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Candidate Genes and the Dopamine System

Possible Implications in Complex Neurological and Psychiatric Disease

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ABSTRACT

The mesencephalic dopamine (DA) system is strongly involved in the pathogenesis of Parkinson's disease (PD), where symptoms occur after the loss of the vast majority of DA neurons. An involvement of this system also in schizophrenia (SZ) and bipolar disease (BD) is suggested by the fact that DA receptor antagonists are among the most potent drugs in clinical use for the treatment of these conditions. Basic science of the past years has provided several clues for how DA cells develop and under which conditions they thrive. Different approaches include research on neurotrophic factors (providing target-derived support for DA cells), neuropeptides (modulators of neurotransmission), transcription factors (activating a large number of "downstream" genes important for development and maintenance of the cell) and endogenous as well as environmental neurotoxins.

This thesis describes an effort to link basic science knowledge of the type described above to clinical samples employing the candidate gene approach. Analyzing genes encoding the above-mentioned proteins in material from patients suffering from PD, SZ and BD aims at establishing direct links between mutations in such genes and susceptibility for the disorders.

The *CALCA* locus is one of the first and by now most well-characterized examples of alternative splicing of genes in humans. While the calcitonin protein is localized to C-cells of the thyroid gland, the alternatively spliced protein CGRP is a neuropeptide with multiple functions within the brain. We identified several new polymorphisms in this locus and investigated association within available materials from PD, SZ and BD patients. No significant associations were found with these disorders. However, the report of the sequence variations identified in the gene may still be valuable markers, since CGRP has been strongly implicated both in hypertension and migraine.

NURR1 is a nuclear receptor being absolutely essential for the development of mesencephalic dopamine neurons. Three different unique missense mutations were identified in patients suffering from psychosis (two in SZ, one in BD). All three mutations were located within 78 bp from each other in a region of exon 3 and disturbed NURR1-mediated transcription similarly when modeled *in vitro*. Since the *NURR1* gene is highly conserved between humans and rodents, introduction of the identified mutations in transgenic animals may lead to a valid model for psychosis susceptibility.

The *ADH4* gene codes for an alcohol dehydrogenase (ADH) that converts retinol to retinal and also acts upon a wealth of other alcohols and aldehydes, some of which have been implicated in the pathogenesis of PD as endogenous or exogenous neurotoxins. We found significant association between one of several polymorphisms identified in this gene and idiopathic PD. In a follow-up study of a larger material several genes within the ADH cluster including *ADH4* were analyzed. The previously found association of *ADH4* with PD remained significant, while other polymorphisms did not show significant association. A nonsense mutation in *ADH1C* was identified in three (of 123) PD patients but none of 127 control individuals.

An effort was also made to characterize the usefulness of available postmortem material for immunohistochemistry. Using a plethora of different antibodies, we could confirm the feasability of postmortem studies on the protein level and found a new marker for corpora amylacea, namely nestin. Since bodies made of aggregated proteins play roles in a number of neurodegenerative disorders, we went on to characterize corpora amylacea further with antibodies against alpha-synuclein, PGP9.5 and other (vimentin, NF)neurofilaments.

Expression of different alcohol and aldehyde dehydrogenases was also studied in postmortem human brain tissue using *in situ* hybridization. High and specific expression of an aldehyde dehydrogenase (ALDH1) was found in human DA cells. Furthermore, *ALDH1* mRNA levels were found to be strongly decreased in remaining dopamine neurons of substantia nigra of PD patients as compared to controls. Other DA neurons (of the ventral tegmental area) showed normal levels of expression, indicating a selective decrease of ALDH1 mRNA levels in neurons degenerating in PD.

To summarize, evidence for the involvement of alcohol- and aldehyde dehydrogenases as well as the nuclear transcription factor NURR1 in the pathogenesis of the diseases of interest was found in the available material. Further studies along these lines using larger materials employing more markers and probes are warranted.

Keywords: Genetic, linkage, association, dopamine, Parkinson's disease, schizophrenia, bipolar disease, retinoid, aldehyde, toxicity.

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That these friends to humanity and medical science, who have already unveiled to us many of the morbid processes by which health and life is abridged, might be excited to extend their researches to this malady, was much desired; and it was hoped, that this might be procured by the publication of these remarks.

Should the necessary information be thus obtained, the writer will repine at no censure which the precipitate publication of mere conjectural suggestions may incur; but shall think himself fully rewarded by having excited the attention of those, who may point out the most appropriate means of relieving a tedious and most distressing malady.

—James Parkinson, *"An Essay on the Shaking Palsy" (1817)*

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ABBREVIATIONS

+/+	Homozygous wildtype
+/-	Heterozygous
-/-	Homozygous mutant
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
APOE	Apolipoprotein E
BD	Bipolar disease (manic depressive disorder, bipolar affective disorder)
CA	Corpora amylacea
COMT	Catechol-o-methyltransferase
CGRP	Calcitonin gene-related peptide
DA	Dopamine
DBH	Dopamine-beta hydroxylase
DOPAL	3,4-Dihydroxyphenylacetaldehyde
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell-line derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
ISH	*In situ* hybridization
MAO	Monoamine oxidase
NF	Neurofilament
NURR1	Nur-related receptor 1 (=NR4A2)
OMIM	The Online mendelian inheritance in man database
PCR	Polymerase chain reaction
PD	Parkinson's disease
PGP9.5	Protein-gene-product 9.5 (=Ubiquitin carboxyl-terminal esterase L1 [UCHL1]
PMI	Postmortem interval
RA	Retinoic acid
RXR	Retinoid-X-receptor
SN	Substantia nigra
SNP	Single nucleotide polymorphism
SZ	Schizophrenia
TH	Tyrosine hydroxylase
TIQ	Tetrahydroisoquinoline
VTA	Ventral tegmental area

NOTES ON THE NOMENCLATURE

Alleles and haplotypes

The expression "allele" was first used to describe a certain variant of a gene that may differ from other variants of the same gene not only in one but in multiple ways. A frequently cited example is the APOE4 allele associated with Alzheimer's disease (Saunders et al. 1993), which differs by two sequence changes (C112R and C158R) from the APOE2 allele. Since one gene (including its promoter and other regulatory sequences) constitutes a functional unit, speaking of alleles in this way emphasizes that single-nucleotide polymorphisms (SNPs) clustered in a certain gene lead to an effect that is the sum of all effects of individual polymorphisms. It is therefore that the combinations of polymorphisms in ADH4 described in this thesis (Paper II) are referred to as "alleles." Haplotypes, according to this nomenclature, refer to combinations of SNPs across several genes but not within the same gene.

Subsequently, however, it has become common practice to refer to any combination of SNPs as a "haplotype," even if the SNPs are located within the same gene. This convention was adopted during the course of the present work, explaining the use of the word "haplotype" rather than "allele" to describe the observed combinations of polymorphisms within the NURR1 promoter (Paper V). The term "allele" is then used to describe the different variants (also called "flavors") of one singlepolymorphism (e.g. the "A" allele or the "G" allele).

Alcohol and aldehyde dehydrogenase nomenclature

The following table aims at resolving the apparent confusion regarding the nomenclature of these enzymes. This thesis employs the recent class-based nomenclature (Duester et al. 1999).

