GENES OF THE SEROTONERGIC SYSTEM & SUSCEPTIBILITY TO PSYCHIATRIC DISORDERS:
A GENE-BASED HAPLOTYPE ANALYSIS APPROACH

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غزال زابلي

Stockholm 2006
The roots of education are bitter,
but the fruit is sweet!

Aristotle
ABSTRACT

Psychiatric disorders are complex, non-mendelian disorders. Complex disorders are ultimately determined by a number of genetic and environmental factors, and the effect of each factor may be obscured or confounded by others. Although detection and precise characterization of the relationship and interactions between genes involved and/or environmental factors may not be ascertained by any available methods, several strategies of investigation may be used. Candidate gene analysis is one disclosing approach, which was used in this thesis to test for associations between a particular gene variant and a disease.

This thesis work aimed to design a simple strategy to construct sets of polymorphic loci limited by gene boundaries, referred to as gene-based haplotypes, to be applied in case-control clinical studies, as opposed to the classical, ancestral haplotype block approach, which is mostly used for genome-wide and large scale population analyses. The gene-based haplotype approach developed here is not influenced by parental transmission data, is cost and time effective, and is more informative than single locus associations. Also, linkage disequilibrium (LD) analysis within the gene and between the markers increases the likelihood to detect concealed causative alleles.

This strategy was applied to two candidate susceptibility genes of the serotonergic system implicated in the pathophysiology of several psychiatric disorders: (i) the serotonin transporter (5-HTT) gene, and (ii) the tryptophan hydroxylase 1 (TPH-1) gene. Focusing on these genes, the strategy was adapted to a classical case-control design to seek for association between specific gene-based haplotypes that are common in the population while potentially carrying risk for the disorder.

Three different psychiatric disorders were explored: Major Depressive Disorder (MDD), Schizophrenia (SCZ) and Borderline Personality Disorder (BPD).

Single locus analyses provided a few positive associations with the disorders, which were mostly weak especially after correction for multiple testing. This was expected, as it is often the case for single-locus association studies. However, when multi-loci sets were constructed using a population genetics algorithm, several haplotypes were identified that displayed strong statistical associations with disease. The significance level obtained for haplotype markers in association studies was substantially higher than that observed for single loci.

Key words: Borderline Personality Disorder, Candidate genes, Case-control, Depression, Gene-based haplotypes, Linkage Disequilibrium, Schizophrenia, SNP.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to by their roman numbers:


# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxy tryptamine, serotonin</td>
</tr>
<tr>
<td>5-HTT</td>
<td>serotonin transporter</td>
</tr>
<tr>
<td>5-HTTLPR</td>
<td>serotonin transporter linked polymorphic region</td>
</tr>
<tr>
<td>APA</td>
<td>American Psychiatric Association</td>
</tr>
<tr>
<td>BPD</td>
<td>borderline personality disorder</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyltransferase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DSM</td>
<td>diagnostic and statistical manual</td>
</tr>
<tr>
<td>DZ</td>
<td>dizygotic</td>
</tr>
<tr>
<td>GAD</td>
<td>generalized anxiety disorder</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
</tr>
<tr>
<td>L allele</td>
<td>long allele of 5-HTTLPR</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MDD</td>
<td>major depressive disorder</td>
</tr>
<tr>
<td>MZ</td>
<td>monozygotic</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>s allele</td>
<td>short allele of 5-HTTLPR</td>
</tr>
<tr>
<td>SDA</td>
<td>serotonin-dopamine antagonists</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitors</td>
</tr>
<tr>
<td>STin2</td>
<td>serotonin transporter intron 2 polymorphism</td>
</tr>
<tr>
<td>TPH</td>
<td>tryptophan hydroxylase</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable number of tandem repeats</td>
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1 INTRODUCTION

1.1 PSYCHIATRIC DISORDERS: PAST & PRESENT

Early psychiatry was concerned with the diagnosis and containment, rather than cure, of conditions which presented a problem to society. People experiencing delusions, hallucinations, periods of mania, etc. were likely to cause aggression in the overcrowded conditions of European cities, and the solution was generally to lock them away. Since depression is, in general, a much quieter condition, people with depression were usually spared the horrors and indignities of the “lunatic asylums”, but it also meant that many were left to suffer in silence, with no hope of a cure.

By the close of the 18th century, doctors shared the consensus that mental illness originated within the brain and were not hereditary. “The cause of madness is seated primarily in the blood vessels of the brain…” wrote American physician Benjamin Rush in 1812. The man who exemplified the uncompromising scientific view of this era was the German psychiatrist Wilhelm Griesinger (1817-1868), who saw mental disorders as somatic diseases. It was necessary to understand the anatomical origins of psychiatric disorders, not their psychological ones. The work of Griesinger was followed by Emil Kraepelin (1855-1926). At first he strongly believed in heredity factors as the cause of mental illness; later he shifted toward a belief in the importance of metabolic factors. Kraepelin’s work can be seen as peak of the neurophysiological approach which began with Griesinger and continued to dominate the scene until Freud’s dynamic motivational approach revived interest in the patient as a unique individual with a unique history. What Freud successfully realized was that neurophysiological and psychological knowledge need not be contradictory. Psychoanalysis predominated until the 1970s, which was followed by renewed interest in genetic, biochemical and neuropathological causes of mental disorder which came to be known as biological psychiatry.

Today, psychiatry is a branch of medicine concerned with the diagnosis, treatment and prevention of mental illness. Psychiatric symptoms may rise upon abnormalities and dysfunctions in the brain (somatogenesis), i.e. cerebral lesions, or through one’s interaction with one’s surroundings (psychogenesis). The latter may emerge due to the individual’s intrapersonal, interpersonal and/or psychosocial dynamics. A clarified description of a disorder’s character accompanied by studies in pathogenesis and
etiology are fundamentally necessary for the development of a rational therapy and prophylaxis.

1.2 PSYCHIATRIC GENETICS

1.2.1 The Gene

In the 1860’s, Gregor Mendel (1822-84) carried out a systematic experimental analysis of plant hybridization and inheritance patterns. The crucial feature of his work was the realization that the gene is a distinct entity. Mendel studied the effect of single genes, in terms of dominant and recessive hereditary traits. His great discoveries were ignored for three decades. At the beginning of the twentieth century, however, “Mendel’s laws” were re-discovered by Hugo Marie de Vries (1848-1935), Karl Correns (1864-1933), and Erich von Tschermak-Seysenegg (1871-1962).

The era of the molecular biology took a new turn in 1944 with the publication of a paper by Oswald Avery (1877-1955) and his colleagues in which they proved that DNA not protein, as many believed at the time, is the agent of heredity. Working with bacteria, Avery and his coworkers purified and tested different bacterial chemicals, eliminating all except DNA as the genetic material. Shortly after, Erwin Schrödinger (1887-1961) developed the view that the properties of the genetic material are stable during countless generations of inheritance. In 1953, the double helix structure was discovered [1], followed by the discovery of the genetic code [2]. It was in 1977 that the desire to determine the exact nucleotide order in every gene became possible when two methods were independently reported by Sanger and by Maxam and Gilbert [3, 4]. Knowledge brought about by these discoveries has led to identification of thousands of genes [5].

Today we estimate that the human genome contains 20,000-25,000 genes composed of 3.165 billion nucleotide bases (adenine, thymine, cytosine, or guanine). The average gene consists of 3,000 bases, but sizes vary greatly. However, genes comprise only about 2% of the human genome. The remainder consists of non-coding regions, providing chromosomal structural integrity, and regulating protein synthesis. The functions for over 50% of discovered genes are yet unknown. The genes discovered so far can be viewed at the continuously updated catalog of human genes and genetic disorders at OMIM, Online Mendelian Inheritance in Man, authored and edited by Dr. Victor A. McKusick and his colleagues (www.ncbi.nlm.nih.gov/omim)

1.2.2 Definitions of Mendelian Genetics

The physical site of a gene is called a locus, and for any particular locus several alternative forms of the gene can be present, which are called alleles. A specific allelic combination at a specific locus for a certain gene is called a genotype. The physical appearance of a particular trait is called a phenotype.
An individual has two alleles at each locus (except for sex chromosomes), inherited one from each parent. When the two alleles are indistinguishable they are referred to as being homozygous, and if it is possible to make a distinction between them, they are heterozygous. Most allelic variations are due to sequence changes that have no measurable functional effect on the expression of the gene. Thus, if the sequence change causes disruption of gene function, in principle it can also cause disease.

### 1.2.3 Mendelian & Complex Disorders

The human body has an extraordinarily complex biology which requires subtle control of a number of biochemical and physiological mechanisms, regulated by genes. Aspects of such systems can be thought of as hierarchies, with genes at the lowest level and clinical endpoints that define disease at the highest [6], which principally consists of two major category of diseases; Mendelian and complex disorders. Mendelian disorders such as cystic fibrosis and sickle cell anemia, show strong correspondence between genotype and phenotype which can be traced by the strong linkage signal in affected families. The reason is that for such diseases, a single gene or a small number of genes carry the mutation and that is sufficient to produce a disease phenotype [7]. However most common disorders, such as psychiatric disorders do not run in families in clearly identifiable Mendelian inheritance pattern, but have a rather more complex model of inheritance.

Complex genetic disorders are also called classical polygenic diseases due to involvement of multiple genes, i.e. a number of genotypes or mutations at different loci must be present to result in a disease [8]. They are common in the general population and carry less than 100% concordance in monozygotic (MZ) twins. This implies that gene products and their phenotypes may be influenced by environmental and non-genetic stimuli which may often be difficult to characterize [7]. The risk of disease is significantly lower in second- and third-degree relatives compared to Mendelian pattern. Illness severity varies greatly among those affected, because different individuals may have different underlying pathologies that lead to similar phenotypic endpoints [8]. Further, a certain mutation or genotype may confer susceptibility in the presence of other mutations or genotypes. Thus, gene-gene interactions (epistasis) involve basic interactive effects of mutations, genotypes, and/or their biologic products [8].

Many factors influencing the course of polygenic disorders can obscure the contribution of a single gene to disease, making the gene’s isolation and characterization of genes difficult. On the other hand, each factor that contributes to a complex disease may only be visible in the presence of other factors.

### 1.2.4 Genetic Markers

Genetic variation can determine disease susceptibility [9] and provides the tools to understand basic biological processes [10]. DNA fragments of variable sequence at
specific positions in the genome, which may also vary between individuals, are called genetic markers. There are several different types of genetic markers, the most common being: short or long insertion/deletion variants, transposable elements, microsatellites, variable number of tandem repeats (VNTR), restriction fragment length polymorphism (RFLP), and single nucleotide polymorphism (SNP).

Transposable elements are also called "jumping genes" or "mobile genetic elements" since they are sequences of DNA that can move around to different positions within the genome of a cell. There are a variety of mobile genetic elements, and they can be grouped based on their mechanism of transposition. Class I mobile genetic elements, or retrotransposons, move in the genome by being transcribed to RNA and then back to DNA by reverse transcriptase. Class II mobile genetic elements move instead directly from one position to another within the genome using a transposase to "cut and paste" them within the genome. Transposons are very useful to researchers as a means to alter DNA inside a living organism. 

