression of *Lmx1a* results in loss of these neurons (Andersson *et al*, 2006b). In support of this, Dreher mice carrying a hypomorphic mutation in the *Lmx1a* gene exhibit neurogenesis defects in mdDA neurons (Ono *et al*, 2007). Moreover, over-expression of *Lmx1a* in mouse or human embryonic stem cells generates mdDA neurons (Andersson *et al*, 2006b; Friling *et al*, 2009; Roybon *et al*, 2008).

Lmx1b is structurally related to *Lmx1a* (Alavian *et al*, 2008) and also important for the development of mdDA neurons (Smidt *et al*, 2000). In *Lmx1b* knockout mice, mdDA neurons are lost around birth (Smidt *et al*, 2000). Interestingly, *LMX1B* expression in post mortem SNc sections is reduced in PD patients, when compared to controls, correlating with the loss of dopaminergic neurons in the area (Smidt *et al*, 2000).

The aim of Paper II was to investigate if genetic variation in LMX1A and LMX1B differs between Swedish PD patients (n=357) and control subjects (n=1428) by genotyping 33 SNPs, covering 183.6 kb, in the *LMX1A* gene and 11 SNPs, covering 52.2 kb, in the *LMX1B* gene. Before correction for multiple comparisons, 3 out of 33 SNPs in *LMX1A* (rs4657411, rs4657412 and rs6668493) and 1 out of 11SNPs in *LMX1B* (rs10987386) were associated with PD. When controlling for gender and home city in a logistic regression model, these polymorphisms were still significant predictors of PD. After splitting for gender, 4 SNPs in *LMX1A* (rs4657411, rs6668493, rs12136019 and rs10753668) and 2 SNPs in *LMX1B* (rs4836551 and rs10448285) were associated with PD in women but not in men, when compared with all controls. Conversely, two SNPs in *LMX1A* (rs1123821 and rs11809911) and two SNPs in *LMX1B* (rs12555176 and rs10987386) were associated with PD in men but not in women, when compared with all controls. The studied SNPs were not found to influence the age at onset. Furthermore, the associated SNPs were found to interfere with several predicted binding sites of transcription factors that are expressed in the human brain.

Due to the large number of SNPs assessed, none of the significant p-values obtained survived correction for multiple comparisons. However, it should be emphasized that many of the studied SNPs were in LD with each other. A regular correction for multiple tests may therefore be inappropriate. More importantly, our findings may be regarded as a confirmation of a previous study by Fuchs and co-workers that found the T-allele of the rs10987386 SNP in *LMX1B* to be associated with PD; due to a problem with DNA amplification, they were however unable to assess this SNP in an independent population, and hence refrained from reporting this association as a main finding in their paper (Fuchs *et al*, 2007).

In two recent GWASs (Fung *et al*, 2006; Maraganore *et al*, 2005), no SNPs in LMX1A or LMX1B were found to be associated with PD. However, in one of the studies, three out of six studied SNPs in LMX1B had p-values of 0.01 or less; after correction for multiple comparisons, these associations were however not significant (Maraganore *et al*, 2005).

Several studies have shown that genetic factors influence both the susceptibility and age at onset of sporadic PD (Belin and Westerlund, 2008; Gasser, 2005; Hicks *et al*, 2002; Sutherland *et al*, 2007). As argued in the discussion concerning Paper I above, given that more than half of the dopaminergic neurons in SNc must degenerate before symptoms of PD occur (Barzilai *et al*, 2003), variation in *LMX1A* and *LMX1B*, which are important for the development and survival of dopaminergic neurons (Ang, 2006; Burbach and Smidt, 2006; Wallen *et al*, 2003), may increase the risk for PD by influencing the individual's reserve capacity with respect to dopaminergic neurons in the SNc. In Paper II, we however observed no association between the studied genes and age at onset, but only with PD *per se*.

In paper II a number of gender-specific associations between the studied SNPs and PD were found. The fact that PD is more common among men than women (Baldereschi *et al*, 2000) has been attributed to the neuroprotective effect of estrogen (Manthey and Behl, 2006; Westberg *et al*, 2004). Moreover, estrogen is recognized as essential for the early development of dopaminergic neurons in the SNc (Kuppers *et al*, 2000). Estrogen also increases the proliferation of dopamine neuronic precursor cells expressing Lmx1a, inducing a higher proportion of dopaminergic neurons (Diaz *et al*, 2009). It is hence possible that estrogen and/or other sex-specific transcription factors interact with genes that influence dopaminergic neuron development (*e.g. LMX1A* and *LMX1B*), and that such interactions may contribute to the sex differences observed in Paper II.

In conclusion, Paper II indicates that genetic variants of *LMX1A* and *LMX1B* may increase the risk of developing PD. Whereas p-values for the observed associations did not survive correction for multiple comparisons, the findings are intriguing given the importance of these genes for dopaminergic neuronal development (Ang, 2006; Burbach *et al*, 2006; Wallen *et al*, 2003), as well as the fact that one of the associations found was a replication of a previously reported finding (Fuchs *et al*, 2007).

Paper III. Polymorphisms in dopamine-related transcription factors LMX1A, LMX1B and PITX3 are associated with schizophrenia.

Whereas several possible mechanisms have been proposed to contribute to the pathophysiology of schizophrenia, the dopamine hypothesis has been one of the most enduring ideas about the disorder (Carlsson, 1988). As mentioned in the introduction above, the hypothesis emerged from studies of antipsychotic drugs that were shown to block dopamine D_2 receptors, whereas dopamine agonists, on the other hand, may induce symptoms that mimic psychosis (Guillin *et al*, 2007), and cause symptoms to worsen in schizophrenic patients (Lieberman *et al*, 1987). Moreover, several imaging studies have shown that psychotic symptoms in schizophrenic patients are associated with exaggerated dopaminergic transmission (Breier *et al*, 1997; Laruelle *et al*, 1996). The hypothesis has later been modified to not only include striatal hyperdopaminergia, but

also frontal hypodopaminergia (Davis *et al*, 1991). Whereas the dopamine hypothesis did not take the neurodevelopmental aspects into account, it has been suggested that dysfunctional development of the mesolimbic and mesocortical dopamine pathways could lead to an imbalance in the dopamine system, later interacting with normal developmental events to generate schizophrenia (Murray *et al*, 1987; Sesack *et al*, 2002).