Gene Name	*Gene symbol in thesis*	*Alternative or old name*	*Subcellular localization*	*Chromosomal localization*	*References*
Alcohol dehydrogenase class I, beta subunit	ADH1B	ADH2	Cytoplasm	Cluster on chromosome 4q21-q23	Duester et al. 1984, 1986
Alcohol dehydrogenase class I, gamma subunit	ADH1C	ADH3	Cytoplasm	Cluster on chromosome 4q21-q23	Duester et al. 1986, Höög et al. 1986
Alcohol dehydrogenase class III	ADH3	ADH5	Cytoplasm and nucleus	Cluster on chromosome 4q21-q23	Sharma et al. 1989
Alcohol dehydrogenase class IV	ADH4	ADH7	Cytoplasm	Cluster on chromosome 4q21-q23	Yokoyama et al. 1994, Zgombic-Knight et al. 1995
Aldehyde dehydrogenase cytosolic isoform	ALDH1	ALDH2 (!)	Cytoplasm	9q21	Hsu et al. 1985, 1986

PAPERS

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

I. Buervenich S, Xiang F, Sydow O, Jönsson EG, Sedvall GC, Anvret M, Olson L. Identification of four novel polymorphisms in the calcitonin/alpha-CGRP (CALCA) gene and an investigation of their possible associations with Parkinson disease, schizophrenia, and manic depression. Human Mutation (2001) 17(5): 435-436

II. Buervenich S, Sydow O, Carmine A, Zhang Z, Anvret M, Olson L. Alcohol dehydrogenase alleles in Parkinson's disease. Movement Disorders (2000) 15(5): 813-828

III. Buervenich S, Carmine A, Galter D, Matsuura T, Ashizawa T, Klockgether T, Wüllner U, Anvret M, Sydow O, Olson L. Further investigations of polymorphisms within the alcohol dehydrogenase cluster on chromosome 4q21-q23 in Parkinson's disease. Manuscript

IV. Buervenich S, Carmine A, Arvidsson M, Xiang F, Zhang Z, Sydow O, Jönsson EG, Sedvall GC, Leonard S, Ross RG, Freedman R, Chowdari KV, Nimgaonkar VL, Perlmann T, Anvret M, Olson L. NURR1 mutations in cases of schizophrenia and manic-depressive disorder. American Journal of Medical Genetics (2000) 96(6): 808-813

V. Carmine A, Buervenich S, Galter D, Jönsson EG, Sedvall GC, Farde L, Gustavsson JP, Bergman H, Anvret M, Olson L. Polymorphisms within the NURR1 (NR4A2) promoter and untranslated regions in schizophrenia and personality traits. Manuscript

VI. Buervenich S, Olson L, Galter D. Nestin-like immunoreactivity of corpora amylacea in aged human brain. Brain Research: Molecular Brain Research (2001) 94(1-2): 204-208

VII. Galter D, Buervenich S, Carmine A, Anvret M, Olson L. Differential expression of cytosolic aldehyde dehydrogenase (ALDH1) in Parkinson's disease, schizophrenia and control subjects. Manuscript

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INTRODUCTION

Complex disease

While longstanding efforts within many fields of research on complex diseases such as Parkinson's disease, schizophrenia and bipolar disease have led to a better understanding of underlying pathologies, the conclusion that "single unifying theories" for the etiologies of these diseases do not exist seems inevitable. Instead, many different factors are likely to contribute and combine to constitute a number of possible disease "pathways." The identification of any such pathway, activated at least in subgroups of individuals diagnosed with often devastating neurological or psychiatric disorders, is a major challenge for multidisciplinary research.

Genetically "simple" versus complex disorders

Genetics is one necessary component of any concerted effort towards a better understanding of the pathology of complex disorders, and as such is not a new one. Long before the arrival of modern molecular genetic techniques and strategies, familial aggregation of disease was observed and documented and heritable traits were identified (Adams 1814). Many such traits were later understood to follow mendelian patterns (Mendel 1865)of inheritance. The collection of descriptions of mendelian disorders (McKusick 1998) has developed into a large database, the Online Mendelian Inheritance in Man (OMIM), which is publicly available (OMIM 2000).

OMIM also contains entries for many characterized genes. Since this dissertation is written in the "post-genomic" era (Lander et al. 2001, Venter et al. 2001), it is worth mentioning that locating a gene within the human genome has not always been as easy as "looking it up in the database" (http://www.ensembl.org/; http://genome.cse.ucsc.edu/; http://www.celera.com). Before the arrival of a physical map (i.e. the exact sequence of the four bases constituting the genetic alphabet) of the human genome, genes were mapped to certain chromosomal locations by hybridization techniques and segregation analysis (Weiss and Green 1967). Segregation of traits (such as eye color) in consecutive generations of a pedigree is caused by segregation of genes; researchers use linkage analysis (see Schulze and McMahon 2002 for a review of different tests for linkage) in order to map genes for disease traits based on this principle.

The identification of the gene for Huntington's disease in 1993 (The Huntington's Disease Collaborative Research Group 1993) constitutes one major success of this approach

in a monogenetic (="simple") disorder. Based on the observation of the autosomal-dominant pattern of inheritance, a small piece of the human genome that followed the trait (chorea) was defined and the pathogenetic expansion of a glutamine-encoding repeat within this sequence was identified by positional cloning. Gene defects have been reported using this approach for a large number of monogenetic disorders.

In complex diseases, a few of the numerous affected individuals belong to kindreds in which the pattern of inheritance appears to follow mendelian patterns. It is therefore that entries for Alzheimer's disease, Parkinson's disease and also for schizophrenia can be found in OMIM. Identification of pedigrees showing regular mendelian inheritance and linkage analysis in these pedigrees is an important step towards identification of genetic defects that may point to pathogenic pathways (Gershon et al. 2001).

Linkage versus association studies – or both?

The power of linkage analysis is limited by several factors. First, the diagnosis of each individual of a pedigree must be accurate. Knowing which individuals are affected is often easier than finding definitely unaffected individuals, and the presence of phenocopies may even lead to "false-positive" diagnoses (Hughes et al. 1992). Exact diagnoses are especially difficult to obtain when the age of onset is variable, as is often the case for complex diseases.

Second, the numbers of individuals in a given pedigree is important, as is the number of generations to which these belong. This limitation can only be overcome with time by collecting DNA material from additional subjects of a given family.

Finally, the number of available markers within the genome is crucial. The time during which the present work was carried out saw a transition from microsatellite (i.e. repeat) markers located in intergenic regions towards single-nucleotide polymorphisms (SNPs), preferably located within coding regions of genes. As a result, the density of markers will soon no longer be a significant limitation.

Linkage analysis can be carried out without an *a priori* suspicion regarding a possible pathogenic pathway for the disorder under investigation ("hypothesis-free research"). However, every linkage study comes to a point where all available samples are investigated using all known markers. The resulting chromosomal region known to contain the disease-causing gene may harbor hundreds of genes rather than one. Without knowing anything about possible disease mechanisms, all genes within the region become equally good candidates for mutation analysis. In this situtation it is rarely feasible to analyze all candidates by mutation screening. Rather, genes within the critical interval are scrutinized for their possible functional relevance in the pathogenesis of the disease of interest. Those genes with the most obvious relationships to affected organ systems are screened first. The smaller the chromosomal interval, the less important it becomes to identify the correct candidate, since even a random sequencing approach will eventually identify the muta-

tion. A successful "intelligent guess" can, however, save labor-intense and cumbersome mutation searches when the candidate interval is large and contains many genes.

In complex disease, linkage analysis can be performed in those pedigrees showing possible mendelian patterns of inheritance, but numbers of available pedigees of this kind are few and diagnoses are difficult to establish with certainty. Linkage regions obtained in complex disorders are therefore often large and contain multiple possible candidate genes.

One important role of the candidate gene approach is thus to help select among genes at a locus identified by linkage analysis, accelerating the identification of disease genes. In addition, the candidate gene approach allows one to omit the first step (=linkage) altogether and instead directly select promising candidate genes based entirely on their presumed functions in systems of interest. In this approach, disease-causing mutations are not followed through generations of pedigrees, but are expected to be found at higher frequencies in affected individuals than in matched controls. This type of candidate gene study thus constitutes a population-based approach as opposed to the family-based design for linkage. The estimated number of functional genes in the human genome has dropped from earlier estimates of about 80,000 possible genes (Antequera and Bird 1993) to about 35,000 today (Ewing and Green 2000). Many of these 35,000 genes, however, generate multiple products due to alternative splicing (see for example the calcitonin/α-CGRP gene investigated in Paper I). Even considering the decreased number of genes, one large disadvantage of candidate studies isthe vast number of possible candidates.

The present work on candidate genes of importance for the dopamine system utilizes a candidate gene approach that takes advantage of linkage studies carried out by other investigators by giving priority to candidate genes located in previously identified chromosomal regions of interest.