Microsatellites are molecular marker loci consisting of very short tandem repeat units of for example di-, tri-, tetra-, or pentanucleotides repeated several to hundreds times along the DNA. Due to their high level of polymorphism, microsatellites are informative markers that can be used for several population genetic purposes, ranging from the individual level (e.g. clone and strain identification) to closely related species. In addition, microsatellites are considered ideal markers in gene mapping studies.

Variable Number of Tandem Repeats (VNTR) or minisatellites are molecular marker loci consisting of tandem repeat units of a 10-50 base motif, flanked by conserved endonuclease restriction sites. VNTR units are considered to be the main cause of length polymorphisms. Due to the high mutation rate of minisatellites, the level of polymorphism is substantial, generally resulting in unique multilocus profiles. Therefore, minisatellites are particularly useful in studies involving genetic identity, parentage, clonal growth and structure, and identification of varieties and cultivars.

Restriction Fragment Length Polymorphisms (RFLP) are fragments of restricted DNA, usually within the 2-10 kb range. Restriction enzymes function as a host modification-restriction system in bacterial cells, and they are the fundamental tool of DNA cloning that can also be used to reveal genetic variation among genomes. Variations detected by this means are called RFLPs, because they are revealed by DNA fragments of variable lengths when fractionated by electrophoresis. The RFLP method can provide sensitive and unequivocal results for genetic mutations. Because of their high genomic abundance and random distribution throughout the genome, RFLPs have frequently been used in gene mapping studies.

Single Nucleotide Polymorphism (SNP) occurs when a single nucleotide has two or sometimes three forms common in a population. A variation must occur in at least 1% of the population to be considered a SNP. Since only about 3 to 5 percent of DNA
A gene-based haplotype approach has coding capability, SNPs are mostly detected in non-coding regions, such as promoter regions, introns, 5’- and 3’-untranslated regions, and intergenic regions [16]. SNPs found within a coding sequence are of particular interest since they are more likely to alter the biological function of a protein. However, SNPs in non-coding regions may alter transcription rates, mRNA processing or stability or level of translation products [16]. Coding SNPs are classified as synonymous if they only change a codon into another that codes for the same amino acid, or non-synonymous if nucleotide change results in a different amino acid [16]. A frequently updated database of recent SNP discoveries is available online which already includes about 10 million such polymorphisms [17, 18].

1.2.5 Genetic Association study

Genetic association studies are investigations designed to determine whether there is a relationship between a genetic marker and the frequency or severity of a specific complex disorder [16]. There are two basic strategies for disclosing and characterizing genes that influence complex diseases: candidate gene analysis and whole-genome searches or “genome scans” [8].

The present thesis work is based on a candidate gene approach. In a candidate gene association analysis, one seeks to test the association between a particular genetic variant (e.g. an allele) and a disease. If the variant is more frequent in subjects with the disease than those without it, then it may be inferred that this is due to a causal relationship between that variant and the disease. Another assumption may be that the gene in question is in linkage disequilibrium with a disease gene at a neighboring locus [8, 19]. Candidate gene analyses are therefore dependent on knowledge about gene variants. However, the complexity of polygenic disorders would hardly be revealed through a single gene variant [18].

1.2.6 Linkage Disequilibrium

Linkage disequilibrium (LD) is defined as the association of sequence variants at different positions along the chromosome. It is a statistical measure obtained within a population of unrelated individuals [19, 20]. In most human populations, LD extends for relatively short distances, on the order of 10s to 100s of kb in most genomic regions [21]. LD is distinct from linkage, which refers to within-family association between markers [16]. The association that LD is based on is probably due to a mutation in the distant past, which resulted in the two (or more) alleles that exist today [19]. The mutation has most likely originated from a specific haplotype (see next section), which in turn belonged to a specific individual of a specific population. Due to recombination or gene conversions the mutation has spread to other haplotypes. Furthermore, the carrier may have spread it to other populations by migration, and its frequency might have come under influence of natural selection or genetic drift [19]. Hence, the amount of LD depends on the age of the mutations involved, and on the history of human population
size and structure \[22\]. In this context, LD data has been vital e.g. in ancestral studies, identifying alleles that have undergone positive selection. Positively selected alleles increase in frequency rapidly and are surrounded by more linkage disequilibrium than other alleles of similar frequency \[19\].

The statistical significance of LD can be tested by standard 2 x 2 contingency table tests. Consider two loci, A and B, with alleles \(A_1/A_2\) and \(B_1/B_2\), respectively. Let \(p_{Ai}\) stand for the frequency of allele \(A_i\), where \(i = 1, 2\), at locus A. More formally, \(p_{A1}\) denotes the frequency of allele \(A_1\) with \(1 - p_{A1}\) being the frequency of \(A_2\). Similarly for locus B; that is to say \(p_{Bj}\) represents the frequency of allele \(B_j\), where \(j = 1, 2\), at locus B. If the alleles at the two loci occur independently of each other in haplotypes, the frequency of the \(A_iB_j\) haplotype is given by

\[
P_{AiBj} = p_{Ai}p_{Bj}
\]

However, if for example allele \(A_1\) is a mutant allele there must be a marked difference of marker allele frequencies between affected and unaffected individuals hence the \(A_1\) and \(B_1\) alleles appear associated so that the \(A_1B_1\) haplotype has the frequency

\[
P_{A1B1} = p_{A1}p_{B1} + D, \quad D = P_{A1B1} - p_{A1}p_{B1},
\]

where, \(D\) is referred to as the disequilibrium parameter. The equilibrium state is characterized by \(D = 0\) and is called gametic phase equilibrium or linkage equilibrium, and values \(D > 0\) are referred to as positive disequilibrium or association. Whether \(D\) is positive or negative depends on the arbitrary labeling of alleles. The maximum value that \(D\) can have depends strongly on allele frequency:

\[
D_{\text{max}} < p_{A1}p_{B2} \text{ or } p_{A2}p_{B1}, \text{ if } D \text{ is positive or,}
\]

\[
D_{\text{max}} < p_{A1}p_{B1} \text{ or } p_{A2}p_{B2}, \text{ if } D \text{ is negative}
\]

To avoid dependence on allele frequency the measure \(D'\) is used:

\[
D' = D/D_{\text{max}}
\]

and since the sign is arbitrary, the absolute value \(|D'|\) is used rather than \(D'\). LD is assumed complete when \(|D'| = 1\), which occurs when only three of the four possible gametic types are present in the population, whereas a value of 0 indicates a lack of LD due to total shuffling among the SNPs on the chromosome \[22\].

One major obstacle for LD or association studies is allelic heterogeneity. When this occurs, while the susceptibility genes may be the same in different probands, mutations will be different in individuals at risk, diminishing remarkably the power of detection \[23\]. Population size, history and structure must be considered before conducting association studies \[24\].

One practical application of LD has been the study of associations between common gene variants and common disease \[25\]. When LD is observed within a disease
population, this may indicate either that a functional variant of the gene carries risk, or that the markers are positioned near a susceptibility locus [26]. If one of the loci is a susceptibility gene, association between an allele and the disease being investigated will be observed [26]. In general, it is easier to detect common variants than rare ones, since they can be detected in smaller sample size. Moreover, they are generally older variants and more geographically dispersed, and may be found in multiple populations. However, they have less LD than rare types cause they have had more time to recombine [22].

1.2.7 Haplotypes

Human genetic diversity appears to be limited not only at the level of individual polymorphisms, but also in the specific combinations of alleles at closely linked sites on an individual chromosome. These specific allelic combinations are referred to as haplotype blocks. Haplotype blocks are considered chromosomal segments descended from a single ancestral chromosome. There is high significant LD between the SNPs within a block due to minimal recombination [27]. Haplotypes are used in different approaches such as genetic linkage mapping and localization of disease genes by LD [18, 27]. The probability to discover a causative variant within a block is mainly dependent on the extent of LD, as it determines the distance within which the causal variant can be mapped [27]. Haplotypes in this context have been the basis for the HapMap project and the whole genome LD mapping. Genomic regions can be tested for association by defining common haplotypes using a dense set of polymorphic markers and evaluating each haplotype for association with disease.

Two more distinct concepts of haplotypes have been recently proposed, namely gene-based haplotypes and gene-based functional haplotypes [27]. Gene-based haplotypes represent combinations of markers within one gene unit. They are simply a collection of specific allele markers but treated as units they are more informative than an individual allele [27, 28], and do not necessarily rely on presence of LD throughout the entire gene, which is a mandatory factor for definition of ancestral haplotypes. Gene-based haplotypes may be integral part of an ancestral haplotype block since they can cover more than one gene unit and are not limited by gene boundaries [27, 28].

The markers comprised in gene-based haplotypes may be of any class, for instance SNPs, microsatellites, RFLPs or VNTRs. If a sufficient number of markers are identified in and around the gene, which can capture disease-relevant LD pattern, multiple correlations with unobserved neighboring and/or embedded variants may be possible [27]. Since higher order of LD is present, gene-based haplotypes are expected to have higher power to detect unobserved variants than a single allelic marker. They may also have significantly more power to predict disease risk and drug response than any individual SNP within a gene [27, 29].

Since in principle gene-based haplotypes may contain all the variations in a gene, this concept can be taken a step forward with the “gene-based functional haplotype”, which is defined as the sequence variant unit that determines structure, function and regulation of the gene and its products. While still entirely theoretical, it should incorporate all
information necessary to characterize a gene variant and the functional or dysfunctional protein coded by the gene, as well as its related regulatory sequences \[27, 29\].

1.2.8 Case-control studies

In order to detect association between a specific allele and the disease, different experimental designs can be performed \[30\]. This thesis has focused on case-control studies. The case-control method compares the frequency of a DNA variant in the proband population (case) with its frequency in the control group. A homogeneous group of probands with a trait of interest are ascertained with a matching (i.e. by gender, age, ethnicity, history, etc) control group. DNA samples are obtained, and subjects are genotyped for a genetic marker believed to be of etiologic relevance. Statistical analyses compare allele or genotype frequencies in cases vs. controls. This type of association study has frequently been used to investigate the impact of environmental risk factors on disease pathogenesis \[31\]. The design is also employed for genetic variability on disease susceptibility. Case-control studies are preferred when no additional knowledge of the genetic model of the disorder is at disposal. They are more sensitive and effective to detect a genetic predisposition factor that carries small risk. However they are potentially prone to bias if cases and controls are not comparable \[30, 31\]. One design advantage is the ability to control the disease phenotype during the study, which is particularly important in psychiatric genetics since psychiatric diagnosis suffers from poor phenotype-genotype correlations. This means that for a particular phenotype it is difficult to select conceivable candidate genes based on a strong biological hypothesis \[32\]. In addition, co-morbidity, etiologic and genetic heterogeneity are frequently to be expected \[23, 31, 32\]. These elements could contribute to false-negative and false-positive results and subsequently give rise to conflicting results, which is what case-control studies are criticized for. On the other hand, the analysis of contradictory results may in fact lead to discovering the etiologic heterogeneity of the disease \[31\].