It is thus possible that genes involved in the survival and maturation of dopaminergic neurons play an important role in schizophrenia. Paper III presents preliminary evidence that genetic variation in *LMX1A* (rs6668493, rs4657411), *LMX1B* (rs10987386) and *PITX3* (rs4919621) are indeed associated with schizophrenia. A sex-specific analysis revealed that women contributed most to the statistical significance of one of the SNPs in *LMX1A* (rs6668493) and men to the SNP in *LMX1B* (rs10987386).

The findings of Paper III did not stay significant after correction for multiple comparisons. However, because of their previous association to PD in Paper I and Paper II, we had a strong *a priori* justification for testing these particular SNPs; as a consequence, our results should not necessarily be Bonferroni-corrected, but interpreted with caution until replicated.

None of the genes under study in Paper III have previously been associated with schizophrenia. However, earlier studies (Fuchs *et al*, 2007; Haubenberger *et al*, 2009; Le *et al*, 2009), including Paper I and II, have found polymorphisms in all three genes to be associated with PD, which, similarly to schizophrenia, involves dysfunction of the dopamine system. Whereas dopamine function is reduced in PD, the conventional view of dopamine involvement in schizophrenia posits hyperactive dopaminergic transmission. Our findings, suggesting that gene variants leading to impaired development and/or survival of dopamine neurons increase the risk to develop psychosis, may appear contradictory given that the same alleles that were associated with PD in Paper I and II, were also found to enhance the risk for schizophrenia in Paper III. However, they are well in line with a more recent theory, suggesting the primary dysfunction in schizophrenia to be a dopaminergic hypofunction, causing *e.g.* cognitive impairement and other negative symptoms, and subsequently leading to a compensatory increase of dopamine release in the ventral striatum eliciting the positive, psychotic symptoms (Carlsson and Carlsson, 2006; Davis *et al*, 1991; Weinberger, 1987).

As discussed in the introduction above, several issues support the notion that schizophrenia is a neurodevelopmental disorder. Several factors may contribute to a dysfunctional development of the mesolimbic and mesocortical dopamine pathways, causing an imbalance in the dopamine system, and later interacting with normal developmental events to generate schizophrenia (Murray *et al*, 1987; Sesack *et al*, 2002). These include perinatal stressors or insults, but also genetic variation in genes such as *LMX1A*, *LMX1B* and *PITX3*, which may disturb normal brain development during perinatal development. An altered expression of these genes during prenatal development, in combination with maturational events during puberty, in which certain brain regions undergo a process of neuronal reduction, may thus contribute to the pathogenesis of schizophrenia (Lewis *et al*, 2002).

A similar example is provided by studies of a hypomorphic mutation in the Tgfα gene, which causes gradual reduction of this protein postnatally, resulting in adolescent-onset

changes in behaviour and brain structure, without any evidence of such aberrations prior to adolescence (Burrows *et al*, 2000). Similarly, structural damage of the ventral hippocampus during the first postnatal week causes significant structural alterations in rats, but changes in measures of performance on behavioural tasks and brain chemistry do not appear until after puberty (Lipska and Weinberger, 2000).

Maternal immune system activation during pregnancy is thought to increase the risk of the baby to develop schizophrenia later in life. Interestingly, expression of *Pitx3* is decreased in mice after *in utero* exposure to an immune challenge (Meyer *et al*, 2008).

In conclusion, Paper III presents preliminary evidence that genetic variation in *LMX1A*, *LMX1B* and *PITX3* may increase the risk of developing schizophrenia. Paper III included a relatively small number of patients (n=213) and observed associations did not survive correction for multiple comparisons. Results should hence be interpreted with caution until replicated in an independent sample.

Paper IV. Association of brain-derived neurotrophic factor polymorphism with the developmental course of attention-deficit/hyperactivity disorder.

BDNF is involved in the differentiation and survival of both dopaminergic and serotonergic neurons (Hyman *et al*, 1991; Knusel *et al*, 1992; Lyons *et al*, 1999), and has been suggested to play a role in the pathogenesis of ADHD (Tsai, 2003). Paper IV assessed parent-reported quantitative measures of hyperactivity-impulsivity and inattention in a community-based cohort of 1236 Swedish twins in order to determine whether the BDNF Val66Met (rs6265) polymorphism is associated with age-specific and/or persistent symptoms of hyperactivity-impulsivity and/or inattention. ADHD symptoms data were collected at three time points, *i.e.* when participants were 8-9 years old, 13-14 years old and 16-17 years old.

Regression analysis revealed that the Met allele was associated with symptoms of hyperactivity-impulsivity at ages 13-14 and 16-17 and with symptoms of inattention at ages 8-9 and 13-14. A multivariate regression analysis revealed that the observed effect of the Met allele on ADHD symptoms reflect an influence on persistent hyperactivity-impulsivity symptoms as well as on age-specific inattention symptoms.

Although the Val66Met SNP is unlikely to alter the activity of the mature protein, it is the only polymorphism in BDNF with a plausible functional effect; the Met allele is thus shown to impair intracellular processing and secretion of BDNF (Chen *et al*, 2004; Egan *et al*, 2003; Hariri *et al*, 2003). Moreover, mice carrying two BDNF Met/Met alleles display defective neuronal secretion of BDNF as well as lowered stress tolerance (Chen *et al*, 2006), and heterozygous *Bdnf*$^{+/-}$ knockout mice, with presumably lowered BDNF levels, are characterized by impaired impulse control and other behavioural and physiological abnormalities (Korte *et al*, 1995; Lyons *et al*, 1999). Furthermore, conditional deletion of *Bdnf* in postnatal mice brain results in hyperactivity after exposure to stressors (Rios *et al*, 2001). Studies in humans suggest that Met allele carriers display decreased volumes of the hippocampus (Szeszko *et al*, 2005), dorsolateral prefrontal cortex, and subcortical regions (Pezawas *et al*, 2004), as well as poorer

episodic memory, when compared with carriers of the Val allele (Egan *et al*, 2003; Hariri *et al*, 2003).

Paper IV raises the possibility that the lowered BDNF activity of Met allele carriers may result in a dysfunctional development of dopaminergic neurons, which have been suggested to play an important part in ADHD pathogenesis (Ernst *et al*, 1999; Jucaite *et al*, 2005; Leo *et al*, 2003; Volkow *et al*, 2009). One previous family study associated the Met allele with increased inattentive symptoms, but only in connection with low socioeconomic status (Lasky-Su *et al*, 2007). In contrast, two previous association studies (Kent *et al*, 2005; Lanktree *et al*, 2008) have reported an increased frequency of the Val allele in ADHD patients. In addition, several studies (Brookes *et al*, 2006; Lee *et al*, 2007a; Sanchez-Mora *et al*, 2009; Schimmelmann *et al*, 2007; Xu *et al*, 2007) have failed to replicate an association between theVal66Met polymorphism and ADHD. Differences between the studied populations with respect to age, gender, ethnic origin, comorbidity, and ADHD subtypes, are examples of factors that may explain the results.