Genes in Parkinson's disease

Parkinson's disease is a neurological disorder that received its name after publication of an essay (Parkinson 1817) aimed at bringing a complex of symptoms ("shaking palsy") which was suggested to belong to one disease entity to the attention of scientists (see quotation on page iii). James Parkinson succeeded with his mission, and considerable research efforts have been undertaken since then to understand what causes this severe neurological disorder. Thus, around the beginning of the last century, it was discovered that substantia nigra in the midbrain degenerates in Parkinson's disease patients (Trétiakov 1919). The identification of dopamine as a transmitter in the human brain in the 1950s (Carlsson et al. 1957, 1958) provided the basis for the observation that degenerating neurons of substantia nigra (SN) use dopamine (DA) as a neurotransmitter, and that DA is lacking in striatum of patients with Parkinson's disease (Ehringer and Hornykiewicz 1960). Introduction of substitution therapy based on L-Dopa (a dopamine precursor that passes the blood-brain barrier) into the clinical treatment of patients was a milestone in the therapy of Parkinson's disease (Birkmayer and Hornykiewicz 1961); L-Dopa has remained the

mainstay therapy. In addition to L-Dopa and complementary neuropharmacological medication, replacement of lost neurons by cell transplantation strategies as well as rescuing affected neurons by treatment with neurotrophic factors holds promise (see Olson 2000). However, to this day it is still not understood why the DA neurons die. Substitution of DA only fights symptoms but does not stop progression of disease.

Figure 1. A classical illustration of the posture of a patient suffering from Parkinson's disease. The cardinal symptoms are bradykinesia, rigidity, tremor and postural instability. (From Gowers, 1893)

One rather optimistic view regarding the pathogenesis of Parkinson's disease was put forward during the middle 1900s. It was assumed that there was a direct link between idiopathic Parkinson's disease and the van Economo epidemic at the beginning of the century that caused post-encephalitic parkinsonism, and that virtually no more Parkinson's disease cases would be seen by the 1980s (Poskanzer and Schwab 1961). Unfortunately this assumption was not correct. Genetic studies may help provide clues to the underlying causes, although it is still a matter of debate whether or not genetic factors are involved at all in the majority of Parkinson's disease cases. Thus, it is difficult to distinguish between true heritable risk factors on the one hand and environmental factors running in families on the other.

It has, however, become clear that defects in genes can cause symptoms that are at least very similar to the cardinal symptoms of idiopathic Parkinson's disease (by definition, cases of familial Parkinson's disease linked to a known genetic defect can no longer be called idiopathic Parkinson's disease!) (Polymeropoulos et al. 1997, Krüger et al. 1998, Kitada et al. 1998, Farrer et al. 1999, West et al. 2001, van Duijn et al. 2001, Valente et al. 2001). Even if heritable genetic factors turn out not to play a role in the majority of cases of Parkinson's disease, identification of genetic defects in a subset of cases may provide evidence that may simplify the identification of environmental influences. Endogenous pathways identified in familial Parkinson's disease that may constitute targets for such exogenous factors have recently been reviewed (Giasson and Lee 2001).

Genes and schizophrenia and bipolar disease

Descriptions of "madness" date back very far, but religious or magic interpretation of symptoms hampered a scientific approach towards understanding of the underlying pathology. Similarly to what was said for Parkinson's disease, systematic description and classification of schizophrenia and bipolar disease in the beginning of the 1900s (Kraepelin 1909, Bleuler 1911, Schneider 1923) triggered multidisciplinary research efforts, and also led to today's distinction of the two diseases as separate entities. There is, however, a considerable overlap of symptoms, and several linkage sites common to the two diseases have been reported (Taylor 1992, Berrettini 2000).

A heritable genetic contribution to the pathogenesis of schizophrenia and affective disorders is more widely accepted (Eisenberg 1968) than such a contribution to Parkinson's disease. Thus, "classical" twin and adoption studies (Essen-Möller 1941, Kallman 1946, Heston 1966, Inouye 1972, Mendlewicz and Rainer 1977, Cadoret 1978) point out a major role for heritable risk factors. Linkage studies in schizophrenia in pedigrees with strong heritability have pointed to many different loci in the human genome (Shastry 2002). Very few of these studies have been reproduced in independent materials, leaving a large number of candidate gene regions in need of further investigation. Moreover, since concordance in monozygotic twins for schizophrenia or bipolar disease does not reach anywhere near 100 percent, it appears that other factors, environmental or psychosocial, must contribute to the pathogenesis.

Because psychotic episodes, hallmarks of schizophrenia but also present in a subset of bipolar disease patients, can be treated by anti-dopaminergic drugs, the DA system has been implicated in the pathogenesis of these disorders (the "dopamine hypothesis") (Carlsson et al. 2001). Many investigators carrying out candidate gene studies have therefore concentrated on DA-related genes, for example DA receptors and the DA transporter (Jönsson et al. 1993, 1999, Li et al. 1994, Arinami et al. 1996, 1997, Griffon et al. 1996, Asherson et al. 1998, Spurlock et al. 1998, Laruelle et al. 2000). In the present study, etiologic involvement of disturbances of dopaminergic neurotransmission is assumed for at least a subset of patients. Thus, candidates are chosen from genes likely to be of importance for the DA neuron system and/or DA neurotransmission.

Figure 2. Early-stage schizophrenia as illustrated by a patient (The Cunningham Dax Collection of Psychiatric Art, 1999.320)

Endophenotypes and "dissection" of genetic disorders

The principle for the search for endophenotypes can be applied to any complex disorder. Thus, if the disorder "as a whole" is too complex to study by linkage analysis, one may try to find phenotypic markers that associate with disease and are inherited according to mendelian patterns. Possible measurable endophenotypes suggested to associate with schizophrenia include an auditory gating deficit (Freedman et al. 1997), DA receptor densities in striatum (Wong et al. 1986) and cerebrospinal fluid DA metabolites (Sedvall and Wode-Helgodt 1980). Furthermore, certain personality traits thought to be heritable have been associated with Parkinson's disease (Todes and Lees 1985) as well as schizophrenia (Stephens et al. 1975) and bipolar disease (Akiskal et al. 1983). Genes determining an endophenotype can be mapped by linkage and/or identified by association studies. Obviously, identification of mutations in genes that code for associated endophenotypes may provide clues regarding pathogenic pathways.

Another approach to genetic subclassification of disease is to map certain symptoms (occurring in affected individuals) rather than whole diseases. Thus, relevant to the present work, psychosis occurs as a symptom both in schizophrenia and in affective disorders. While presence of psychosis is a necessary criterion for the diagnosis of schizophrenia, it is not seen in all individuals diagnosed with affective diseases. Selecting those individuals with bipolar disease who exhibit psychotic symptoms and the correlating linkage regions found in their families to linkage regions found in schizophrenia kindreds has recently been suggested as a possible road to identification of susceptibility genes for psychosis (Potash et al. 2001). Once such genes have been identified, one could proceed with this method and discover other influences (environmental or genetic) that lead to the full picture of disease seen in the patient.

Finally, attempts are being made to find susceptibility genes for subdiagnoses of disease, such as catatonic versus paranoid schizophrenia (Stober et al. 2000) and type I versus type II bipolar disease (Foroud et al. 2000). Naturally, the subdivision of patient material reduces the power of genetic studies, demanding large collections of well-characterized samples.

The special vulnerability of the dopamine system

Based on its proven (Parkinson's disease) or likely (psychosis) involvement in severe human disease, the DA neuron system has remained the focus of attention for many neuroscientists. A quest into the causes for disturbances of this system should begin by defining the unique characteristics of DA neurons and how they may interact with environmental factors to cause disease.

The present work emphasizes two distinct features of DA neurons that may constitute inherent weak links in neurodegenerative or psychiatric disease. First, DA cells contain a large amount of DA. Second, these neurons express retinoid-related genes at high levels and may be especially dependent on synthesis of retinoic acid (RA) and RA-mediated signalling. Links between these two properties of DA neurons and Parkinson's disease and schizophrenia are discussed below.