Fundamentally, cases and controls should represent "identical" samples from a single population except for the diagnostic differences. However, misclassification errors are common and have a heavy impact on the genetic analysis. The choice of study population must consequently be based on strict inclusion/exclusion criteria to prevent admixtures and dilutions of the groups \[31\].

Control groups are theoretically randomly sampled from the general population. The ideal control group should have the same genetic background and ethnicity as the affected group. Control groups should preferably be older than the mean age of onset for the disease under study, and have no personal and family history of the spectrum of the disease \[33\]. However in reality the uncertainties around the genetic validity of etiology, pathogenesis and symptoms of the disorders, especially in case of psychiatric disorders, make the random selected population less than ideal.
1.3 CLASSIFICATION IN PSYCHIATRY

Psychiatric Diagnoses are currently categorized by two systems; the Diagnostic and Statistical Manual of Mental Disorders (DSM) and the International Classification of Diseases (ICD).

1.3.1 Diagnostic and Statistical Manual of Mental Disorders (DSM)

DSM is published by the American Psychiatric Association (APA). It was first published in 1952 (DSM-I), and the version used today was issued in 1994 (DSM-IV) and revised in year 2000 (DSM-IV-TR). DSM covers all mental health disorders for both children and adults, with each disorder supported by a list of criteria that describe particular behaviors and subjective experiences. These behaviors and experiences are regarded as symptoms of the disorder. It also lists known causes of these disorders, statistics in terms of gender, age of onset, and prognosis as well as some research concerning the optimal treatment approaches. DSM uses a multiaxial or multidimensional approach for diagnostics because generally other factors in a person's life have high impact on their mental health. It assesses five dimensions as described below:

Axis I: Clinical syndromes
Axis II: Personality and developmental disorders
Axis III: Physical conditions
Axis IV: Severity of psychosocial stressors
Axis V: GAF: Global Assessment of Functioning, or the overall level at which an individual functions includes social, occupational, academic, and other areas of personal performance.

1.3.2 International Classification of Diseases (ICD)

The first edition (1893), known as the International List of Causes of Death, was adopted by the International Statistical Institute in Chicago. American Public Health Association (APHA) recommended revising the system every ten years to ensure that the system remained current with medical practice advances. As a result, the first international conference to revise the International Classification of Causes of Death convened in 1900; with revisions occurring every ten years thereafter. The revisions that followed contained minor changes, until the sixth revision of the classification system. The sixth revision included morbidity and mortality conditions, and its title was modified to reflect the changes: *Manual of International Statistical Classification of Diseases, Injuries and Causes of Death (ICD)*. In 1948, the World Health Organization (WHO) assumed responsibility for preparing and publishing the revisions to the ICD every ten years.

The ICD has become the international standard diagnostic classification for all general epidemiological and several health management purposes. These include the analysis of the general health situation of population groups, and the monitoring of the incidence
and prevalence of diseases and other health problems in relation to other variables such as the characteristics and circumstances of the individuals affected. Currently the tenth version, ICD-10, is in use and is distinguished from DSM by covering only clinical syndromes and personality disorders.

1.3.3 Endophenotypes

Psychiatric diagnoses are based on observable symptoms and there is considerable heterogeneity within any given diagnosis. Because there is likely a complex cascade of events between the genetic pathogenesis of a disorder and the eventual manifestation of symptoms, it is not clear that clinical diagnoses are the best phenotype for use in genetic analyses. A more optimal phenotype for genetic analyses might be an intermediary measure that would bridge the gap between the clinical and experimental approaches [34]. These intermediary phenotypes have been termed endophenotypes. The concept of endophenotypes was first applied to psychiatric disorders by Gottesman and Shields [35]. The concept was based on the assumption that the number of genes involved in the variation of endophenotypes representing relatively straightforward and more elementary phenomena are fewer than those involved in producing a psychiatric disorder entity [36]. The use of endophenotypes has been proposed as a strategy to aid gene identification efforts for complex phenotypes. Psychiatric endophenotypes can include measurements obtained through various methodologies: neurophysiological, biochemical, endocrinological, neuroanatomical, cognitive, and neuropsychological measures [37]. Of particular relevance today are advanced tools of neuroimaging, which have greatly enhanced the range of findings [38]. When biological markers are considered as endophenotype candidates, there are several criteria to comply with [36]; (i) the marker should be associated with the disease; (ii) it should be found in higher rates in the unaffected relatives of affected individuals than in the general population; (iii) the marker should be heritable, and there should be a genetic correlation between the trait and disorder, indicating that shared genes are contributing to the observed relationship [36].
1.4 PSYCHIATRIC DISORDERS COVERED IN THIS THESIS

Three distinct psychiatric disorders assemble the basis of this thesis, namely Major Depressive Disorder (MDD), Schizophrenia (SCZ), and Borderline Personality Disorder (BPD).

1.5 MAJOR DEPRESSIVE DISORDER (MDD)

1.5.1 Epidemiology

Major depressive disorder, also known as unipolar depression, is classified under the category of “mood disorders”, and is one of the most common psychiatric diseases with prevalence estimates ranging from 5-20% \[^{[39]}\]. It is associated with increased mortality mainly because of a high suicide rate \[^{[40]}\]. The mean age of onset for major depression is 40 years of age, with 50% of the patients having an onset between ages 20 and 50 \[^{[41]}\]. For more than 75% of patients, major depression is a recurrent, lifetime illness characterized by repeated remissions. Over 50% who recover the first episode will have a relapse within 6 months unless they are given maintenance antidepressant treatment \[^{[42]}\].

1.5.2 Clinical Diagnosis & Treatment

MDD is characterized by one (single episode) or more major depressive episodes (recurrent episodes) without a history of manic, mixed, or hypomanic episodes. Depression affects biological processes that control metabolic activity and neuroendocrine regulation, which in turn affect cognition and emotions, sleep regulation, activation of the hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system \[^{[43]}\]. Therefore MDD is a clinically heterogeneous disorder and comprises different symptoms such as disturbances in mood or loss of interest or pleasure in nearly all activities which should be distinguished from normal behavior toward loss. The symptoms need to be persistent for more than two weeks to signify a diagnosis. Four additional symptoms must also be present including changes in appetite, weight, sleep, and psychomotor activity; decreased energy; feelings of worthlessness or guilt; difficulty thinking, concentrating, or making decisions; or recurrent thoughts of death or suicidal ideation, plans, or attempts. The episode must be accompanied by distress or impairment in social, occupational, or other important areas of functioning \[^{[44,45]}\]. The DSM-IV-TR diagnostic criteria for MDD are listed in Box 1 \[^{[45]}\].

Suicidal thoughts or intentions occur in different degrees of intensity in depressed individuals. Up to 15% of individuals with severe major depressive disorder die by suicide. There is a 4-fold increase in death rate of individuals with major depressive disorder over 55 years of age \[^{[45]}\]. Furthermore, MDD is considered a leading cause of disability among individuals between 15-44 years of age \[^{[40]}\].

Treatment can either combine both pharmacotherapy and psychotherapy or utilize one or the other individually. Psychotherapy is useful in helping the patient understand the factors involved in either creating or exacerbating the depressive symptomatology.
MDD has a better prognosis than other mood disorders for which, medication and therapy have been very successful in alleviating symptomatology [41].

**Box 1. DSM-IV-TR Criteria for Major Depressive Disorder**

A. At least one of the following three abnormal moods which significantly interfered with the person’s life:
   1. Abnormal depressed mood most of the day, nearly every day, for at least 2 weeks.
   2. Abnormal loss of all interest and pleasure most of the day, nearly every day, for at least 2 weeks.
   3. If 18 or younger, abnormal irritable mood most of the day, nearly every day, for at least 2 weeks.

B. At least five of the following symptoms have been present during the same 2 week depressed period.
   1. Abnormal depressed mood (or irritable mood if a child or adolescent) [as defined in criterion A].
   2. Abnormal loss of all interest and pleasure [as defined in criterion A2].
   3. Appetite or weight disturbance, either:
      - Abnormal weight loss (when not dieting) or decrease in appetite.
      - Abnormal weight gain or increase in appetite.
   4. Sleep disturbance, either abnormal insomnia or abnormal hypersomnia.
   5. Activity disturbance, either abnormal agitation or abnormal slowing (observable by others).
   6. Abnormal fatigue or loss of energy.
   7. Abnormal self-reproach or inappropriate guilt.
   8. Abnormal poor concentration or indecisiveness.
   9. Abnormal morbid thoughts of death (not just fear of dying) or suicide.

C. The symptoms are not due to a mood-incongruent psychosis.

D. There has never been a Manic Episode, a Mixed Episode, or a Hypomanic Episode.

E. The symptoms are not due to physical illness, alcohol, medication, or street drugs.

F. The symptoms are not due to normal bereavement.

1.5.3 Heritability

MDD is a familial disorder as a result of genetic factors [46]. Although epidemiological studies indicate an environmental component in the etiology of MDD, a large genetic component has also been found. The heritability of major depressive disorder is estimated at a range of 30-40% [47-50]. Recently Kendler and colleagues performed the largest twin study to date, including 15,000 pairs of twins from the national Swedish Twin Registry [46]. In this study the heritability of life time MDD was found to be similar to previous findings being around 40%. Moreover the heritability of liability to depression was found to be approximately twice in women than in men [46]. The reason has been hypothesized to involve hormonal differences, the effects of childbirth, variety of psychosocial stressors and different behavioral models of learned helplessness [41]. Several studies have found the risk of MDD in first-degree relatives of probands to be 2 to 4 times that of controls [51, 52], and 4 to 8 times greater in relatives of proband with recurrent, early-onset major depressive disorder [53].

1.5.4 Etiology

Major depression is believed to result from interplay of multiple genes interacting with environmental and developmental epigenetic components [39]. Potent environmental
factors may include a history of abuse (physical, emotional, and/or sexual), childhood neglect, and life stress such as poor social support and difficulties related to finances or employment \cite{54, 55}. Genetic risk factors for stressful life events are positively correlated to genetic risk factors for major depression \cite{55}.

A complete comprehensive model of vulnerability to depression including the neurobiological and environmental factors is not available yet. However, the stress regulatory HPA axis subsequently involving several signal transductions pathways, such as the serotonergic system, are believed to be involved in etiology of depression \cite{55}. The HPA axis has been implicated in the causality of affective disorders, such as major depression and post-traumatic stress disorder \cite{56, 57}, along with neurodegenerative disorders (for example Alzheimer) and systemic disorders (for example asthma, hypertension) \cite{58}. Evidence suggest that prolongation of the adaptive stress responses in form of inadequate control of glucocorticoid regulation can promote the development of disease \cite{58}.