In contrast to previous studies, which have been based on clinical samples defined by using a categorical definition of ADHD, Paper IV used a dimensional approach based on symptom counts in a population-based sample. Whereas the lack of clinical diagnosis may be regarded as a limitation, it is also possible that the dimensional approach may enhance the possibility of identifying gene variants influencing ADHD traits, since case-control studies, to a greater extent than population-based studies, may be confounded by subjects with severe ADHD caused by non-genetic factors (*e.g.* prenatal environmental risk factors).

In conclusion, the findings of Paper IV support the hypothesis that BDNF is involved in the pathogenesis of ADHD. The results highlight the importance of distinguishing between hyperactivity-impulsivity and inattention, respectively, and demonstrate the value of using a longitudinal approach in genetic studies of ADHD.

Paper V. Amygdala function is associated with a dopamine transporter gene polymorphism in patients with social anxiety disorder and healthy controls.

Fundamental for detection of biologically relevant stimuli and for fear generation, the amygdala is under excitatory influence of dopamine (Grace *et al*, 2002; Kroner *et al*, 2005; Salgado-Pineda *et al*, 2005). The dopamine transporter (DAT) mediates reuptake inactivation of extracellular dopamine and is consequently very important for the regulation of dopamine transmission. Paper V investigates if a VNTR polymorphism in the DAT gene (SLC6A3/DAT1) influences amygdala function during processing of aversive emotional stimuli.

In the first part of the study, 95 patients with social anxiety disorder (SAD) and 17 controls, all from Sweden, were genotyped and studied with respect to rCBF by means of positron emission tomography (PET) during a stressful public speaking task, in which subjects held a newly prepared speech during scanning. A surrounding audience consisting of 6-8 individuals observed the participants in order to enhance their level of anxiety. In addition, a subgroup of 32 patients and 17 controls from this cohort were assessed during exposure to black and white photographs of angry and neutral faces taken

from the set of Ekman and Friesen (Ekman and Friesen, 1976). In a separate study, amygdala function was assessed using fMRI during an affective face processing task in which subjects viewed faces expressing either anger or fear or neutral stimuli (*i.e.* geometrical figures) in an independent group of 78 healthy subjects from US.

As assessed by PET, SAD patients carrying at least one 9-repeat allele had higher blood flow when performing the public speaking task, in both left and right amygdala, as compared with carriers of two 10-repeat alleles. In contrast, controls with one or two 9-repeat alleles had lower blood flow in both left and right amygdala, as compared to controls carrying two 10-repeats. Similarly, during exposure to either neutral or angry faces, SAD patients carrying one or two 9-repeat alleles displayed higher blood flow in both left and right amygdala as compared to carriers of two 10-repeats, whereas controls with one or two 9-repeats, on the other hand, displayed lower blood flow in both left and right amygdala as compared to carriers of two 10-repeats.

On the other hand, amygdala reactivity (*i.e.* amygdala activity during the processing of angry faces as compared to that during the processing of neutral faces) was positively associated with one or two 9-repeat alleles in both controls and SAD patients. The latter finding was replicated using fMRI, adding support to the notion that the 9-repeat allele is associated with enhanced reactivity. Right amygdala reactivity did not differ between genotypes in neither of the two samples. Amygdala lateralization is not uncommon in human neuroimaging studies, maybe due to left amygdala activation being more common and more sustained than activation of the right amygdala (Sergerie *et al*, 2008). Distribution of genotypes did not differ between SAD patients and controls and there was no association between genotype and the various anxiety ratings.

DAT is a fundamental regulator of dopamine neurotransmission by determining the duration and amplitude of dopamine action. The SLC6A3 VNTR polymorphism has previously been associated with increased severity of symptoms during alcohol withdrawal in alcoholic patients (Sander *et al*, 1997; Schmidt *et al*, 1998), with PD (Haddley *et al*, 2008), with angry-impulsive personality traits (Joyce *et al*, 2009), and with attention deficit hyperactivity disorder (ADHD) (Yang *et al*, 2007). While results from previous studies on the possible effect of the SLC6A3 VNTR polymorphism on DAT protein expression have been contradictory (Fuke *et al*, 2001; Heinz *et al*, 2000; Jacobsen *et al*, 2000; Michelhaugh *et al*, 2001; Mill *et al*, 2002; Mill *et al*, 2005; Miller *et al*, 2002; van de Giessen *et al*, 2009; van Dyck *et al*, 2005; VanNess *et al*, 2005), functional PET and MRI studies unanimously suggest that carriers of the 9-repeat allele, as compared to carriers of the 10-repeat allele, display higher dopamine release (Brody *et al*, 2006) and enhanced activation of brain regions innervated by dopamine afferents (Bertolino *et al*, 2008; Bertolino *et al*, 2009; Caldu *et al*, 2007; Dreher *et al*, 2009; Forbes *et al*, 2009; Franklin *et al*, 2009; Schott *et al*, 2006), in situations where increased dopamine release may be expected.

An influence of dopamine on amygdala function is well established (Grace *et al*, 2002; Kroner *et al*, 2005; Salgado-Pineda *et al*, 2005). The current findings that SAD patients with the 9-repeat allele display enhanced amygdala activity as compared to carriers of two 10-repeat alleles during observation of neutral faces, during observation of angry faces, with respect to emotional reactivity (*i.e.* the response to angry faces as compared to neutral faces), and during the public speaking task, are in line with studies suggesting

dopamine to exert a stimulatory influence on amygdala (Hariri *et al*, 2002; Kienast *et al*, 2008; Tessitore *et al*, 2002). The responses observed in healthy controls were however more complex than those seen in SAD patients. Similarly to patients, controls carrying the 9-repeat allele displayed enhanced amygdala reactivity, lending additional support for the notion that the 9-repeat allele is associated with enhanced dopamine activity, and that dopamine exerts a stimulatory influence on amygdala function. However, the observation that controls carrying the 9 repeat allele displayed decreased amygdala activity in other experimental conditions is contrary to what was found in SAD patients, and to what could be expected given the alleged facilatory influence of dopamine on the amygdala.