Dopamine as a precursor for endogenous neurotoxic molecules

Dopamine is a catecholamine that can be converted into toxic compounds via several pathways. The ability of DA to condense with formaldehyde is utilized in the Falck-Hillarp method (Falck et al. 1962), which provided a breakthrough in catecholamine research. In this reaction, tetrahydroisoquinolines (TIQs) are formed and then transformed into fluorescent 3,4-dihydroisoquinolines. Using fluorescence microscopy, DA-containing neurons (as well as noradrenaline, adrenaline and serotonin neurons) can be identified and mapped within the brain (Dahlström and Fuxe 1964, Andén et al. 1965). Condensation reactions of DA with other aldehydes such as acetaldehyde lead to the formation of other TIQs, such as salsolinol (Yamanaka et al. 1970). Salsolinol is a neurotoxic substance related to

the parkinsonism-inducing agent MPTP (Niwa et al. 1992). Elevated levels of methyl-salsoninol and a higher activity of salsolinol-methylating enzymes have been reported in Parkinson's disease (Naoi et al. 2000). One conclusion from this observation may be that high levels of reactive aldehydes are especially dangerous for DA neurons, since large amounts of DA are available in the cytoplasm for deleterious condensation reactions. A similar sensitivity may be associated with noradrenaline neurons, for example in locus coeruleus, another nucleus strongly affected in Parkinson's disease pathology.

Another mechanism by which DA may be converted into a toxic agent is oxidation into cytotoxic DA-o-quinones. These highly reactive substances can react with proteins. Antibodies against such proteins have been reported in plasma from patients with Parkinson's disease (Rowe et al. 1998).

Finally, dopamine is metabolized by monoamine oxidase (Blashko and Burn 1951, Blashko 1952) to form dihydroxyphenylacetaldehyde (DOPAL), an aldehyde that has been reported to be toxic to DA neurons *in vitro* (Kristal et al. 2001). This strengthens the hypothesis that a defense system against aldehydes is of particular importance in DA neurons. Such defense systems include aldehyde-detoxifying enzymes located within the DA neurons themselves (to neutralize endogenous aldehydes), as well as enzymes in the circulation and in the gastrointestinal tract (to prevent exogenous aldehydes from entering the brain).

Retinoic acid and dopamine neurons

Retinoids are suggested to be of importance for the DA system because retinoid-handling proteins and enzymes are expressed at high levels there (Zetterström et al. 1994, 1996a,b, 1999). Presence of the retinoid-related receptor Nurr1 is required for DA neurogenesis (Zetterström et al. 1997), and the retinoic acid-generating enzyme Aldh1 is expressed in DA neurons at high levels throughout life (McCaffery and Drager 1994). Insufficient amounts of active vitamin A may lead to reduced activity of retinoic acid receptor-mediated transcription in striatum and, following that, decreased trophic support (e.g. by decreased release of the neurotrophic factor GDNF) for DA neurons. Possible connections between vitamin A-related disease and schizophrenia at genetic and anatomical levels have recently been pointed out (Goodman 1998).

Linking nature (genes) and nurture (environment) in diseases of the dopamine system

Based on assumptions outlined above, genes encoding proteins involved in retinoic acid-related or aldehyde-detoxification pathways were considered candidate genes for Parkinson's disease, schizophrenia and bipolar affective disorder.

Alcohol and aldehyde dehydrogenases are involved in both pathways (Duester 1994, 2001). Due to the broad substrate specificity of these rather promiscuous enzymes, physiologically important pathways may become out-competed by an abundance of toxic agents.

For example, the defects seen in fetal alcohol syndrome may be caused by an occupancy of alcohol and aldehyde dehydrogenases with the metabolism of ethanol, leading to a competitive decrease in retinol metabolism (to retinoic acid) by the same enzymes (Duester 1994). One may speculate that a long-term competitive disturbance of alcohol and aldehyde dehydrogenases may lead to disturbances in retinoic acid synthesis, DA metabolism and toxic aldehyde degradation. Genetic defects in any of these genes may leave the individual with an impaired defense system against such competitors, leading to higher susceptibility for disease.

Nurr1 is a nuclear orphan receptor that can heterodimerize with the retinoic receptor RXR, but can also be active as a homodimer (Perlmann and Jansson 1995, Zetterström et al. 1996a). No ligand has yet been identified, but may well exist, since the sequence of the messenger RNA contains a conserved ligand-binding domain. Nurr1 acts as a transcription factor and belongs to the group of "immediate early genes" (Law et al. 1992, Mages et al. 1994). It is expressed in DA neurons throughout life, and mice lacking Nurr1 fail to develop DA neurons in mesencephalon (Zetterström et al. 1997). Other expression areas include layer VI of the cerebral cortex and the hippocampal formation (Zetterström et al. 1996b). Disturbances in NURR1-mediated transcription may therefore lead to disturbances in the DA system as well as in cortical and hippocampal areas, all of which have been implicated in the neuropathology of schizophrenia. Nurr1 expression is strongly induced by hypoxia in hippocampus (Honkaniemi and Sharp 1996). Perinatal ischemia may thus lead to increased expression of NURR1 causing an imbalance in the amounts of expressed downstream targets. The gene for tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis, has been shown to be regulated by Nurr1 (Iwawaki et al. 2000). Similarly, Nurr1 regulates the gene encoding the DA transporter (Sacchetti et al. 2001). Genetic mutations in NURR1 may thus disturb NURR1-mediated transcription in a manner similar to that of exogenous stressors or may aggravate the effects of ischemia or other types of stress.

In addition to the candidate choices described above, the gene coding for calcitonin and the neuropeptide α-CGRP was investigated at the molecular level. Neuropeptides may exert indirect trophic effects via their neuromodulatory properties. Increasing the number of synaptic contacts may "stabilize" neurons and protect them from degenerative events seen for example during aging.

CGRP has been suggested as a trait marker for major affective disorder (Mathé et al. 1994). Furthermore, several ways in which calcitonin or CGRP may interact with DA systems have been described in the literature (Nicoletti et al. 1982, Deutch and Roth 1987, Clementi et al. 1992, Mathé et al. 1996, Gruber et al. 2001). Genetic susceptibility towards dysregulation of CGRP may thus lead to secondary dysregulation of DA signalling, which in turn might be treatable with dopaminomimetic (L-Dopa) or antidopaminergic (haldol) medication. In this model, the genetic defect responsible for susceptibility to disease would be located outside the DA system itself.

The biochemical and physiological rationales for candidates were matched with available linkage data. Thus, the ones alcohol dehydrogenases were investigated first were those located within the genome close to the markers identified in the "synuclein family" of Parkinson's disease (Polymeropoulos et al. 1997, Markopoulou et al. 1999). Furthermore, it has been pointed out that several of the known schizophrenia loci established by linkage analysis correlate with locations of genes involved in retinoic acid metabolism (Goodman 1998). These loci include the human NURR1 and ALDH1 genes. Linkage of bipolar disease has been reported once for the chromosomal region 11p15, which is the genomic location of the calcitonin/a-CGRP gene (Law et al. 1992).

AIMS

The specific aims of the present study were to

- Identify new molecular genetic markers within genes that may relate to the dopamine system
- Determine frequencies of such markers in the general population versus samples of individuals affected by Parkinson's disease, schizophrenia and bipolar disease, and test for association of any such marker with disease
- Screen for mutations in candidate genes
- Determine if identified mutations disturb gene function
- Establish a protocol for human postmortem *in situ* hybridization
- Characterize postmortem material regarding its utility for immunohistochemical and *in situ* hybridization studies
- Measure expression levels of candidate genes by *in situ* hybridization.

MATERIAL AND METHODS

The following materials and methods are described in the indicated papers. For detailed descriptions of these methods the reader is referred to the indicated papers and to references within these papers.

Method	*Papers*
DNA samples	I through V
Manual sequencing	I, II and IV
Automated sequencing	III, IV and V
RFLP-analysis	I, II and IV
Pyrosequencing	III and V
In vitro mutagenesis assay	IV
Immunohistochemistry	VI
In situ hybridization	VII
Statistical analysis	I through V

Samples

Well-characterized patient materials are prerequisites for any genetic study. Association studies are, as mentioned earlier, population- rather than family-based and require case-control materials of unrelated individuals.