Additionally, the HPA axis is connected to the serotonin system by direct neural connections through raphe nucleus and hippocampus but also indirectly via the amygdala \cite{59}. Alterations in serotonergic neuronal function in the central nervous system (CNS) are observed in patients with MDD\cite{60, 61}. The hypothesis is based on several findings that include reduced cerebrospinal fluid (CSF) concentrations of 5-hydroxyindoleacetic acid (5-HIAA) \cite{62}, which is the major metabolite of serotonin (5-HT). Reduced concentrations of 5-HT and 5-HIAA in postmortem brain tissues of depressed and/or suicidal patients have been found \cite{63}. Further decreased plasma tryptophan concentrations has been found in depressed patients \cite{64}. Also, clinically efficacious antidepressants such as selective serotonin reuptake inhibitors (SSRIs) \cite{65}, enhance 5-HT neurotransmission following inhibiting 5-HT uptake \cite{66}. Other evidence suggest that number of 5-HT transporter binding sites are decreased in postmortem brain tissues of depressed patients and in platelets of drug-free depressed patients \cite{60, 67}.

The noradrenergic system has also been implicated in pathophysiology of depression \cite{41}. There are indications of both down- and up-regulation of noradrenergic system including increased density of α2-adrenergic receptors in locus coeruleus, the major brain norepinephrine containing nucleus \cite{55}. α2-adrenergic receptors act as inhibitory autoreceptors on noradrenergic terminals in the brain and periphery. Occupation of these autoreceptors by agonists, including noradrenaline itself, attenuates noradrenaline release from sympathetic nerves \cite{68}. A gene variant of α2-adrenergic receptor type c has in its homozygous form been associated to anxiogenic effects.

Another hypothesis about the etiology of MDD is that neurotoxic effects damage hippocampal cells which in turn mediate many depressive symptoms, with deficient neuroprotective peptides. Genetic factors may alter balance of neurototoxic/neuroprotective responses to stress \cite{69}. Reduced serum brain-derived neurotrophic factor (BDNF), a neuroprotective peptide has been reported in MDD and bipolar disorder \cite{70} but the subsequent reports have not confirmed these data \cite{71}.
1.5.5 Molecular Genetics

Etiological research in major depressive disorder has also focused on the investigation of genetic factors. The serotonergic system has become a high priority target of these studies. Especially the serotonin transporter (5-HTT) has received particular attention since it is the target of antidepressant drugs [72]. Two susceptibility loci on 5-HTT gene have been identified, one located in the promoter region (5-HTTLPR) and the second in intron 2 (STin2). The genetic properties and association studies on these two polymorphisms are described in detail in section 1.8 as they are the focus of this thesis.

Stressful life events are suggested to predispose and predict subsequent depression, and this relationship might be mediated by the polymorphism 5-HTTLPR. However, it appears that the associated genotype influences stress reactivity and sensitivity to the pathogenic effects of the environment rather than causing depression [73]. Lifetime diagnoses of depression and anxiety show extensive comorbidity. It has been estimated that depressed patients have an overall rate of about 55% for any anxiety disorder [74]. However, this rate varies widely among various diagnoses, for example it is about 65% in panic/agoraphobia but only 20% in social and simple phobias. It has also been estimated that the likelihood for someone with a mood disorder to get an anxiety-disorder diagnosis (either concurrently or subsequently) appears to be greater than the reverse [74]. The first major analysis on self-reported anxious and depressive symptoms indicated that the phenotypic covariation between the two types of symptoms was largely due to shared genetic factors [75, 76]. These analyses were further confirmed by twin studies that showed generalized anxiety disorder (GAD) and MDD share the same genetic factors but that their environmental determinants are mostly distinct [77]. However, the precise nature of the genetic factors remains unclear [74]. Moreover, these genetic factors were shared with neuroticism, a broad personality trait that reflects individual differences in subjective distress and dissatisfaction [75, 78]. Neuroticism and MDD have been associated in clinical, family and twin studies, although with controversial outcome [79]. High neuroticism scores may predict onset of MDD in healthy individuals [79]. Anxiety, depression, and neuroticism may be linked to a single genetic diathesis representing an underlying vulnerability to subjective distress and negative affectivity [74]. Several reports are relevant to the 5-HTT gene polymorphism and anxiety disorders [80, 81]. The frequency of the long variant of the 5-HTT polymorphism in intron 2 (see section 1.8) was found significantly higher in patients with GAD or obsessive compulsive disorder (OCD) [81]. Anxiety-related traits, including neuroticism, tension and harm avoidance have been correlated to 5-HTT promoter polymorphism as well [82].

Monoamine oxidase B (MAO-B), a mitochondrial enzyme responsible for the oxidative deamination of catecholamines and indolamines found in platelets has also been studied as a possible biological marker for MDD and GAD [83]. Although the MAO-B activity involvement has not always been replicated, lower MAO-B activity is suggested to be correlated to earlier onset [83] and to severity of depression [84]. Moreover, low MAO-B activity has been associated with violent criminality and suicide [85, 86].

In the context of serotonin dysregulation a SNP (G1463A) in the rate-limiting enzyme of neuronal serotonin synthesis, tryptophan hydroxylase-2 (TPH-2) was identified [87]. The functional SNP in TPH-2 replaces the highly conserved arg441 with his, which resulted in about 80% loss of function in serotonin production.
Identification of a loss-of-function mutation suggests that defect in brain serotonin synthesis may represent an important risk factor for unipolar major depression \[^{[87]}\]. The stress hormone regulating HPA axis has been implicated in susceptibility to depression \[^{[88]}\]. SNPs in FKBP5, a glucocorticoid receptor-regulating of hsp-90 protein associated with faster response to antidepressants and the recurrence of depressive episodes in 2 independent samples. FKBP5 triggers adaptive changes in glucocorticoid receptor and, thereby, HPA-axis regulation. Individuals carrying the associated genotypes had less HPA-axis hyperactivity during the depressive episode \[^{[88]}\].

A genome wide linkage survey for genetic loci that influence the development of unipolar mood disorders was conducted in 81 families identified through individuals with recurrent early-onset MDD \[^{[89]}\]. The findings indicate the region of chromosome 2q33-q35 that includes the CREB1 gene as a gender-specific susceptibility locus. Nineteen loci were found to predominantly affect the risk of depression in women. Analyses further show that the loci typically affect the risk of a spectrum of depressive disorders as well as alcoholism and other addictions \[^{[89]}\]. The role of the 48-bp repeat polymorphism in the dopamine D4 receptor (DRD4) gene on chromosome 11p15 has also been studied in mood disorders. An association has been found between the DRD4 2-repeat allele and unipolar depression \[^{[90]}\].

### 1.5.6 Endophenotypes

The complex nature and heterogeneity of major depression has led to identification of several endophenotypes; REM sleep abnormalities, hippocampal volume reduction, tryptophan depletion, catecholamine depletion CRH dysfunction and increased amygdala activity that may result in amygdala volume reduction are suggested biological endophenotypes related to MDD \[^{[91]}\].
1.6 SCHIZOPHRENIA (SCZ)

The term schizophrenia was originally introduced by Eugen Bleuler (1857-1939), a Swiss psychiatrist and psychologist. Bleuler identified specific primary symptoms of SCZ to develop his theory about the internal mental breakdown of patients. These symptoms included associational and affective disturbances, autism, and ambivalence, summarized at the four A’s: Association, Affect, Autism, and Ambivalence.

1.6.1 Epidemiology

SCZ is the major illness under the category of “psychotic disorders”. It is relatively common affecting approximately 1% of the general population, a chronic and frequently devastating neuropsychiatric disorder. The age of onset ranges from mid to late adolescence and the disease persists throughout life. It is more or less equally prevalent in both genders. However, there is a gender specific age of onset: males 18-25 years and females 25-35 years. Suicide is the leading factor of mortality in SCZ patients. Elements such as depressive illness, a history of suicide attempts, and poor social situations are considered to enhance the risk of suicide.

1.6.2 Clinical Diagnosis & Treatment

SCZ is a psychosis and it affects emotions although it is distinguished from mood disorders, in which such disturbances are primary. SCZ is characterized by psychotic features (delusions and hallucinations), disorganization, dysfunction in normal affective responses, and impaired cognition. The DSM-IV-TR diagnostic criteria for SCZ are listed in Box 2. A patient’s disorder is diagnosed as SCZ when the patient exhibits two of the symptoms listed as symptoms 3-5 in criterion A. At least one of the following must be present: a) thought echo, b) delusions of control, influence or passivity, c) hallucinatory voices. Symptoms must persist for a minimum of 6 months and a diagnosis of schizoaffective disorder or mood disorder must be absent. There is no cure for this disorder thus the prognosis is considered to be poor. However, medication is the most important part of treatment as it can reduce and sometimes eliminate the psychotic symptoms. Case management is often needed to assist with daily living skills, financial matters, and housing and therapy can help in coping skills to improve social and occupational abilities.

1.6.3 Heritability

SCZ aggregates in families and appears to have a significant genetic component. Twins and adoption studies have shown that this familiality is due to genetic factors rather than shared environmental factors. The prevalence among siblings and parents is approximately 10%. Twin studies estimate the concordance rate for monozygotic (MZ) twins at 41-65% compared with dizygotic (DZ) concordance of 0-28%, with a heritability estimate of 80-85%. Twin studies are also valuable for
investigating the etiological relationships between SCZ and other disorders, and the genetic basis of clinical heterogeneity within SCZ [96].

Moldin et al (1998) reviewed family and twin studies published between 1920 and 1987 and found the recurrence risk ratios to be 48 for monozygotic twins, 11 for first-degree relatives, 4.25 for second-degree relatives, and 2 for third-degree relatives [97]. He also found that concordance rates for monozygotic twins averaged 46%, even when reared in different families, whereas the concordance rates for dizygotic twins averaged only 14% [97]. Further studies on different classes of relatives show that the recurrence risk of SCZ decreases too rapidly with increasing distance from the proband to be a single-gene disorder [98], i.e. it does not follow classical Mendelian patterns of inheritance. SCZ often co-exists in familial pedigrees with other psychotic illnesses and severe mood disorders [95].

Box 2. DSM-IV-TR Criteria for Schizophrenia

A. Characteristic symptoms: Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated):
   1. delusions
   2. hallucinations
   3. disorganized speech (e.g., frequent derailment or incoherence)
   4. grossly disorganized or catatonic behavior
   5. negative symptoms (i.e., affective flattening, alogia, or avolition)

B. Social/occupational dysfunction: For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

C. Duration: Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

D. Schizoaffective and Mood Disorder exclusion: Schizoaffective Disorder and Mood Disorder With Psychotic Features have been ruled out because either (1) no Major Depressive Episode, Manic Episode, or Mixed Episode have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.