One possible explanation to the observed difference between patients and controls in Paper V would be that the two groups may differ with respect to dopamine function, and consequently also with respect to how the DAT VNTR polymorphism influences various phenotypes. The idea that SAD may be associated with reduced dopamine activity has been put forward by many authors (Furmark, 2009; Stein and Vythilingum, 2007; Stein *et al*, 2008), and gains support from studies which have shown that SAD patients respond to treatment with pro-dopminergic drugs (*i.e.* irreversible non-selective monoamine oxidase inhibitors) (Blanco *et al*, 2002), as well as from the observation that treatment with dopamine-blocking antipsychotic drugs increases social anxiety symptoms (see above) (Stein *et al*, 2002a). Also of importance in this context is a study showing an increased prevalence of SAD in patients with PD (Richard, 2005), and another one reporting decreased levels of homovanillic acid, the main metabolite of dopamine, in the cerebrospinal fluid of patients with comorbid SAD and panic disorder, but not in patients with panic disorder alone (Johnson *et al*, 1994). Moreover, one study suggests that persons carrying the Val allele of the COMT Val158Met SNP, which is associated with lower dopamine levels, display increased symptoms of phobic anxiety (McGrath *et al*, 2004). Also, SAD has been associated with lowered D_2 receptor binding (Schneier *et al*, 2000), although this finding was not possible to replicate in a subsequent investigation (Schneier *et al*, 2009). Finally, SAD patients have been reported to display lowered striatal dopamine reuptake site densities when compared with controls (Tiihonen *et al*, 1997).

That genotypes of certain polymorphisms may have opposite effects on brain function in patients and controls, respectively, has been observed in other studies as well. A SLC6A4 polymorphism thus has been shown to excert opposite effects on amygdala responsiveness in adolescents with anxiety or depression as compared to in controls (Lau *et al*, 2009). In the same notion, opposite effects of the COMT Val158Met polymorphism on cortical function was observed in healthy subjects as compared to in patients with schizophrenia (Prata *et al*, 2009).

However, it is also possible that the different outcome between patients and controls in Paper V merely reflects phenotypic differences with respect to how the subjects perceived the test situation. The amygdala is activated not only by fear- and anxiety-related events but also by neutral stimuli (Sergerie *et al*, 2008) and by attention-related cognitive tasks (Kiehl and Liddle, 2001; Laurens *et al*, 2005; Ousdal *et al*, 2008; Schaefer *et al*, 2006), which gains support from the fact that amygdala activity in Paper V was not higher during the public speaking task (which was associated with marked anxiety in SAD patients) than during the processing of facial expression (when anxiety levels were

considerably lower). Amygdala activity in patients may hence to a large extent be associated with the anxiety experienced by the SAD patients in the test situation, whereas the enhanced activity observed in controls may measure attention rather than fear. That dopamine may play different roles during stress-induced and attention-induced amygdala activation, respectively, might help to explain the different outcomes observed in patients and controls.

In conclusion, Paper V demonstrates that variation in the SLC6A3 VNTR polymorphism is important for challenge-induced amygdala activity during performance of public speaking and during processing of angry or neutral faces. This effect was diagnosis-dependent; SAD patients with at least one 9-repeat, and controls with two 10-repeats, thus displayed significantly higher blood flow in both left and right amygdala. On the other hand, carriers of the 9-repeat, regardless of diagnosis, displayed higher left amygdala reactivity than carriers of two 10-repeats, both in the PET study and in the fMRI study. The results, and especially those in patients, are well in line with previous data suggesting the 9-repeat allele to be associated with enhanced dopamine function, and amygdala to be under stimulatory influence of dopamine.

Concluding remarks

The data presented in this thesis suggest that variation in dopamine-related genes is of importance for dopamine-related disorders as well as for amygdala function. The findings lend support, *e.g.*, to the following conclusions: *i)* that variants in genes influencing dopamine function are indeed an important source of variation with respect to dopamine-related traits and disorders, *ii)* that genes influencing the early development of dopamine neurons deserve increase attention in this context, *iii)* that schizophrenia and ADHD should probably be regarded as neurodevelopmental conditions, and that the primary dopaminergic dysfunction in schizophrenia may be hypofunction rather than hyperfunction, and *iv)* that not only the serotonin transporter, but also the dopamine transporter, is an important regulator of amygdala activity.

All of the findings presented herein stem from association studies using a candidate gene approach. With the advancement of GWASs, candidate gene association studies might appear less relevant than before. However, there are several drawbacks to the GWAS approach, including the fact that statistical analysis of several thousand genotypes requires consideration for multiple testing, hence increasing the risk of missing true associations with small effect size (Ziegler *et al*, 2008), which is a major problem when studying complex disorders caused by many vulnerability polymorphisms that interact to cause a certain disorder. Moreover, in order to be adequately powered to study complex traits, GWASs require very large sample sizes. The old-fashioned way of conducting research - *i.e.* to formulate an hypothesis based on available knowledge, and then designing an experiment specifically testing this hypothesis, rather than just measuring everything that can be measured - may hence still be a feasible strategy also in genetic research.

Replication is usually the best way of confirming that an association is "true". The finding that a PITX3 variant is associated with PD in Paper I is an example of a finding that *i)* is based on a very reasonable a priori hypoyhesis and *ii)* has been replicated by others. That this finding nevertheless should be accidental, *i.e.* "untrue", seems hence

highly unlikely; yet, there are studies failing to confirm it, illustrating that also associations that are probably "true" in one cohort may not always be replicated in another.

The association between SLC6A3 and amygdala function reported in Paper V also is very much in line with previous knowledge (on how amygdala is influenced by dopamine), and was observed in two independent populations. Again it seems highly unlikely that this should be a falsely positive finding.

The previous literature on the possible association between BDNF in ADHD is an example of a field where association studies have yielded divergent results. Concluding that all these results have been "untrue", and that ADHD is unrelated to BDNF function, would however probably be premature, given that many groups have indeed found significant associations (though not always in the same direction). Paper IV can hopefully help to shed some light the characteristics of this association by using a longitudinal approach and by distinguishing between hyperactivity-impulsivity and inattention, respectively.