Diagnostic criteria used to acquire our samples are discussed in the respective manuscpapersripts. The average age of onset of Parkinson's disease in our sample (63 years) and age at DNA sampling (69 years) are both at the upper limit of ranges reported in other international samples (Marttila 1992). Family history was determined by interviewing the proband. This method is likely to underestimate the true number of familial cases. Thus, it is known that when interviewed index cases sometimes claim that relatives are asymptomatic, considerable numbers are found to actually be affected when examined by trained neurologists (Uitti et al. 1997).

Collecting control material raises the question of how to match control individuals to cases. Geographical matching is of utmost importance in order to provide a genetic background as similar as possible so that markers that may be neutral to disease but specific to a geographic region do not confound the study. Whether or not to match for age is more difficult to decide. Especially in studies of Parkinson's disease, age-matching of "healthy" controls may exclude genetic mutations from the control material that are present in Parkinson's disease patients, unless those patients that also suffer from other, apparently unrelated, disorders are excluded. Without such precaution there is a risk of population stratification. While age-matching can thus lead to a larger number of false-positive re-

sults, non-age matching decreases the power of a given study, because some of the controls involved will become affected later during life, causing the difference in allele frequency between controls and patients to be underestimated. Ideally, prospective follow-up of a younger control material should eventually eliminate such an underestimation.

In all studies included in the present thesis controls were age- and sex-matched only for schizophrenia, since these studies also included endophenotype investigations. Measurements of DA metabolites, for example, may be age-dependent. Studies in Parkinson's disease were performed purely on the level of genomic DNA, and controls were thus not matched for age.

Molecular genetic methods: Bigger, better, faster, more?

Molecular genetics is a young science currently undergoing rapid technical development. Within the time span of the present thesis work, this development is reflected by thedramatic increase in speed of analysis from the first to the last papers included. Once an interesting mutation has been identified with whatever methodology at hand at the time, one may proceed to study it in larger materials, using additional markers and novel methodology. Two of the investigations included in this thesis work were carried out using "old" techniques, but triggered further analysis of related polymorphisms and genes, both carried out later when a high-throughput genotyping method (pyrosequencing) became available. Figure 3 illustrates output from sequencing and genotyping methods employed.

From manual sequencing to pyrosequencing

Manual (radioactive) sequencing has many disadvantages if used for SNP discovery, the most prominent of these being the great amount of "hands-on" time for mixing of reactions, pouring and running gels. Nevertheless, manual sequencing still has some advantages over other techniques. First, deletions or insertions detected in polymerase-chain reaction (PCR) fragments that lead to double bands on sequencing gels can still be read and the size of the deletion can be determined from the gel, information which is often difficult or impossible to obtain from electrophorograms created by automated sequence analysis (see for example the 3-basepair deletion in Paper II). Furthermore, one can load all lanes of a given letter from different individuals beside each other (Figure 4), so that even rare mutations can be identified in large numbers of samples within seconds simply by looking at the resulting autoradiogram.

Automated capillary sequencing is another method used for SNP and mutation discovery. Here, the advantage lies in the short "hands-on" time for analysis of large materials; the disadvantage is that mutation detection is more difficult. Identification of mutations by comparing electrophorograms of different individuals is very cumbersome. Software for automatic detection of mutations from electrophorograms has been improved during the past years, but in order to be certain to identify all mutations, specificity must often be sacrificed for sensitivity by adjusting the threshold for a "positive" finding to such low levels that numerous false-positive findings must be excluded by human review.

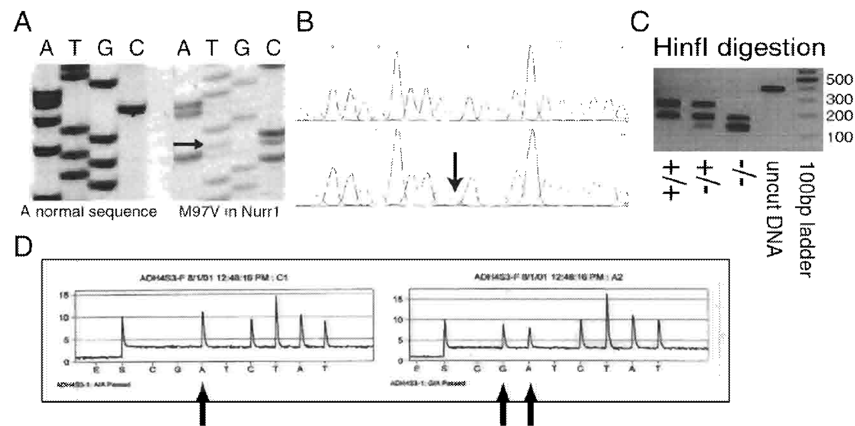

Figure 3. Methods of mutation detection and genotyping. **A** *Manual sequencing.* Mutations produce clear double bands (arrow) and let the original band appear half as strong. **B** *Automated sequencing* (output from the sequencing machine is in color for better distinction of the four letters). Sequences can easily be read and exported into other software. Heterozygous mutations (arrow) can be missed if the base-calling sensitivity of the system is insufficient. **C** *Restriction fragment length polymorphism analysis (RFLP).* As illustrated here, an internal control restriction site should always be present in the PCR-product in order to check for sufficient activity of the enzyme (in this case HinfI). Heterozygosity leads to additional cleavage of one of the bands and homozygosity to complete cleavage. **D** *Pyrosequencing.* Signal intensity grows linearly with gene dosage. On the left side, signal from an individual homozygous for the "A" allele is shown (arrow). On the right side, heterozygosity leads to a reduction of the "A" peak by 50 percent and an additional "G" peak of about the same size (pair of arrows). Before and after the two candidate alleles, letters that do not appear in the sequence (C and T, respectively) are tested in order to exclude unspecific signal. The sequence ends with CTTAT.

Pyrosequencing is very rapid and requires very short preparation times, but sequences obtained by this method are only readable up to a maximum length of 20 bases. This method is therefore not feasible to use for mutation or polymorphism detection, but instead is designed for high-throughput genotyping, i.e. determination of presence or absence of known mutations.

High-throughput genotyping

Pyrosequencing, used in this study, as well as several other high-throughput genotyping methods rely on hybridization of rather short stretches of nucleotides to a target sequence. This accelerates the procedure dramatically, but also creates a problem when investigating genes belonging to multi-gene families. These problems can be circumvented by specificity testing using restriction enzymes. In homozygous mutant individuals, all of a given PCR product should be digested when the restriction enzyme is specific for the

mutated sequence. If no restriction sites are available, pyrosequencing results should be routinely checked by automated sequencing, where much longer PCR products can be worked with, enabling primers for PCR to be designed within promoter or intronic regions that show a lesser degree of conservation.

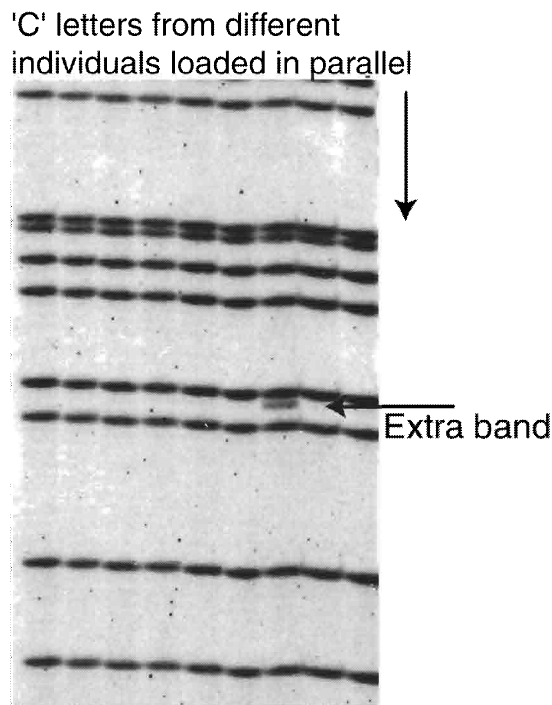

Figure 4. Detail from the seqencing gel on which the M97V mutation was identified, illustrating the advantage of parallel loading of samples from different individuals.