E. Substance/general medical condition exclusion: The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.

F. Relationship to a Pervasive Developmental Disorder: If there is a history of Autistic Disorder or another Pervasive Developmental Disorder, the additional diagnosis of Schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

1.6.4 Etiology

SCZ is probably a heterogeneous group of disorders with mixed biopathology. Several brain areas including the limbic system, the frontal cortex, cerebellum and the basal ganglia are suggested to have pathophysiological role in development of SCZ [41]. The classical dopamine hypothesis of SCZ postulates a hyperactivity of
dopaminergic transmission at the dopamine D₂ receptor in the mesencephalic projections to the limbic stratum \[99\]. The hypothesis is based on pharmacologic evidence of conventional antipsychotic drugs with high affinity for D₂ receptors and dopamine agonists (cocaine, amphetamine) that can induce psychotic-like features in healthy subjects \[100\].

Other nondopaminergic mechanisms such as the serotonergic and GABAergic systems may have possible pathogenetic importance in subgroups of SCZ \[41, 99\]. Serotonin has received attention since it was discovered that serotonin-dopamine antagonist (SDA) drugs have serotonin-related activities \[41\]. Serotonin receptors are also involved in psychotogenic properties of hallucinogens and the amount of cortical serotonin receptors (5-HT₂A and 5-HT₁A) are shown to be altered in schizophrenic brains \[100\]. The receptors are further suggested to play an important role in therapeutic and/or in developing side-effects of antipsychotics \[100\]. Decreased NMDA (N-methyl d-aspartate) subtype of glutamate receptor may also be a predisposing factor in SCZ, since it is involved in inducing SCZ-like symptoms in healthy individuals \[101\]. Although genetic risk factors play a key role in etiology of SCZ, research on non-genetic risk factors is abundant. Obstetric studies reveal that pre- and perinatal brain development is vulnerable to elements such as inflammatory responses to maternal infection and cytokines that regulate neurodevelopmental processes \[102\]. Population-based studies demonstrate that complications of pregnancy, delivery and/or abnormal fetal growth are significantly related to SCZ \[103\]. There are also reports of viral infection, urban birth and late-winter/early-spring birth as risk factors for developing SCZ \[100\].

### 1.6.5 Molecular Genetics

The fact that neuroleptics act through the dopaminergic and serotonergic system have provided a number of leads for SCZ susceptibility loci \[93\]. Various dopamine receptors have been cloned due to the high affinity of neuroleptics, and subdivided into D₁ (D₁ and D₅ receptors) and D₂ (D₂, D₃ and D₄ receptors) families based on their biochemical and pharmacological properties \[104\]. Among these, the D₂, D₃ and 5-HT₂A receptor genes have generated the most promising data \[93\]. A polymorphism in the promoter region of D₂ was found to alter receptor expression *in vitro* but no correlation was observed in single association studies \[98\]. Significant decrease in number of 5-HT₂A receptor and association with a functional polymorphism in D₃ exon 1 have also been reported but overall they do not seem to play a major role in predisposition to SCZ \[98\]. The unclear role of these genes in producing or modifying SCZ phenotypes and the complex pathophysiology of the disease has stimulated much work aimed at identifying other susceptibility genes.

Recently new putative susceptibility genes have been proposed, such as those encoding dysbindin (DTNBP1), neuregulin 1 (NRG1), D-amino acid oxidase (DAO), D-amino acid oxidase activator (DAOA), and regulator of G protein signaling-4 (RGS4). There are strong implications regarding DTNBP1 and NRG1, but the proofing data for DAO, DAOA and RGS4 are not as compelling \[105\].
DTNBP1 was identified mapping chromosome 6p22. The encoded protein binds to a complex in synaptic terminals in the cerebellum and hippocampus. Evidence in favor of DTNBP1 as a susceptibility gene is strong and significant associations have been published by several studies. Despite the strong association, the specific risk alleles and haplotypes are not consistent between the different studies.

NRG1 encodes for fifteen proteins with diverse range of functions in the brain areas, such as synaptogenesis, glial differentiation, myelination and neurotransmission, and the gene has been mapped to 8p21-p22. No specific susceptibility variants have yet been identified but several studies have replicated the initial Icelandic haplotype at the 5' end of NRG1.

Transcription of the RGS4 gene was found diminished in schizophrenic patients. Four SNPs within a 10-kb span of RGS4 were identified with significant association to SCZ, on chromosome 1q23. There is also correlation with structural alterations in the dorsolateral prefrontal cortex of SCZ patients.

DAO and DAOA are suggested to influence the risk of SCZ through similar pathways, mediated through alterations of NMDA receptor function. Although there are a number of studies on DAO and DAOA, there is no consensus concerning the specific risk alleles or the haplotypes across the studies.

Several lines of evidence have implicated chromosomal abnormalities associated with SCZ. A high-susceptibility locus has been confirmed on chromosome 22q12, associated with velo-cardio-facial syndrome (VCFS), which includes symptoms of SCZ. The region codes for high density lipoproteins which play a central role in cholesterol transport. Cholesterol is important in cellular processes such as modulating gene transcription and signal transduction both in the adult brain and during neurodevelopment. Other chromosomal abnormalities are micro-deletions of chromosome 22q11 which occur approximately 100 times more frequent in adult schizophrenic patients than in the general population. Proline dehydrogenase (PRODH) gene and catechol-O-methyltransferase (COMT) are other candidate genes in the 22q11 region that have been implicated in susceptibility to SCZ. The association between val158/108met polymorphism of the COMT gene and SCZ has been evaluated in a meta-analysis of 14 case-control and 5 family-based studies published between 1996 and 2002. The case-control studies showed no indication of an association, but in the family-based studies modest evidence was found implicating the val allele as a risk for SCZ. Emphasizing on population stratification bias it was suggested that the val allele may be a small but reliable risk factor for SCZ for people of European descent. However another large-scale association study also covering a meta-analysis of the COMT val/met polymorphism, did not show any associations in Asian nor European populations.

Another major chromosomal abnormality involves a translocation which disrupts two genes on chromosome 1, DISC1 and DISC2 (Disrupted-in-SCZ). DISC2 contains no open reading frame and can regulate DISC1 expression but the function of DISC1 is not completely understood. Its function may be related to cytoskeletal regulation during neuronal migration. Four studies have attempted to find association between DISC1/DISC2 and SCZ. Positive data have only been reported in two cases of Finnish and US schizophrenic samples.
A role has been proposed for the AKT1/GSK3β (kinases implicated in synaptic plasticity) signaling pathway in SCZ \[117\]. This signaling pathway is a known target of lithium, which is used for the treatment of certain mood disorders. Reduced levels of AKT1 protein in the brains of individuals with SCZ have been reported, indicating AKT1 as a potential SCZ susceptibility gene \[117\].

The role of synapsin II variants in susceptibility to SCZ has been suggested by reduced synapsin II mRNA expression in the brains of SCZ patients. Synapsin II encodes a neuron-specific phosphoprotein that selectively binds to small synaptic vesicles in the presynaptic nerve terminal (PSYN) \[118\]. Moreover genes of the PSYN group showed decreased expression in the prefrontal cortex of schizophrenic patients \[119\].

The brain-derived neurotrophic factor (BDNF) gene has also been studied as a risk factor for SCZ. The 66 val/met polymorphism has showed association for valine (allele G) with SCZ \[120\].

### 1.6.6 Endophenotypes

Neurobiological abnormalities in SCZ were initially assessed using histopathological studies on postmortem brains of SCZ subjects, but led to inconclusive findings \[38\]. Inconclusive results in postmortem studies might have been due to variables such as age, postmortem changes, and natural limitations to perform longitudinal studies. Technical advances that permit the imaging of the whole brain and all its tissues in live human subjects have partially overcome these limitations \[38\].

Abnormalities due to early stage of psychosis with a range of brain structure changes including deficits in temporal lobe, reduced whole brain volume, reduced prefrontal cortical grey matter volume, significant enlargement of lateral and third ventricles and reduced thalamic volume have been identified by recent MRI research \[100\]. fMRI and PET studies have provided identification of neural structures associated with observed cognitive problems such as working memory tasks. These studies further suggest that auditory hallucinatory states are associated with activation in areas such as the anterior cingulate, bilateral temporal cortex, right thalamus, left hippocampus and parahippocampal cortex \[100\].
1.7 BORDERLINE PERSONALITY DISORDER (BPD)

The origin of the term "BPD" dates back to the early 1900’s. At this time people with mental health disabilities were either categorized as neurotic or psychotic. In 1938, the term was first introduced by Adolph Stern, referred to patients on the "borderline" between neuroses and psychoses [121].

1.7.1 Epidemiology

Approximately 1-2% of the population meet the criteria for BPD [122, 123]. Up to 10% of the patients commit suicide, a rate almost 50 times higher than in the general population [124]. The disorder is more common in women (70%) than in men (30%) [125].

1.7.2 Clinical Diagnosis & Treatment

In 1975, Gunderson and Singer identified putative descriptions of BPD features in the areas of dysphoric affects, impulsive action, psychotic-like cognition, interpersonal relationships and social maladaptation [126]. It was only in DSM-III, based on the systematic description of these characteristics, that BPD became a diagnosis consisting of eight criteria. The most significant revision in DSM-IV was the addition of a ninth criterion to DSM-IV, "transient, stress-related severe dissociative symptoms or paranoid ideation." [121]. According to the latest version, DSM-IV-TR, the diagnosis of borderline personality disorder is made on the Axis II when the patient shows at least five of the nine criteria listed in Box 3 [44].

Four core elements are suggested to play a major role in the development of BPD: interpersonal stress, affective instability, impulsivity and dissociation [127]. Affective disturbance involve experiencing several dysphoric states and periods of euthymia with various intensities during the course of a day. Their impulsivity is categorized in two types, with type one including deliberate physical self-destruction, suicidal communications and suicide attempts. Substance abuse, disordered eating, and/or reckless driving are examples of the other type of impulsivity. Further, BPD patients have disturbed cognition with a variety of non-psychotic or psychotic-like symptoms and are engaged in intense unstable relationships [128].

The disorder is fairly stable, making prognosis difficult to assess. Longitudinal studies show that patients have a high incidence of major depressive disorder episodes. While the disorder is chronic in nature, gradual improvements with work are definitely seen. While it is difficult for anyone to change major aspects of their personality, the symptoms of this disorder can be reduced in both number and intensity. Long term treatment is almost always required [41].

1.7.3 Co-morbidity

Although BPD can exist as the sole diagnosis, it often co-occurs with Axis I disorders such as major depression and substance abuse [126]. MDD may be primary and lead to
the development of traits and behaviors found in BPD as secondary complications. Conversely, BPD psychopathology may be primary and predispose patients to the development of superimposed Axis I disorders. In a study of patients diagnosed with BPD, 39% met criteria for at least one mood disorder, of which 31% were diagnosed with major depression \([129]\). BPD and certain Axis I disorders may share some common etiologic factors that increase their co-occurrence. However, they may even be unrelated, but still tend to co-occur and influence each other’s symptom expression or course \([126]\).