Previous reports on the possible relationship between LMX1A and LMX1B on the one hand, and PD and schizophrenia on the other, and between PITX3 and schizophrenia, are sparse or non-existing. However, given that these genes are essential for the development of mdDA neurons, we had a strong *a priori* hypothesis regarding their possible involvement in dopamine-associated disorders like PD and psychosis, and we hence believe that our preliminary findings do deserve replication attempts. As mentioned above, preliminary data supporting an association between LMX1B and PD are already at hand.

The often uncertain phenomenological nature of psychiatric disorders such as ADHD, SAD and schizophrenia, call for the use of endophenotypes that are thought to be closer to the pathogenic genotype than is the clinical phenotype itself. In this vein, the genetic influence on amygdala function was studied in Paper V, the rational being that amygdala reactivity may be of importance not only for the disorder in focus in paper V, *i.e.* SAD, but for a number of anxiety disorders, as well as for depression. Similarly, the method used in Paper IV also reduces phenotypic heterogeneity by analysing ADHD symptom dimensions separately and over time. It should be mentioned, however, that the relationship between endophenotype and clinical manifestations of a disorder is not always apparent. Thus, case-control studies still have an important role to play in establishing susceptibility genes for psychiatric and other complex disorders.

One might ask what good it does to identify associations with small effect size. Needless to say, there is no way of removing detrimental genetic variants from the population; instead the answer is that elucidation of genes influencing the risk for a certain disorder, also when they are exerting only a minor influence, may aid to shed light on pathophysiological mechanisms, hence paving the way for future treatments that will address the core pathophysiology of the disorder.

APPENDIX: SUBJECTS AND METHODS

Subjects

Patients with Parkinson's disease

The PD patients (n=361: 216 males and 145 females) were recruited from hospitals and care centres in Sweden (Gothenburg, Stockholm, Falköping and Skövde). All patients fulfilled the PD Society Brain Bank criteria for idiopathic PD (Daniel and Lees, 1993), except that the presence of more than one relative with the disease was not regarded as an exclusion criterion. All subjects were of Caucasian origin. Moreover, mean age at onset of PD was 60 years. Several studies suggest that genetic factors may be more important for the etiology of early onset PD, as compared to late onset PD. By defining early onset of PD as debut ≤50 years of age (Hakansson *et al*, 2005), 69 patients were categorized as having early onset PD, while 283 were categorized as suffering from late onset PD. In 9 patients the age of onset could not be established. In addition, control subjects (n=333: 125 males, 179 females, and 29 subjects of unknown gender) were recruited from hospitals and care centres in Gothenburg and Stockholm, Sweden. All subjects provided informed consent and the study was approved by the ethical committees at Göteborg University and at Karolinska Institutet.

Patients with PD are studied in Paper I and II.

The Kungsholmen population

Individuals from the Kungsholmen project (n=1090) were used as controls to PD patients in Paper II. The Kungsholmen project is a longitudinal population-based study that started in 1987, which has gathered data on aging from a multidisciplinary perspective during a 12-year long period. The Kungsholmen population includes persons living in the Kungsholmen district in Stockholm. Individuals born before 1913 were invited to participate (n=2368 in 1987). Data was subsequently collected in five phases and included extensive clinical screenings by nurses, physicians, and psychologists. Comprehensive assessment of memory and other cognitive functions, collection of DNA, physical, neurological, and psychiatric examinations were performed. After examinations, diagnoses of current diseases were made according to standardized criteria. A family interview with a next-of-kin concerning past and current health status of the subject, as well as selective risk factors of the most common chronic neurodegenerative diseases (*e.g.* PD) was also performed. The Ethics Committee of the Karolinska Institutet has approved the Kungsholmen Project (Fratiglioni *et al*, 1992).

Individuals from the Kungsholmen project were used as controls in Paper II.

Patients with schizophrenia

Patients with schizophrenia (n=213) were recruited from care centres in the city of Umeå, Sweden. All patients fulfilled the DSM-IV criteria for schizophrenia. The schizophrenic patients consisted of 110 men (51.6%) and 103 women (48.4%). Mean age of patients at the time of DNA sampling was 49.3 years and mean age at onset was 25.3 years. The ethical committee at the University of Umeå has approved the study.

Schizophrenic patients are examined in Paper III.

The Betula study

Paper III used control subjects recruited via the Betula project (n=2718) consisting of 1222 men (45.0%) and 1496 women (55.0%), mean age was 58.6 years. The Betula study is a prospective cohort study involving a total of 3,000 subjects aged 35-85 years. Subjects living in the city of Umeå, Sweden, were randomly selected from the population registry and contacted by mail. Only volunteers without severe mental disorders were included in the study. Subjects participated in extensive examinations of their health and memory. The main objectives of the study were to study health and memory in adulthood and old age, and determine early preclinical signs and risk factors of dementia. The ethical committee at the University of Umeå approved the study (Nilsson *et al*, 1997).

Individuals from the Betula project were used as controls in Paper III.

ADHD symptoms in the Twin study of Child and Adolescent Development

Paper IV is based on data from the Twin study of Child and Adolescent Development (TCHAD), a longitudinal study on the health and behaviour of 1480 Swedish twin pairs born between May 1985 and December 1986 (Lichtenstein *et al*, 2007). TCHAD used data regarding ADHD-related symptoms from three different time points. Parents completed a binary-scaled checklist containing 14 items based on the DSM-IV. Symptoms persisting for more than 6 months were scored as 0 if the item was not true and 1 if it was true. A hyperactivity-impulsivity scale was created from the sum of 8 symptoms of hyperactivity-impulsivity listed in DSM-IV and an inattention scale was created from the sum of 6 DSM-IV items related to inattention (Larsson *et al*, 2006). At wave 1 in 1994, 1106 (75%) of parents to twins 8-9 years old replied to the questionnaire mailed to them. At wave 2 in 1999 (13-14 years old) 73% and at wave 3 in 2002 (16-17 years old) 74% of the parents responded to the questionnaire. All participants provided informed consent and the ethics committee at Karolinska Institutet approved the study (Lichtenstein *et al*, 2007).

Individuals from TCHAD with ADHD symptoms are investigated in Paper IV.