Bioinformatics

Computer-aided primer and probe design have been used in Papers I–V and VII. In human studies, giving priority to avoidance of repeat sequences (some of which, such as Alu repeats, have very high copy numbers within the human genome) has yielded the most specific results. The xprimer-based primer design program provided by the Alces Virtual Genome Center (http://alces.med.umn.edu/rawprimer.html) was used, followed by tests for possible RNA-folding using the mfold program (http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi) (SantaLucia, Jr. 1998). Probes that were folded in exothermic reactions

and/or were subject to hairpin formations including the 3 prime end of the sequence were excluded.The BLAST SNP program (http://www.ncbi.nlm.nih.gov/SNP/snpblastByChr.html) was used for "in silico" SNP discovery.

In situ hybridization and immunohistochemistry: Modifications for postmortem human tissue

Based on an established protocol for *in situ* hybridization using unfixed cryosections (Dagerlind et al. 1992), adaptations were made aimed at optimization for human studies. Probe design was carried out as described under "Bioinformatics" above. Sections were thawed onto SuperFrostPlus slides (VWR international, Stockholm, Sweden), the isotop ^{33}P was used instead of ^{35}S and unincorporated nucleotides were removed by a different column-absorption method (ProbeQuant G-50 microcolumns). These changes led to a significant reduction of overall background signal and also to a reduction of "sticky spots" on the slides.

Immunohistochemistry on fresh tissue sections subjected to short post-sectioning formalin fixation produced good results using an established protocol. We were not able to further improve results by any of several tested modifications, such as blocking with different serums or changing incubation times or temperatures.

Statistics

Correction for multiple comparisons constitutes a critical issue for any kind of biological research, not only genetic studies. For example, if twenty independent investigators search for survival-promoting activity of a certain growth factor and one of them finds it at a confidence level of $\alpha=0.05$ while no one else does, the one positive finding is likely to be the first (and often the only) study to be published. Negative results have a tendency to be neglected by investigators as well as scientific journals.

In candidate gene studies, reporting negative findings may seem especially unattractive. Excluding a gene completely is very difficult, and even if a negative study has enough power to "very likely" exclude contribution of a certain gene in a disorder, there are still 34,999 or so other genes left to study, making the result appear to be a very minor scientific achievement (Figure 5).

Actually, publication of negative results in candidate gene studies is extremely important. No study in any material held by one single group is likely to have enough power to definitely prove or reject influence of a certain genetic locus. First, the effect of a single locus is often expected to be small; second, most association studies include numerous markers that are investigated in parallel, demanding correction for multiple testing. Therefore, most judgements regarding certain loci are likely to be made in metastudies. This strongly calls for the publication of all acquired results in order to provide as representative a picture as possible.

Figure 5. The "shotgun approach" to negative results in genetic studies
Source: Nick D. Kim, used by permission.

DISCUSSION OF RESULTS

Identification of new polymorphic sites (Papers I–III and V)

As indicated by way of introduction, the number of available polymorphic markers within the human genome is an important factor in linkage and association studies. The denser the coverage of markers and the more available information on common haplotypes (i.e. the combined appearance of several sequential polymorphic sites) within a population, the better the resolution that can be obtained for mapping of disease genes within a sample. Once polymorphic sites have been identified in a given gene, they can be used in association studies for all kinds of diseases, and a number of genes have indeed been suggested to be candidates for several different disorders. The APOE polymorphism mentioned above, for example, was initially identified in studies of genetic factors in hyperlipidemia (Utermann et al. 1977) before its association with Alzheimer's disease was discovered (Saunders et al. 1993).

During the present thesis work, new polymorphic sites were identified in the genomic sequences coding for α-CGRP, alcohol dehydrogenase class IV and NURR1. Furthermore, polymorphic sites derived from large-scale SNP discovery efforts and stored in public databases were included in the present work. Many of these had previously not been characterized regarding their frequency in control populations, and none had been studied with regard to the disorders that are the topic of the present thesis.

Complete absence of SNPs from coding regions of the NURR1 gene constituted a surprising finding, since large numbers of individuals have been investigated by us (Paper IV) and later by others (Chen et al. 2001, Ishiguro et al. 2002). Why not even one silent sequence change (i.e. a sequence change not affecting the amino acid sequence and thus presumably not subject to evolutionary selection pressure) was found is difficult to explain. The strong conservation of the NURR1 gene between individuals and between mice and humans indicates little freedom for variation in the sequence, points to the importance of NURR1, and makes the three sequence changes that *were* found in patients with psychiatric disease appear even more severe.

Polymorphisms at the CALCA locus and diseases of the dopamine system (Paper I)

(The online version of this paper contains two figures which, due to decreases in size and resolution made by the journal, are not quite in focus. Therefore, both these figures are reproduced here once again.)

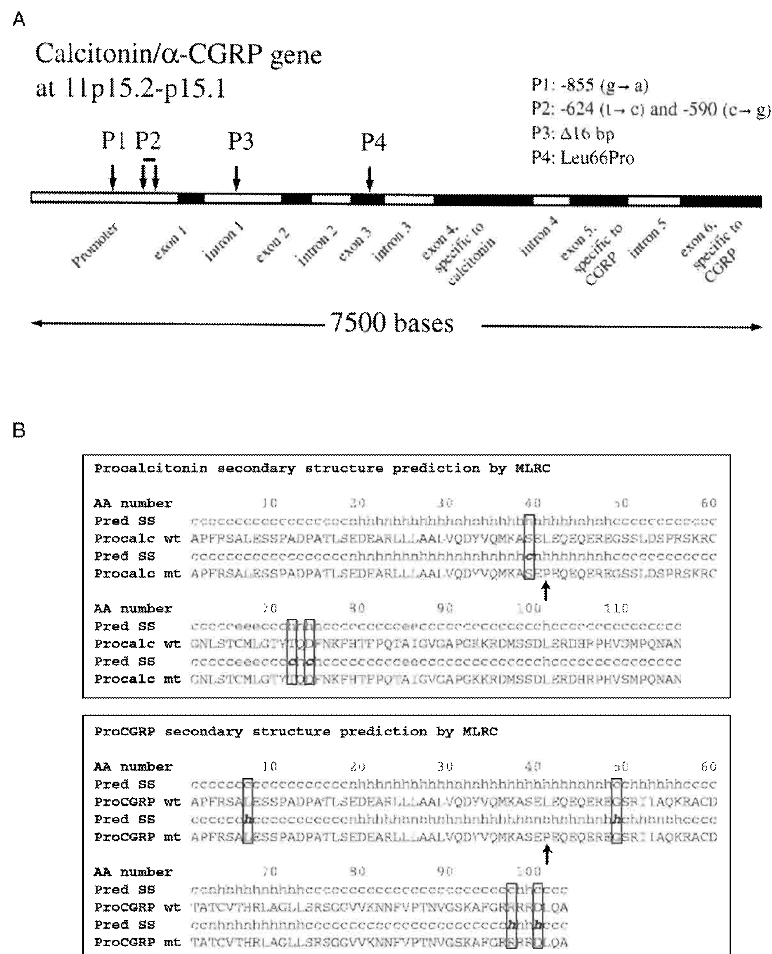

Figure 6. **A** Identified mutations at the CALCA locus and **B** effect of the Leu66Pro substitution on calcitonin and CGRP propeptide secondary structures based on computer simulation. Predicted structure changes are boxed. Arrows indicate mutated sites. AA = amino acid; Pred SS = predicted secondary structure; Procalc = procalcitonin; Pro CGRP = Pro-a-CGRP; wt = wildtype; mt = mutated; h = a-helix; c = random coil; e = extended strand.