1.7.4 Heritability

BPD heritability is estimated at almost 50% \([126, 130]\). Prospective studies show a range of 15-23% of morbid risk of BPD in first-degree relatives of probands \([123, 131]\). The role of a predisposing genetic background has been investigated in twin studies \([130]\). In a Norwegian twin study, the concordance for BPD was 35% in MZ pairs and 7% in DZ pairs and the best-fitting model estimated the heritability to 69% \([132]\). Earlier studies from this group on a small sample of twins indicated that environment is important for development of BPD while genetics is not \([133]\). Their more recent and larger studies suggest instead a strong genetic effect in the development of the disorder \([132]\).

Impulsive aggression is a liability component in the heritability of suicide and suicidal behavior \([134]\). Twin studies have shown an increased suicide risk among co-twins even after controlling for other risk factors \([135, 136]\). In a large Australian twin study, genetic factors accounted for 45% of the variance in suicide thoughts and behavior \([136]\). Several twin and adoption studies indicate that suicidal behavior is heritable, and heritability includes both suicide attempts and suicide completion \([134]\). Both completed suicide and suicidal behavior have been reported to have higher concordance rates in MZ than in DZ twins (14.9% vs. 0.7% and 23% vs. 0.7% respectively) \([137]\). Some overlap between heritability of suicidal ideation and actual suicidal behavior has also been noted in twin studies \([135]\).

1.7.5 Etiology

Several environmental and genetic factors are considered predisposing \([130, 138]\). Environmental factors such as sexual abuse or situations of abandonment are proposed to induce dysfunctional behaviors and psychosocial conflicts, which in turn might cause emotional dysregulation and impulsivity \([128]\). Single traumatic events may lead to post traumatic stress disorder, whereas long term exposure to stressors such as sexual abuse in combination with other emotional neglects may contribute to development of BPD \([127]\). A history of physical or sexual trauma during childhood is reported by 40-70% of inpatients. The severity of borderline psychopathology has also been linked to severity of childhood sexual abuse \([139, 140]\). Impulsive aggression and affective instability are suggested to be major contributing elements of BPD \([130]\). While each feature may also individually contribute to certain other personality disorders (impulsive aggression to antisocial personality disorder, and
affective instability to histrionic personality disorder) their co-occurrence may particularly predispose to BPD \cite{122}.

The 5-HT system has been implicated in the etiology of both self-directed and non-self-directed impulsive aggression \cite{141, 142}. Structural and functional neuroimaging data show dysfunctional areas in brain networks such as the anterior cingulated cortex, the hippocampus, and the amygdala which are also involved in dysfunctional 5-HT neurotransmission \cite{143}. In female patients with BPD, reduction of the volumes of the hippocampus have been observed, but it is not clear if the association of volume reduction is under influence of duration of traumatic experiences \cite{144}.

### 1.7.6 Molecular Genetics

Features of BPD such as affective instability and impulsive aggression has been associated with the cholinergic system and the serotonergic activity respectively \cite{130}. The evidence for serotonergic involvement in impulsive aggression, violent behaviors and various suicidal behaviors rely on correlation with 5-HT metabolites such as 5-HIAA \cite{145}. Violent offenders who committed impulsive crimes have shown lower levels of 5-HIAA than nonviolent offenders \cite{145}. Decreased levels of 5-HIAA have also been observed in self-directed aggression such as violent suicidal behavior \cite{146}. Several studies have found lower platelet MAO activity which has been associated with various forms of impulsive behavior \cite{147}. Certain personality traits such as impulsivity have also been positively correlated with the number of platelet serotonin binding sites in patients with BPD \cite{127}.

Studies showing reduced serotonergic activity in association to impulsive aggression in BPD patients have led to studies of serotonergic candidate genes \cite{130}. One of the 5-HT system genes that have been studied in relationship to aggression, anger, impulsivity, and suicidal behavior is TPH-1 gene \cite{148-151}. Significant associations have been reported between SNP A779C on TPH-1 gene intron 7 and CSF 5-HIAA concentrations among Finnish impulsive offenders \cite{152}. The polymorphism was also associated with violent offenders who had a history of suicide attempts \cite{153}. While allele 799C was associated with the Finnish group, the allele 799A was more represented in depressed suicide attempters \cite{150, 151}. In an extensive study, seven TPH-1 polymorphisms were investigated in relationship to suicidal behavior. The strongest associations were found for violent suicidal attempts with no history of depression \cite{154}.

Most studies have also investigated A218C, another TPH-1 SNP in intron 7 in strong linkage disequilibrium with the A779C SNP. Several Caucasian studies have reported an excess of 218A in suicide related behavior while others have not \cite{155}. A meta-analysis of 9 association studies evaluated the relationship between the A218C polymorphism of the TPH gene and suicidal behavior. A significant association was observed between this polymorphism and suicidal behavior \cite{156}.

In a study of women with bulimic syndrome co-morbidity with personality disorder was observed in individuals carrying the s allele of 5-HTT promoter polymorphism. The carriers of the s allele also showed significantly more affective instability,
interpersonal insecurity and behavioral impulsivity \[157\]. There are other studies showing the involvement of the serotonergic system: a G allele polymorphism in 5-HT1B receptor has been associated to suicidal patients with personality disorder, and 5-HT2A receptor gene variations have been correlated to impulsive aggression \[122\]. Another dimension of borderline personality disorder is the striking emotional reactivity to environmental stimuli, defined as affective instability. The neurochemistry underlying affective instability has not been investigated as widely as that for impulsive aggression \[130\]. However, clinical research suggest that cholinergic dysregulation may play a role in developing affective instability \[122\]. The limbic areas involved in emotional regulation (amygdala, hippocampus and cingulate cortex) are rich in cholinergic neurons. Studies show that procaine, a cholinergic agonist, selectively activates limbic structures \[158\]. More specifically, using PET imaging activation of the left amygdala has been correlated with affective response to procaine \[159\]. Another evidence of cholinergic involvement in BPD is based on decreased REM sleep which in response to cholinergic inhibitors diminished even more in its latency \[160\]. Further, intravenous administration of cholinergic inhibitors has been shown to shift the patients’ affection toward depression, and the magnitude of the depression was correlated with the number of affective instability traits (i.e. affective instability per se, identity disturbance, and unstable relationship) \[161\].

### Box 3. DSM-IV-TR Criteria for Borderline Personality Disorder

A pervasive pattern of instability of interpersonal relationships, self-image, and affects, and marked impulsivity beginning by early adulthood and present in a variety of contexts, as indicated by five (or more) of the following:

A. Frantic efforts to avoid real or imagined abandonment.

   **Note:** Do not include suicidal or self-mutilating behavior covered in Criterion E.

B. A pattern of unstable and intense interpersonal relationships characterized by alternating between extremes of idealization and devaluation

C. Identity disturbance: markedly and persistently unstable self-image or sense of self

D. Impulsivity in at least two areas that are potentially self-damaging (e.g., spending, sex, substance abuse, reckless driving, binge eating).

   **Note:** Do not include suicidal or self-mutilating behavior covered in Criterion E.

E. Recurrent suicidal behavior, gestures, or threats, or self-mutilating behavior

F. Affective instability due to a marked reactivity of mood (e.g., intense episodic dysphoria, irritability, or anxiety usually lasting a few hours and only rarely more than a few days)

G. Chronic feelings of emptiness

H. Inappropriate, intense anger or difficulty controlling anger (e.g., frequent displays of temper, constant anger, recurrent physical fights)

I. Transient, stress-related paranoid ideation or severe dissociative symptoms

### 1.7.7 Endophenotypes

The endophenotype approach has not been as successful for BPD as for other psychiatric diagnosis such as SCZ \[138\]. However, suicide has been widely examined, being a highly rated characteristic of BPD. Postmortem studies of suicidal victims have
revealed reduced level of brain 5-HT and higher receptor density across all cortical layers in prefrontal cortex \cite{162}. Imaging studies have linked reduced serotonergic responsiveness to reduced activity in limbic brain areas including orbital frontal cortex and related ventral medial cortex and cingulated cortex \cite{130}.

1.8 THE SEROTONERGIC SYSTEM

Serotonergic neurons are widely distributed in the mammalian brain. Therefore, 5-HT influences almost every sphere of mammalian physiology, from cardiovascular regulation \cite{163, 164}, respiration, the gastrointestinal system \cite{165}, pain sensitivity, and thermoregulation to more centrally controlled functions. The latter include the maintenance of circadian rhythm, appetite, aggression, sensorimotor activity, sexual behavior, mood, cognition, learning, and memory \cite{166}.

This neuronal network is one of the earliest to develop in the embryo \cite{167}. Serotonin-containing neurons projecting to the forebrain originate in four brain stem nuclei, the principal of these being median and dorsal raphe nuclei. The dorsal raphe nucleus projects thin serotonin fibers, which are more abundant in the cortex, whereas the median raphe nucleus provides thick serotonin fibers with large varicosities that, are relatively sparse and more abundant in the hippocampus \cite{168}. The hippocampus receives fibers following a dorsomedial course through the cingulate cortex \cite{169}.

Serotonin neurons innervate almost all areas of the brain but mostly they are localized along the midline of the brainstem \cite{170}. Their projections and targets are summarized in the figure below.

Figure 1. Projections and targets of the serotonergic neurons.

1.8.1 The Discovery of Serotonin

The chemical 5-hydroxytryptamine was first isolated in serum and because of its powerful vasoconstrictive effects, was dubbed “serotonin” \cite{171}. Serotonin was later
detected in brain extracts that produced peripheral vasoconstriction\textsuperscript{[172]}. Later on, the existence of multiple serotonin receptor subtypes was reported which were named 5-HT-M and 5-HT-D after their antagonists, morphine and diabenzyline, respectively \textsuperscript{[173]}. The 5-HT receptors were later reclassified by Peroutka and Snyder based on radioligand-binding studies in brain homogenates \textsuperscript{[174]}. Since the late 1980s, the application of molecular cloning techniques has contributed in cloning of 14 distinct subtypes of serotonin receptors \textsuperscript{[175]}.

1.8.2 Serotonin Biosynthesis, Function & Regulation

Serotonin (5-hydroxytryptamine, 5-HT), the “happiness hormone” has a phylogenetically ancient role in neural transmission \textsuperscript{[176]}. Prior to its vital role as neurotransmitter in the mature brain, 5-HT acts as a regulator of brain development. Because its turnover is higher in the immature mammalian brain than any other stage, it has been proposed that serotonin plays a key role in brain development process \textsuperscript{[177]}. Once serotonergic cells have developed, serotonin release may influence neurogenesis, neuronal apoptosis, cell migration and synaptic plasticity \textsuperscript{[178]}. Serotonin acts as a developmental signal for brainstem serotonin neurons and their target cells through the activation of serotonin receptors \textsuperscript{[178]}. Disruption of serotonergic development can leave permanent alterations in brain function and behavior, as in cases of human developmental illnesses like autism and Down Syndrome \textsuperscript{[177]}.