Social anxiety disorder patients

Paper V is a PET study of patients with SAD (n=97, 37 men, 60 women, mean age 35.9 years) who were recruited through newspaper advertisements in Sweden. Following a short telephone interview, participants were asked to answer a battery of SAD questionnaires, and psychiatric status was evaluated by a clinical psychologist using face-to-face structured clinical diagnostic interviews (SCID) (First *et al*, 1998). Criteria for exclusion included depressive disorder and other major psychiatric conditions (other than co-morbid anxiety disorders), treatment for SAD during the past six months, chronic use of prescribed medication, substance abuse, pregnancy, menopause, left-handedness, and any disorders that could be expected to influence the outcome of the study. All patients fulfilled the DSM-IV criteria for SAD and had marked anxiety for

public speaking. In addition, eighteen healthy individuals (9 men, 9 women, mean age 34.5 years) were recruited to function as controls in the PET study. The Uppsala University medical faculty ethical review board and the Uppsala University isotope committee approved the PET study. All participants gave informed written consent after having the procedure and its consequences explained.

Paper V reports on amygdala activity in patients with SAD and healthy controls.

The adult health and behaviour (AHAB) project

Paper V uses fMRI in order to study amygdala activity in 103 control subjects who were recruited from the adult health and behavior (AHAB) project, a community sample of 1379 middle-aged volunteers. Subjects were nonpatient, middle-aged volunteers and assessed for a wide range of behavioural and biological traits. All participants were in good general health, exclusion criteria included medical diagnoses of cancer, stroke, diabetes, chronic kidney or liver disease, or a lifetime history of psychotic symptoms, use of psychotropic, glucocorticoid, or hypolipidemic medication, conditions affecting cerebral blood flow and metabolism (*e.g.* hypertension) and DSM-IV psychopathology. All participants provided informed consent according to the guidelines of the University of Pittsburgh Institutional Review Board.

Paper V reports on amygdala activity in individuals from the AHAB project.

Methods for genotyping and sequencing

DNA amplification

Prior to sequencing or genotyping, DNA sequences of interest were amplified with the polymerase chain reaction (PCR). The PCR consists of a series of repeated temperature cycles that usually consists of three discrete temperature steps. The temperatures and time of these cycles depend on what Taq polymerase (thermostable DNA polymerase) is used, the melting temperature of the primers and concentration of dNTPs in the reaction. The first step of the reaction consists of heating a mixture containg the DNA template, primers, dNTP, and buffer to a temperature of 94–98 °C. This causes the two strands of the DNA template to separate, by disrupting the hydrogen bonds between complementary bases. The temperature of the reaction is then lowered to 50-65 °C (depending on melting temperature of primers), allowing the two primers to anneal to the single stranded DNA template. The Taq polymerase will subsequently begin DNA synthesis, depending on what Taq polymerase used, the temperature is raised to around 72 °C, allowing the synthesis of a new DNA strand, which is complementary to the DNA. The time of this extension step depends on the length of the amplified DNA sequence. The procedure is then repeated and the amount of amplified DNA is doubled during each cycle, leading to an exponential increase in DNA copies.

DNA sequencing by capillary electrophoresis

DNA sequencing by capillary electrophoresis is used to determine the precise order of nucleotide bases in a molecule of DNA. Before capillary electrophoresis, the DNA is

amplified using a method called cycle sequencing, involving successive rounds of denaturation, annealing, and extension in a thermal cycler. Different dyes are attached to the dideoxyribonucleotides (ddNTPs) that are present in each reaction mix. The fluorescently labeled ddNTPs randomly terminate DNA synthesis, creating DNA fragments of varying lengths. DNA template, primer, buffer, the four dNTPs, the four fluorescently labeled ddNTPs, and Taq polymerase are added to the reaction tube. Fluorescent fragments are generated by incorporation of dye-labeled ddNTPs, each ddNTP (A, C, G and T) carries a different dye color, and all terminated fragments contain a dye at their 3' end. After a sufficient number of cycles to allow for optimal generation of extended products, the reaction is purified, before elecrophoresis is undertaken on a capillary electrophoresis-based Genetic Analyzer from Applied Biosystems. During capillary electrophoresis, products of the sequencing reaction are injected into polymer-filled capillaries, and high voltage is applied in order for the negatively charged DNA fragments to move through the polymer toward the positive electrode. Shortly prior to reaching the positive electrode, the fluorescently labelled DNA fragments, which are separated by size, move through the path of a laser beam, which causes the dyes on the fragments to fluoresce. The fluorescence is detected and the data is subsequently collected and converted to digital data, and saved as a sample file of raw data. Using software from Applied Biosystems, the data is then translated into the corresponding nucleotide bases.

Paper I uses sequencing to investigate the PITX3 gene in 23 PD patients.

Sequenom genotyping

The Sequenom (San Diego, CA, USA) iPLEX Gold assay is a universal method for detecting polymorphisms in genetic material (Little *et al*, 1997). The Sequenom MassARRAY Designer software was used to design both PCR and MassEXTEND primers for the multiplexed reactions. After the multiplex PCR (containing DNA, buffer, $MgCl_2$, dNTP, PCR primers and enzyme) unincorporated dNTPs in the PCR product were neutralized using shrimp alkaline phosphatase (SAP). The SAP cleaves a phosphate from the unincorporated dNTPs, converting them to dNDPs and rendering them unavailable to future reaction. Thereafter, a single base primer extension reaction (MassEXTEND primer) is used to detect differences at the SNP level: a reaction cocktail containing primer, enzyme, buffer, and mass-modified nucleotides is added to the amplification products and subsequently thermocycled. During theromcycling, mass-modified nucleotides are added into the SNP site by primer extension by one of the nucleotides. The resulting allele-specific extension products of different masses are desalted and analysed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Mass spectrometry genotyping typically provides a high signal-to-noise ratio that results in a low rate of false-positives. Nevertheless, genotype results were assessed and verified using Sequenom MassARRAY TyperAnalyzer software, version 1.0.1.46, prior to statistical analysis (Ehrich *et al*, 2005; Gabriel *et al*, 2009; Stanssens *et al*, 2004).

Paper II, III and V use Sequenom technology to genotype SNPs in LMX1A, LMX1B, BDNF and PITX3.