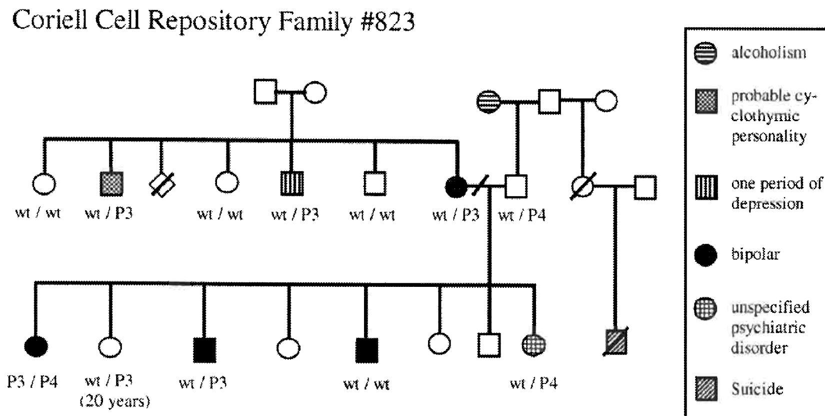

Figure 7. Investigation of linkage of P3 and P4 to affective disorder. Wt = wildype.

None of the four novel polymorphisms described in this study (Figure 6) showed significant association with any of the diseases of interest. We also investigated the distribution of polymorphisms found in the CALCA gene in a pedigree with marked presence of affective disorder. This pedigree (Figure 7) has also been used in association studies of the insulin gene in bipolar disease (Detera-Wadleigh et al. 1987). The fact that we could identify common haplotypes composed of polymorphisms identified by us in the CALCA gene in combination with those in the insulin gene identified in the previous study illustrates the advantage of public DNA and cell repositories. Thus, accumulated genotyping data acquired in many different studies will facilitate identification of common haplotypes in different families or populations. By reconstructing these haplotypes the power of genetic studies will increase, since the large numbers of polymorphisms that are available today will no longer lead to numerous "independent observations" but rather serve as markers for larger chromosomal areas that can be followed through generations and within populations. Populations in which large common haplotypes were identified are the Evenki and Saami of northern Eurasia, which therefore recently have been suggested to constitute particularly valuable populations for mapping of complex disease (Kaessmann et al. 2002) .

Since CGRP has been strongly implicated in the pathophysiology of migraine, it is surprising that no association study of migraine has yet been published using the above-identified or other polymorphisms. It may, however, be that such studies have been carried out and undergone the previously described "shotgun fate" for negative results in complex genetics.

Alcohol dehydrogenases in Parkinson's disease (Papers II and III)

Sequencing the gene for class IV alcohol dehydrogenase revealed seven polymorphic sites, constituting five different alleles (now called haplotypes). A significant association of one allele with Parkinson's disease was found (Paper II). It was later demonstrated that one mutation of this allelic variant of ADH4 (called P1) leads to a significant reduction of ADH4 transcription, while another promoter polymorphism not associated with disease (called P2) did not cause any significant effect (Figure 8).

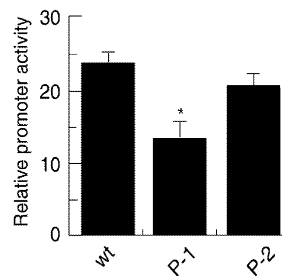

Figure 8. *In vitro* effect of ADH4 promoter polymorphisms on promoter activity. The human stomach derived cell line AGS was transfected with a plasmid containing the reporter gene firefly luciferase under the control of either wildtype or mutated forms of the alcohol dehydrogenase class IV (ADH4) promoter. To exclude transfection variability, cells were cotransfected with the control reporter gene renilla luciferase. The panel above shows the results of one representative experiment (means +/– standard error of the mean of 8 parallel cultures. * indicates $p<0.05$).

The association identified by us could, however, not be confirmed by another group studying a Caucasian material originating from the east coast of the USA (Tan et al. 2001). One reason for discordance of findings in different population-based association studies is the possible presence of a true disease-causing mutation in a nearby gene in linkage disequilibrium with the mutation that shows positive association in one population but not in the other.

In order to establish whether such a "linked" polymorphism may be present in another gene of the alcohol dehydrogenase family in our material and to check for association of further sequence changes in alcohol dehydrogenase genes, thirteen known polymorphisms were selected across the ADH cluster (in ADH1B, ADH1C, ADH3 and ADH4)and investigated for their possible associations with Parkinson's disease (Paper III). While the previously identified association of ADH4 with Parkinson's disease remained significant also in this larger material, no other polymorphisms in ADH genes showed significant association. Interestingly, a (rare) nonsense mutation in ADH1C was found to be present in three patients with Parkinson's disease but not in controls. Whether this rather dramatic sequence change indeed may be involved in Parkinson's disease will be established by screening of large independent materials. The confirmation of the actual existence of this SNP in a Caucasian population may also trigger investigation of association of this polymorphic site with other disorders, such as alcoholism.

If the identified association of an alcohol dehydrogenase variant with Parkinson's disease holds true, this may be one piece of evidence for a disease pathway originating outside the DA neurons and even outside the brain. Thus, alcohol dehydrogenases of the ADH cluster on chromosome four are mainly expressed in the gastrointestinal system. There, they may constitute a first line of defense against toxic aldehydes absorbed from food. A second line of defense may be present in the blood. Thus, we recently confirmed that a molecular variant of the HDL-bound enzyme paraoxonase 1 (PON1) appears associated with Parkinson's disease in our sample (Carmine et al. 2002). The physiological function of paraoxonase is likely to be a lipoprotein-bound aldehyde dehydrogenase. Other (environmental) factors may influence activity of these enzymes in the gastrointestinal tract and blood, providing yet another basis for gene–environment interactions in the pathogenesis of Parkinson's disease

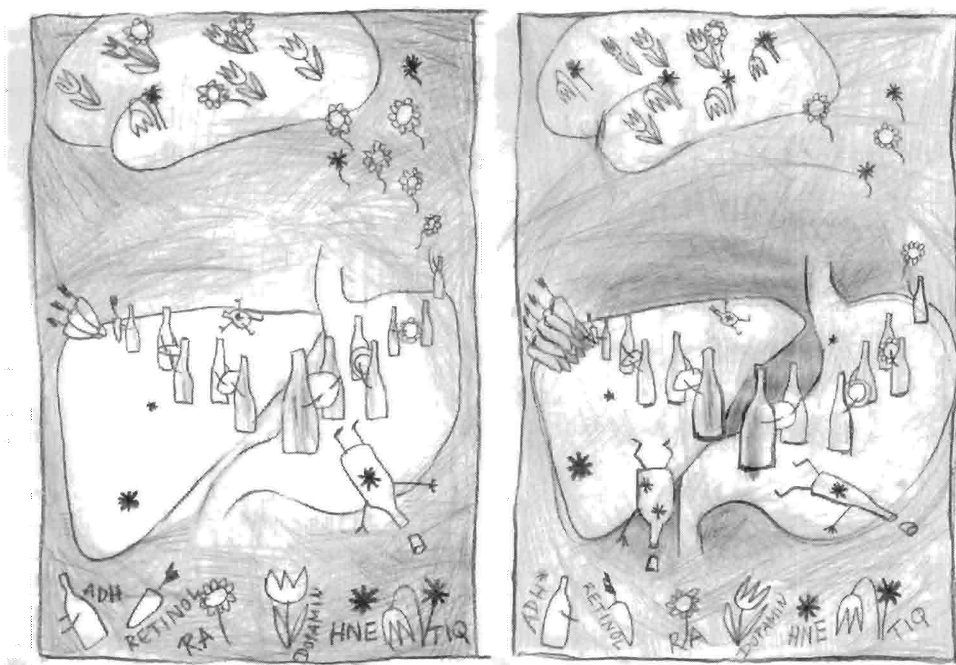

Figure 9. The GI model of Parkinson's disease. Under normal conditions (displayed on the left side), alcohol- and aldehyde-dehydrogenases (ADH) synthesize sufficient amounts of retinoic acid (RA) from exogenous retinol. The same enzymes also detoxify the aldehyde 4-hydroxynonenal (HNE). Only occasionally does HNE reach the brain and condense with dopamine forming toxic tetrahydroisoquinolines (TIQs). If genetic defects are present in alcohol- and aldehyde-dehydrogenases (ADH*, illustrated on the right), RA is synthesized less efficiently and more toxic HNE also reaches the brain. HNE toxicity affects the entire brain but is most dangerous for catecholaminergic (e.g. dopaminergic) neurons. Drawings were kindly provided by Dr. Tena Frank (© 2002).