Serotonin is principally found stored in three main cell types: (a) serotonergic neurons in the CNS and in the intestinal myenteric plexus, (b) in the mucosa of the gastrointestinal tract and (c) in blood platelets. In the human body serotonin is biosynthesized in the postganglionic serotonergic neurons from the dietary precursor L-tryptophan. The biochemical pathway involves two steps, the first of which being the

\[
\begin{align*}
\text{Tryptophan hydroxylase} & \rightarrow \\
\text{Dopa decarboxylase} & \rightarrow
\end{align*}
\]
conversion of tryptophan catalyzed by tryptophan hydroxylase (TPH) to yield 5-hydroxytryptophan. This step is the rate limiting step of serotonin synthesis. 5-hydroxytryptophan is then decarboxylated by the dopa decarboxylase (aromatic L-amino acid decarboxylase, L-DOPA) to 5-hydroxytryptamine (5-HT), also known as serotonin. Inhibitors of this enzyme include the drugs benserazide and carbidopa, which do not cross the blood brain barrier, and are used clinically to prevent peripheral decarboxylation of the L-DOPA administered as a precursor for central dopamine formation in patients with Parkinson disease [179].

Serotonin breakdown is carried out primarily by monoamine oxidase (MAO), which occurs as two subtypes called MAO-A and MAO-B, differing in their tissue and cellular distributions. MAO-A is more selective for serotonin oxidation, and its activity inhibition in the CNS is linked to the antidepressant properties of a number of subtype selective and nonselective MAO inhibitors. The action of MAO converts serotonin to 5-hydroxyindole acetaldehyde which in turn is readily metabolized by aldehyde dehydrogenase to produce 5-HIAA as the major excreted metabolite of serotonin [179].

As a neurotransmitter, 5-HT is released by raphe nuclei neurons, distributed along the brainstem. Upon its release into the synaptic cleft, it activates 5-HT receptors positioned on the dendrites, cell bodies and postsynaptic terminals. The serotonergic action is terminated via uptake of 5-HT via a specific monoamine, Na⁺/Cl⁻-dependent transporter, the serotonin transporter located at the presynaptic neuron. The serotonin transporter (5-HTT) is hence the major regulatory of serotonin concentration in the synaptic cleft [180]. Serotonin is thereafter recycled into the neurotransmitter pool [66]. The uptake may be inhibited by various agents and substances such MDMA (ecstasy), cocaine, tricyclic antidepressants (TCAs), and selective serotonin reuptake inhibitors (SSRIs). An imbalance in the central 5-HT concentration, due to diverse inhibitory mechanisms and/or regulatory dysfunctions, is believed to be associated with various psychiatric conditions [40, 55, 122, 146, 181].

1.9 THE SUSCEPTIBILITY GENES OF THE SEROTONERGIC SYSTEM

Serotonergic function in the CNS and peripheral organs, tissues and cells is determined by complex interactions among proteins involved in serotonin synthesis and breakdown mediated by various enzymatic reactions, its release and reuptake, as well as its effect on a large family of receptors and transporters located on pre- and postsynaptic neurons. Many of the genes encoding for these proteins have been identified and investigated to associate their polymorphisms with psychiatric disorders. Two widely investigated genes of the serotonergic system are the serotonin transporter (5-HTT) and the tryptophan hydroxylase 1 (TPH-1).

1.9.1 Serotonin Transporter (5-HTT) Gene

The serotonin transporter gene, also known as solute carrier family 6 member 4, (SLC6A4) spans 37.8 kb and consists of 15 exons including 2 noncoding exons,
designated 1A and 1B \[182, 183\]. The single gene encoding the human 5-HT transporter was mapped to chromosome 17q11.1-q12, carrying 630 aa residues with about 2.5 kbp mRNA \[66\]. The homologous mouse gene has been located on mouse chromosome 11 \[184\].

Dysregulation of 5-HTT has been reported in various complex behavioral traits and disorders such as depression, bipolar, anxiety, obsessive-compulsive, schizophrenic, and neurodegenerate disorders, in addition to substance abuse and eating disorders \[60, 61, 185\]. Clinically, reduced 5-HT uptake and reduced inhibitor binding to 5-HTT have been associated with depression and anxiety \[60, 67\]. 5-HTT is also the target of SSRIs, common drugs used to treat various conditions including depression, anxiety, post-traumatic stress disorder, obsessive compulsive disorder, and others \[65\].

In 1996, two polymorphisms were identified on the transporter gene. Ogilvie et al recognized 3 novel alleles of the variable number tandem repeat (VNTR) region within intron 2 \[186\], hence referred to as STin2. Each VNTR element consists of 17 bp and may exist in 10 and 12 repeats. However other rare types such as seven- and nine-repeat units have also been reported \[187\]. Functional studies on this polymorphism have shown that the amount of 5-HTT protein is significantly higher in cells carrying the 12-repeat allele when activated with morphogens \[188\], and in developing mouse brains STin2 acts as a transcriptional regulator in an allele-dependent way \[189\]. No significant genotype effect has been found on platelet 5-HT uptake \[190\] or on 5-HIAA levels in CSF \[191\] in native-expressing cells. STin2 variants have been associated with risk for major depression and bipolar disorder \[186, 192, 193\]. The association between unipolar depression and STin2 polymorphism was largely explained by an excess of the allele with 9 copies of the VTNR \[186\]. However, this association has only been replicated once using the same set of data \[194\], and no other study has been able to show any association with MDD \[65, 195-198\]. On the other hand, association studies in bipolar disorder has been more successful and overall evidence implicates STin2.12 allele with bipolar disorder \[65\]. Also, family- and population based studies have shown that the STin2.12 allele is significantly associated with SCZ \[187, 199, 200\].

The second reported polymorphism was identified by Heils et al in the promoter region of 5-HTT \[61\]. The polymorphism is located approximately 1 kb upstream of the transcription initiation site of the SLC6A4 gene, resulting in two common alleles: the short (s) variant containing 14 copies of a 20-23 bp GC rich repeat units, and the long (L) variant comprising of 16 copies. More specifically, the polymorphism consists of a 43-bp (initially described as 44bp \[201\]) insertion or deletion (indel) involving repeat units number 6 to 8 \[61\]. Findings across studies of different populations show variation
in the frequency of the L allele across populations, ranging from more than 70% in Africa and African Americans to 50% in Europeans to less than 30% in Japan [202]. Today totally 27 variants in the SLC6A4 locus have been identified, comprising 25 SNPs and 2 indels [203], 14 of which have been reported in Japanese and Caucasian populations with ethnic differences in allelic frequencies [204]. However they have not been found functionally significant in reporter gene assays [201]. Recently an A/G SNP has been denoted within the 5-HTTLPR L form, where the L_A variant yields high mRNA levels while the G type behaves equivalent to the low expressing s allele [205]. Lesch and colleagues, who referred to the polymorphism as 5-HTTLPR, found that the basal activity of the long variant was more than twice that of the short form in addition to genotype-dependent differences in mRNA concentrations, membrane binding and serotonin uptake [206]. The subjects were also evaluated for anxiety-related personality traits and the only association found was with the neuroticism factor. Individuals with either 1 or 2 copies of the s form had higher neuroticism scores than did individuals homozygous for the L variant [206].

Carriers of the s allele have been described to have reduced gray matter volume of the perigenual anterior cingulate cortex (pACC) and amygdala compared to those with the L allele. The differences may underlie normal emotional reactivity and influence genetic susceptibility for depression [207]. In addition, individuals with one or two copies of the s allele, which has been associated with increased fear and anxiety-related behaviors, exhibit greater amygdala neuronal activity, in response to fearful stimuli. These data would indicate that genetically driven variations in excitability of the amygdala to emotional stimuli may contribute to the increased fear and anxiety typically associated with the s allele [208]. Early MDD age of onset has been associated with smaller hippocampal volumes in individuals homozygous for the s allele, while individuals with the L/L genotype and smaller hippocampal volumes would tend to develop late-life depression [209]. A recent meta-analysis of 26 studies of various ethnic groups, aimed at establishing the correlation between 5-HTTLPR and anxiety traits, provided no support for the presence of the short form of the promoter polymorphism in relation to the traits. However, there was strong evidence for the presence of moderating variables, and subsequent analysis revealed that choice of the measure of trait anxiety was significant [210]. Individuals carrying the s form were however found to exhibit more depressive symptoms, diagnosable depression, and suicidality in relation to stressful life events than individuals homozygous for the long allele [73]. However, in an association study the short allele of the promoter polymorphism was significantly more frequent in MDD patients of German descent than in control subjects [211]. In an extensive study, among 1,932 individuals, comprising 539 with unipolar affective disorder, 572 with bipolar

![5-HTT (SLC6A4) Gene](attachment:image.jpg)
disorder, and 821 controls, no significant differences in genotype or allele frequencies of the 5-HTTLPR polymorphism were found [212]. Another recent meta-analysis of case-control studies on the association between the polymorphisms STin2 and 5-HTTLPR and affective disorders (bipolar disorder and unipolar depression) concluded that the short alleles of the 43-bp indel polymorphism was in significant association with bipolar disorder but not unipolar disorder whereas VNTR had no association with either disorders [213].

Another meta-analysis was conducted to evaluate the association of the serotonin transporter promoter polymorphism with alcohol dependence, which included data from 17 published studies including 3,489 alcoholics and 2,325 controls. The frequency of the short allele was significantly associated with alcohol dependence, and an even greater association was seen with either a co-morbid psychiatric condition or an early-onset or more severe alcoholism subtype [214]. 5-HTTLPR has also been implicated in the regulation of cardiovascular responses to mental stress. Individuals carrying one or two copies of the long 5-HTTLPR allele (L/L and L/s) had greater blood pressure and heart rate responses to mental stress [215]. This may be related to the fact that the 5-HTT promoter contains a glucocorticoid response element that makes it responsive to stress-induced levels of corticosteroids. This effect is particularly evident among carriers of the s form [216]. 5-HTTLPR has been also studied in a number of other psychiatric disorders, such as autism, where results have been contradictory [217, 218], and Alzheimer, where the L/L genotype has been associated with aggression [219]. Response to SSRIs in depressed patients has been widely investigated [220].

1.9.2 Tryphtophan hydroxylase 1 (TPH-1) Gene

TPH-1 is localized on the human chromosome 11p15.3-p14, it is about 29 kb long, and includes 10-11 exons [221].

Two informative SNPs, A218C (rs1800532) and A779C (rs1799913) are both located in intron 7 and are in linkage disequilibrium with each other [153]. TPH variants might be associated to pathogenesis events involving dysfunction of the 5-HT system and has therefore been investigated widely as a candidate gene for psychiatric and behavioral disorders [222, 223]. SNP A779C (rs1799913) has been associated with 5-HIAA concentrations in CSF [224]. A Japanese study analyzed one penta-nucleotide repeat polymorphism in intron 1 b and SNP A779C in schizophrenic patients and control subjects without evidence for case-control differences [225]. However, they reported an association between the latter polymorphism and negative and total symptom scores in men. Another study reported
an association between SNP A779C and violence among schizophrenic men\textsuperscript{[226]}. In an Indian case-control sample Semwal \textit{et al} (2001) reported association between this SNP and SCZ, although a family based association study was not able to replicate the findings\textsuperscript{[227]}. In addition, this group analyzed a promoter variant (A6526G) without evidence for association with SCZ\textsuperscript{[227, 228]}. Polymorphisms in the promoter region may influence the function of the TPH-1 gene and further influence the tendency of alcohol dependence in one ethnic group in Taiwan\textsuperscript{[229]}. This study was performed consequent to the findings that related the intron 7 polymorphism to alcoholism\textsuperscript{[151]} and to 5-HIAA levels in CSF in a cohort of impulsive Finnish alcoholics\textsuperscript{[152]}.