Pyrosequencing genotyping

Pyrosequencing (Qiagen, Valencia, CA, USA) is a DNA sequencing technique that relies on detection of DNA polymerase activity together with the chemiluminescent enzyme luciferase. Pyrosequencing performs sequencing of a single DNA strand by synthesizing the complementary strand, one base pair at a time. Since the added nucleotide is known, the sequence of the template DNA can be determined. All primers used for pyrosequencing were designed with Biotage Assay Design Software, version 1.0.6. Following PCR amplification of the DNA template containing the sequence to be analyzed, single stranded DNA template is immobilized on streptavidin-coated beads and hybridized to a sequencing primer. A vacuum preparation workstation is used to process samples from PCR products to single-stranded sequencing templates in a 96 well format. Nucleotides are then added and incorporated in the complementary strand by DNA polymerase, releasing pyrophosphate in the process. ATP sulfurylase converts the pyrophosphate to ATP in the presence of adenosine 5´ phosphosulfate. The ATP provides the energy for luciferase to oxidase luciferin, generating visible light in the process. This light is proportional to the number of incorporated nucleotides, which are added and removed, by degradation with apyrase, after each reaction. Light is only produced when the nucleotide added complements the first unpaired base of the template. A Pyrosequencing PSQ 96MA machine, containing a light detection system capable of analyzing small amounts of DNA, was used to read DNA (Ronaghi *et al*, 1996; Ronaghi *et al*, 1998).

Pyrosequencing was used to genotype SNPs in the PITX3 gene in Paper I.

Agarose gel electrophoresis

Gel electrophoresis is a technique that is used to separate DNA fragments according to size. When DNA fragments containing a repeat polymorphism is amplified using PCR, the resulting products differ in size according to number of repeats, which can be used to genotype repeat polymorphisms. PCR products are mixed with a loading buffer (bromophenol blue) and placed in wells in the agarose polymer gel, positioned in a buffer, and an electric current is applied. A molecular weight marker, consisting of DNA fragments of known molecular weight, is added for reference. Because of the negative charge of the DNA molecules, the electric charge causes the DNA to move through the gel matrix towards the positive electrode. Smaller DNA fragments travel farther in the gel, and PCR products can thus be separated according to size. DNA bands in the gel are then visualized by ethidium bromide, which together with DNA fluoresces under ultraviolet light (Serwer, 1989).

Paper V uses gel electrophoresis to genotype the SLC6A3 repeat polymorphism.

Neuroimaging methods

Positron emission tomography

Positron emission tomography (PET) is an imaging technique that detects gamma rays emitted indirectly by a positron-emitting radionuclide (or tracer). Prior to PET scanning, subjects are placed in the scanner and inserted with a venous catheter for tracer

injections. Tracers are typically radioactive isotopes with short half-lives, in neuroimaging ^{15}O is usually used, which has a half-life of around two minutes. The radioactive isotopes are subsequently incorporated into a biologically active molecule (*e.g.* H_2O) before being injected into the body of the patient. PET using the tracer [$H_2{}^{15}O$] is able to measure brain activity since the binding potential of [$H_2{}^{15}O$] is correlated with regional cerebral blood flow (rCBF), which in turn is closely linked to neuronal activity. PET recording starts after a brief waiting period when the tracer molecule becomes concentrated in the tissues under study. When the radioactive tracer decays, it emits a positron that, when colliding with an electron, releases two gamma rays in exact opposite directions. These are detected when they reach a scintillator in the scanner, emitting light in the process, which is subsequently detected by the scanning device. The relatively short half-life of the tracer ^{15}O is an advantage since less time (*i.e.* around 30 minutes) is needed in order to record a sufficient number of signals. A map, illustrating the parts of the brain where the tracer has become concentrated, is then constructed and normalized according to a stereotactic template.

Paper V uses PET to investigate the effect of a SLC6A3 polymorphism on amygdala activity.

Functional magnetic resonance imaging

fMRI is a non-invasive neuroimaging method, with a relatively high spatiotemporal resolution. It relies on the paramagnetic properties of oxygenated and deoxygenated hemoglobin in order to visualize changes in blood flow that is associated with neuronal activity in the brain. Many nuclei possess a quantum mechanical property called spin, which are normally randomly oriented. However, a powerful external magnetic field can be applied to align the nuclear magnetization (*e.g.* of hydrogen atoms in water) in the body. Radio frequency fields are used to systematically alter the alignment of this magnetization, causing the nuclei to produce a rotating magnetic field detectable by the scanner. Activity in the brain can be detected with MRI via direct measurements of blood oxygenation. Active neurons require a lot of oxygen and blood releases oxygen to active neurons at a greater rate than to inactive neurons (*i.e.* hemodynamic response). The hemoglobin of the blood is diamagnetic when it is oxygenated and paramagnetic when it is deoxygenated. Therefore, the magnetic resonance signal of blood differs according to the level of oxygenation. This form of MRI is known as blood oxygenation level dependent (BOLD) fMRI. By collecting data with an MRI scanner that is receptive to changes in magnetic susceptibility one can assess changes in BOLD contrast. These changes can be either positive or negative depending on the relative changes in both blood flow and oxygen consumption. BOLD functional images can be acquired with relatively good temporal and spatial resolution (Logothetis, 2008).

Paper V uses fMRI to investigate the effect of a SLC6A3 polymorphism on amygdala activity

Statistics

The p-value

In statistical hypothesis testing, the output is accompanied by a significance level called p-value, which is the probability of obtaining the observed results (*e.g.* genotype frequencies), assuming that the null hypothesis is true. In order to reject the null hypothesis this probability needs to be low, since a high probability for the observed deviation from the expected values would suggest the null hypothesis to be true. A threshold is usually set prior to testing; if the test results in a p-value smaller than this value, the null hypothesis can be rejected and the alternative hypothesis can be accepted. The threshold is often set to 0.05 or 0.01, corresponding to a 5% or 1% chance of an identical outcome to the observed one, given the null hypothesis to be true. A threshold set too high increases the risk of false positives. In contrast, if set too low, the power of finding true effects will decrease.

Hardy-Weinberg principle

The Hardy-Weinberg principle is a concept in population genetics that relates the gene frequency to the genotype frequency. It states that both allele and genotype frequencies in a population are in equilibrium, which can be calculated with the equation $p^2+2pq+q^2=1$, where p and q indicate frequency of allele A and a, respectively.

Chi-square test (Paper I, II and V)

A chi-square test for independence is used to explore the relationship between two categorical variables (*e.g.* genotype and diagnosis), each containing two or more categories. A test chi-square test for independence determines whether paired observations on two variables are independent of each other. It tests whether the null hypothesis that the frequency distribution observed in a sample is consistent with a theoretical chi-square distribution, *i.e.* the test compares the observed proportions of cases that occur in each category with the expected value if there is no association between the two variables. One limitation for chi-square tests is that the approximation to the chi-square distribution is not valid if expected frequencies are too low (*i.e.* expected frequencies below 5). Moreover, chi-square tests are not optimal for analysis of 2 by 2 tables, in which case we have used Fisher's exact test. SPSS software, version 17.0 (SPSS 17.0; SPSS Inc., Chicago Il.) or Haploview software, version 4.1 was used in order to perform the tests.