Nurr1 in psychiatric and Parkinson's disease (Papers IV and V)

In the human gene coding for NURR1, rare mutations were found in three patients (two with schizophrenia and one with bipolar disease) having experienced at least one psychotic episode each. All three sequence changes appeared to decrease NURR1 transcriptional activity in an in vitro assay. These molecular changes may therefore lead to impaired activation of NURR1-controlled genes. Although these changes are very rare even among psychotic patients, the total absence of these mutations or any sequence change whatsoever in the coding regions of NURR1 in controls and patients with Parkinson's disease is intriguing.

If NURR1 signalling is indeed involved in a pathogenic pathway leading to schizophrenia, the three identified cases may represent rare cases of the disorder in which genetic mutations have a major role with only a minor contribution of environmental factors (if the environmental contribution was large, it would be very unlikely to find as many as three cases with NURR1 mutations). Therefore, and supported by the strong conservation of NURR1 between mice and humans, introduction of the identified sequence changes into transgenic mice may provide a "true" animal model for schizophrenia susceptibility. Such model animals may then be used to test for different environmental stressors (hypoxia, ischemia, psychosocial stress, etc) that have been suggested to be involved in schizophrenia, but that, without the necessary genetic component, may not be sufficient to cause symptoms. Although it will be difficult to predict how a "schizophrenic mouse" would behave, several markers are testable and have been used in previously described models of the disease (Mohn et al. 1999).

The lack of association of the identified NURR1 polymorphisms with schizophrenia and the few positive results in the screening for possible endophenotypes speak against an involvement of these sequence changes in the majority of cases. The negative results provided here may however facilitate further molecular analysis of NURR1 and may be used in metastudies of this locus. We did not find any mutations of NURR1 in patients with Parkinson's disease. Recently, other investigators reported association of homozygosity for the BseRI polymorphism described by us with Parkinson's disease (Xu et al. 2002). Whether or not homozygosity for this or any of the promoter polymorphisms described in Paper V are associated with Parkinson's disease in our material remains to be determined.

Immunohistochemical studies of human postmortem brain material (Paper VI)

The quality of human postmortem brain material is subject to variation depending mainly on conditions before death, the cause of death and the length of the postmortem interval (PMI). In order to judge whether available postmortem specimens were suitable for studies on the protein level, postfixed sections of fresh-frozen midbrain tissue underwent investigation using an array of antibodies. Those specimens that did not show strong and specific signals according to expected patterns were excluded from further studies.

In brain sections from Parkinson patients and controls, large numbers of corpora amylacea (CA) were identified in subcortical and, most numerously, subependymal locations. Neither the origin of these remarkable bodies nor their possible function is yet known (Cavanagh 1999). Whether or not their presence is increased in human neurodegenerative disease remains to be established. This is not an easy task due to the large variability of their occurrence in different individuals as well as in different sections of material from the same individual.

Accumulation of proteinaceous material in Parkinson's disease (intracellular Lewy bodies) (Lewy 1912) may either be toxic to neurons or, possibly, protective by clustering toxic molecules that otherwise would be harmful to the neurons. By analogy, the question of whether corpora amylacea constitute packages of "waste" that cannot be disposed of otherwise, are evidence of some form of a degenerative process, or even some some other unknown function cannot yet be answered. The only well-established fact regarding their biogenesis is that they are found in increasing numbers with age (Cavanagh 1999).

The cellular origin of CA is not known. The fact that they were found to be immunoreactive for NeuN, a marker for neuronal nuclei, may speak in favor of a neuronal background. Furthermore, CA were found to be labeled by an antibody against nestin. The very same antibody from the same manufacturer had previously been used in order to identify neuronal stem cells and expanding them in order to repair spinal cord injury (Akiyama et al. 2001). Very speculatively, it may be conjectured that the presence of CA under the ependyma (where stem cells reside), immunoreactive for nestin (while being completely negative for other markers such as neurofilament or vimentin) may indicate that these enigmatic bodies originate from stem cells that failed to divide into proper neurons. Recent research has indicated that neurogenesis indeed occurs in subependymal locations throughout life (Doetsch 1999, Johansson 1999), which could explain the increases in the number of CA with age. It was also found that CA close to the epenyma and those at a greater distance differed in their affinity for antibodies for GFAP or synuclein, possibly indicating different stages in the formation of CA.

Protein aggregates are however notoriously "sticky," and the observed labelling with the nestin antibody may be entirely unrelated to nestin. In this case, it may still be used for identification of CA, providing the advantage that no other structure except CA is labelled with this particular antibody in the aged human brain. This property may facilitate computer-aided counting of these enigmatic bodies.

Differential ALDH1 expression in Parkinson's disease (Paper VII)

There are different ways of carrying out expression studies, with microarray-based techniques having become very popular recently. For human postmortem brain expression studies, however, use of mRNA extraction from tissue blocks is a questionable basis from which to start, due to the variable quality of tissue samples. This is especially problematic in comparative studies of patients with Parkinson's disease and controls, when the numbers of surviving cells differ dramatically among individuals.

In situ hybridization provides the opportunity to assess expression levels at the level of the individual cell while also securing specificity of the signal by checking its correct cellular localization. After testing the quality of different tissue specimens in this way with a variety of markers, expression of cytosolic aldehyde dehydrogenase (ALDH1) was assessed semiquantitatively in DA cells of control individuals and Parkinson patients.

The observed lower signal for ALDH1 mRNA in surviving DA cells in patients with Parkinson's disease may only be a sign that these neurons are sick rather than an indication of disease etiology. On the other hand, this finding may also be part of a progressive pathogenic pathway leading to disease. Thus, the toxic properties of DA may not be dangerous to healthy DA cells containing normal amounts of detoxifying enzymes, such as ALDH1, a view supported by the observation that L-Dopa therapy in non-parkinsonian individuals does not seem to kill DA neurons (Rajput 2001). However, during the course of the disease, mechanisms not currently understood may lead to a downregulation of ALDH1 and possibly also other important defense systems. Under these pathological circumstances, the toxicity of DA and its metabolite DOPAL may increase dramatically, accelerating further DA neuron decay.

Due to the absence of SNPs within the coding region of ALDH1, no molecular association studies have so far been carried out. However, based on the observed findings on differential expression of ALDH1 in parkinsonian brains, promoter studies similar to those described for the NURR1 promoter included in this thesis are warranted.

CONCLUSIONS AND OUTLOOK

Polymorphic sites in the gene encoding calcitonin and calcitonin gene-related peptides show no significant association with Parkinson's disease or schizophrenia, but may be used as tools in investigations of other candidate disorders such as migraine or hypertonia.

Genetic mutations in alcohol or aldehyde dehydrogenases may constitute heritable risk factors for Parkinson's disease and provide a link between endogenous metabolic pathways and the outside world (such as dietary retinol and toxic aldehydes). Transgenic mice with deficiencies in these genes will be exposed to environmental (dietary) challenges in order to study the combined impact of these two factors on the status of the DA system, with special emphasis on events during aging.

The NURR1 gene shows no variability in the coding regions but some variability in the promoter and intronic sequences. None of the polymorphic sites in the promoter or sixth intron showed significant association with the diseases of interest. Screening for association of putative endophenotypes of schizophrenia with the identified molecular variants revealed positive findings at a frequency that can likely be explained by chance events.

Three unique mutations were identified in exon 3 of NURR1 in patients with psychotic symptoms. In order to respond to the current need of valid animal models for psychosis, introduction of these sequence changes into the highly similar mouse Nurr1 genomic sequence should be attempted.

A protocol has been established for in *situ* hybridization on human postmortem material. This method will be used in order to map candidate genes within the human brain under normal and disease conditions.

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