Fisher's exact test (Paper I and V)

Fisher's exact test is a statistical test of independence that tests the independence of rows and columns in a 2 by 2 contingency table of categorical data. It is based on the exact sampling distribution of the observed frequencies. It is called an exact test since the significance of the deviation from a null hypothesis can be calculated exactly. SPSS software, version 17.0 was used in order to perform the test.

Kaplan-Meier analysis (Paper I)

The Kaplan-Meier test is usually used to estimate survival function from life-time data (*e.g.* survival time after treatment), and used in order to compare the survival of two or more groups. A Kaplan–Meier plot will normally have percent survival on the y-axis and elapsed time after treatment on the x-axis, survival curves showing, for each time plotted on the x-axis, the portion of all individuals surviving as of that time. In Paper I, a Kaplan–Meier analysis was performed using GraphPad Prism software, version 4.0, to clarify differences in age at onset of PD as a result of PITX3 rs4919621 genotype. PD patients were grouped into categories according to age at onset and genotype. The result confirmed that the AA genotype is associated with an earlier age at onset than the other two genotypes of the rs4919621 SNP.

Logistic regression (Paper II)

Regression analysis is a method for obtaining a regression equation, in which the dependent variable is a function of the independent (explanatory) variables, and parameters are estimated (usually by the least squares method) as to best fit the different values of the dependent and independent variables. Logistic regression is useful in describing a relationship between one or more risk factors (*e.g.* age, allele) and a categorical outcome. The independent variable can be either continuous or categorical. When a dichotomous dependent variable is included, as in Paper IV, an SPSS procedure labelled binary logistic regression was used. It is used for predicting the probability of occurrence of an event by fitting data to a logistic curve, *i.e.* it assesses a set of predictor variables to see if these predict or explain a categorical dependent variable, by assessing goodness of fit. It allows for the calculation of both the sensitivity and specificity of the model and the predictive values, in the form of odds ratios. The odds ratio represents the change in odds of being in one of the categories of outcome when the value of a predictor increases by one unit. Odds ratios are usually reported with a 95 per cent confidence interval, which is the range of values that with 95 per cent confidence encompasses the true odds ratio value.

Multivariate regression (Paper IV)

Multivariate regression is used in situations that include more than one dependent variable, while taking into account several predictive variables simultaneously. The dependent variables in multivariate regression should be related in some way (*e.g.* different aspects of some variable). In contrast to multiple regression analysis, which includes one dependent variable, the model in multivariate regression contains matrices (in stead of vectors). Multivariate regression is defined by the general linear model: Y=XB+E. The first column in matrix Y gives the scores of the first dependent variable, second column gives the score of the second dependent variable etc. The X matrix contains the factors, the B matrix contains the set of factor coefficients (model parameters) and the E matrix contains the noise terms. Paper IV used multivariate regression models to investigate associations between the BDNF Val66Met genotype and continuous scales of hyperactivity-impulsivity and inattention, using SAS software, version 9.2 (SAS Institute, Inc., Cary, NC).

Analysis of variance (Paper V)

Analysis of variance (ANOVA) is an inferential statistical test that is founded on the general linear model. ANOVA allows for the comparison of mean scores of a continuous dependent variable in three or more groups of a categorical independent factor (*e.g.* genotype). An ANOVA compares the variance in scores between the different groups with the variability within each of the groups. An F ratio is calculated that represents the variance between the groups, divided by the variance within groups. A large F ratio thus indicates more variability between groups, which is caused by the independent variable, than chance variability within groups. Given a significant F value, the null hypothesis stating that the group means are equal, can be rejected. Analysis of covariance (ANCOVA) is an extension of ANOVA that allows one to explore differences between groups while controlling for an additional variable, called covariate. ANCOVA removes the variation in the dependent variable that is caused by the covariate and then performs an ANOVA on the corrected scores.

Statistical procedures for neuroimaging data (Paper V)

Data in Paper V as well as from other neuroimaging studies involving PET or fMRI are usually analysed using statistical parametric mapping (SPM). SPM identifies specific regional effects (*e.g.* brain activity) when studying function or disease-related changes. The end result is a statistical parametric map that shows the significance of regional effects. SPM analysis is voxel-based, each voxel is a volume element that represents a value on a regular grid in three dimensional space. Prior to statistical analysis, data are realigned, spatially normalised into a standard anatomical space and smoothed. After smoothing, a general linear model is employed to estimate the parameters of a temporal model (encoded by a design matrix that contains the explanatory variables or covariates) and derive the appropriate test statistic at every voxel. The general linear model can be used to apply a number of statistical analyses. The SPM test statistic (*e.g.* t-test) allows one to test the null hypothesis that a given contrast of means is zero. The final stage is to make statistical conclusions on the basis of the SPM, using random field theory to provide a method for p-value adjustment (Friston *et al*, 1994).

ACKNOWLEDGEMENTS

I hereby express my sincere gratitude to my supervisor Elias Eriksson, and co-supervisors Hans Nissbrandt and Lars Westberg, for the support and advice they have provided throughout the duration of my PhD, for which I am truly grateful.

I am also indebted to the other members of Elias Eriksson's research group with whom I have had the privilege of working with over the years:

Agneta Ekman, Inger Oscarsson, Petra Suchankova-Karlsson, Jonas Melke, Jessica Bah-Rösman, Kristina Annerbrink, Gunilla Bourghardt, Monika Hellstrand, Britt-Marie Benbow, Benita Gezelius, Carolyn Johnson, Jakob Näslund, Erik Studer and Susanne Henningsson.

I also direct my gratitude to collaborators and co-authors for their help and advice, and would particularly like to thank:

Anna Zettergren, Andrea Carmine Belin, Mats Fredrikson, Tomas Furmark, Fredrik Åhs, Paul Lichtenstein and Henrik Larsson.

Moreover, I would like to thank all my colleagues at the Department of Pharmacology that I have had the great pleasure of getting to know, especially:

Erik Pålsson, Daniel Klamer, Caroline Wass, Kim Fejgin, Elisabet Jerlhag, Elin Löf, Sara Landgren and Daniel Andersson.

Finally, I would like to thank Kristina and Edvin for being the light of my life.

This PhD project was supported by the Swedish Brain Power initiative.

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