Association between the TPH-1 SNP A218C (rs1800532) and bipolar disorder has been reported\textsuperscript{[230]}, however such findings have not been confirmed for bipolar disorder or for other mood disorders\textsuperscript{[231, 232]}. The TPH-1 218A allele has been reported to be associated with SCZ in some\textsuperscript{[233]} but not all studies\textsuperscript{[234-236]}. The TPH-1 A218C polymorphism was also reported to interact with a serotonin transporter polymorphism, giving rise to case-control differences in SCZ\textsuperscript{[237]}. In one of the studies the A218C polymorphism was associated with suicidal behavior\textsuperscript{[234]}. A meta-analysis of the relationship between the A218C polymorphism and suicidal behavior was conducted on 9 association studies\textsuperscript{[156]}. A significant association was observed between this polymorphism and suicidal behavior with a dose-dependent effect of the A allele on the risk for suicidal behavior was observed\textsuperscript{[156]}. TPH-1 variants were shown to be a potential liability factor for suicidal behavior in alcoholics\textsuperscript{[151]}, and in patients with unipolar disorder, substance abuse, and cluster B personality disorders\textsuperscript{[238]}. Further association studies of the intron 7 variants have either partially confirmed the findings in other populations\textsuperscript{[150, 234]} or not found any association\textsuperscript{[239-241]}. Chao and Richardson investigated the two intronic TPH-1 polymorphisms in a small African-American sample without evidence for case-control differences\textsuperscript{[242]}

A second isoform of TPH, thereby named TPH-2, has recently been described\textsuperscript{[243]}. Its gene is located on chromosome 12 and shows 71% homology to TPH-1 on the amino acid level\textsuperscript{[243]}. TPH-1 and TPH-2 are expressed in nearly equal amounts in several human brain regions such as frontal cortex, thalamus, hippocampus, hypothalamus, and amygdala. TPH-2 is predominantly expressed in the brain stem whereas it is absent from peripheral tissues such as heart, lung, kidney, duodenum and adrenal gland, where only TPH-1 is expressed\textsuperscript{[244, 245]}. Haplotype analysis of TPH-2 recently showed a significant association with MDD\textsuperscript{[244]}, suggesting that variants of this gene could represent risk factors in the development of MDD or others psychiatric disorders.
2 AIMS

The general aim of this study was to identify gene variants of the serotonergic system that may be implicated in susceptibility to different psychiatric disorders.

To achieve this objective, more specific goals were formulated:

A. To set up a sensitive and specific methodology aimed to select appropriate and informative polymorphisms along a gene unit;

B. To design a simple strategy for haplotype reconstruction that could be used for clinical association studies;

C. To apply the strategy to studies on the psychiatric disorders chosen for this thesis work.
3 MATERIALS & METHODS

3.1 HUMAN SUBJECTS

All studies were approved by the Ethics Committee of the Karolinska Hospital. All subjects were unrelated Caucasians living in the Stockholm County. Cases and controls were matched for age, ethnicity, and geographical distribution. Subjects were interviewed using the Structured Clinical Interview for DSM-III-R [246, 247] or DSM-IV [248, 249].

Paper I
Letters were sent to potential patients on sick leave because of a non-psychotic psychiatric diagnosis. They were then approached by telephone, and invited to participate in a clinical study. All patients were ambulatory, and none had received inpatient care for their current illness. Patients meeting DSM-IV criteria for Major Depressive Disorder at any time during the current sick-leave period (over three months) were included; totally 228 subjects (mean age ± standard deviation: 46.8 ± 8.6, 166 women and 62 men). The control group included 253 individuals (mean age ± standard deviation: 42.6 ± 13.7 years, 98 women and 155 men). At the time of blood collection and diagnostic interview, none of the individuals in this group had suffered from any psychiatric condition.

Paper II
The control group included 98 female individuals (mean age ± standard deviation: 44.0 ± 13.8 years) recruited either as re-examined healthy individuals, mainly staff and medical students, or subjects drawn from the general population for previous biological psychiatric studies performed at the Karolinska Institute. At the time of blood collection and diagnostic interview, none of the individuals in this group had any psychiatric history. For the recruitment of the BPD group, information regarding a dialectic behavioral therapy (DBT) project was distributed to all Stockholm County Council psychiatric clinics, serving a population of approximately 1.8 million inhabitants. These clinics referred 162 patients, all women, with a DSM-IV BPD diagnosis. One inclusion criterion for recruitment of patients in this study was at least two potentially life-
threatening suicide attempts, with at least one attempt made less than six month prior to referral. Lethal intent was defined according to the patient’s belief that the attempt could have been lethal. Exclusion criteria were: (i) a current diagnosis of substance dependence; (ii) evidence of dementia or other irreversible organic brain syndrome; (iii) a current diagnosis of a psychotic disorder or major depressive illness with melancholic features; (iv) a life-threatening eating disorder. Ninety-five patients (mean age ± standard deviation: 30.5 ± 8.1 years) were included in the study.

The latest suicide attempt before study inclusion was carried out with the following methods: drug overdose (70.5%), penetrating lesions (21%), hanging or strangulation (6.3%), jumping (1.1%), poisoning (1.1%). Most patients also met DSM-IV criteria for a diagnosis of major depression or anxiety disorders. Specifically, 68 patients (72%) had a diagnosis of major depression, 62 (65%) a diagnosis of an anxiety disorder, and 37 (39%) had both diagnoses.

**Paper III**

Control subjects were either re-examined healthy individuals, mainly staff and medical students, or subjects drawn from the general population for biological psychiatric studies performed at the Karolinska Institute. The control group included 253 individuals (39% women) with a mean age ± standard deviation of 42.6 ± 13.7 years. At the time of blood collection and diagnostic interviews, none of the control individuals met criteria for any psychiatric condition. The patient group was composed of 155 individuals (39% women) diagnosed with SCZ (n=139), schizoaffective disorder (n=10), or schizophreniform disorder (n=6). Their mean age ± standard deviation at interview and onset of illness was 43.8 ± 16.5 and 24.2 ± 7.3 years, respectively.

**Paper IV**

This study included the same subjects as in Paper III, except for the control group which was decreased from 253 to 246 individuals (mean age ± standard deviation: 41.0 ± 11.2 years, 95 women and 151 men) due to repeated failure in PCR amplification in seven samples.

**Paper V**

Except for a few differences, in this study the same subjects were studied as in Paper I. 5-HTT is known to associate with anxiety disorders and anxiety-related personality traits. Therefore for the present study patients co-morbid for other psychiatric or personality disorders were excluded. The patient group was thus reduced to 194 subjects (mean age ± standard deviation: 45.5 ± 9.3, 141 women and 53 men). The control group was the same as in Paper IV.

**Paper VI**

The patient group examined in this study consisted of 48 subjects with a current BPD diagnosis. Inclusion and exclusion criteria were the same as for BPD subjects in Paper II. Control subjects were 30 women, reasonably well matched for age and education, recruited through public advertisement and paid for their participation. They filled out
the SCID II-screening personality questionnaire (a comprehensive self-report version of the SCID-II interview for DSM-IV) in order to exclude subjects who scored five or more points on the BPD scale.

### 3.2 EXPERIMENTAL ANALYSES

#### 3.2.1 DNA extraction

Venous blood was drawn from all patients and controls and immediately frozen in aliquots at −70°C or below until analyzed. Genomic DNA was prepared from whole blood by use QIAamp® DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany). Upon concentration determination, purified DNA samples were stored at +4°C, awaiting further analysis.

#### 3.2.2 Designing primers

OLIGO 6 Primer Analysis Software version 6.31 (Molecular Biology Insights, Inc., Cascade, CO USA) was used for designing oligonucleotide primers for genotyping purposes. A part of the sequence in query identified from NCBI database is uploaded to the program, covering usually ~ 200-300 bp down- and upstream of the investigating SNP. Based on nearest neighbor thermodynamics, Oligo's search algorithms find optimal primers for PCR (including multiplex, consensus or degenerate primers, inverse PCR, and site directed mutagenesis), sequencing, and hybridization probes. For each primer or primer pair, Oligo's various analysis windows show a multitude of useful data. A typical Oligo outcome window is presented, showing essential information such as primer and product lengths and positions, GC content, annealing temperature, and differences in Tm.

#### 3.2.3 Polymerase Chain Reaction (PCR)

The purpose of a PCR is to amplify copies of a DNA sequence. There are three major steps in a PCR, which are repeated for 30 to 50 cycles. During the first step,
denaturation at 95°C, the double strand melts open to single stranded DNA. During the second step, annealing, ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. Once there are a few bases built, the ionic bond is so strong between the template and the primer, that it does not break anymore. This step is then followed by an extension reaction at 72°C. This is the ideal temperature for the polymerase to couple the template complementary bases to the primer on the 3’ side. The polymerase adds deoxynucleotide triphosphates (dNTP) from 5’ to 3’ direction, i.e. reading the template from 3’ to 5’ side, bases are added complementary to the template.

### 3.2.4 PCR Conditions

DNA (50 ng/reaction) was amplified by polymerase chain reaction (PCR), carried out in a T3 Thermocycler (Biometra® GmbH, Göttingen, Germany) in a total volume of 25 µl. The reaction buffer was composed of 1.5 to 2.5 mM MgCl₂, 67 mM Tris-HCl pH 9.2, 16.6 mM (NH₄)₂SO₄, 0.1% v/v Tween 20 (Sigma, Stockholm, Sweden), 200 µM dNTPs (Amersham Pharmacia Biotech, Uppsala, Sweden), 20 pmol of each primers (MWG Biotech AG, Ebersberg, Germany), and 0.75 units of Taq DNA polymerase (Roche Diagnostic GmbH, Mannheim, Germany). Thermal cycling was performed with an initial denaturation for 8 minutes at 96°C, followed by 50 cycles of denaturation for 30 seconds at 96°C, primer annealing for 30 seconds (for annealing temperatures see Table 1), and extension for 30 seconds at 72°C. A final primer extension was conducted for 5 minutes at 72°C. All PCR products were separated by electrophoresis on a 2% agarose gel (Roche Diagnostic GmbH) and visualized by ethidium bromide staining.

![Figure 3. 5-HTTLPR](image1.png)

![Figure 4. STin2](image2.png)

Figure 3. A 2% agarose gel showing 5-HTTLPR PCR products, separating the long and short alleles

Figure 4. A 2% agarose gel showing the PCR products of STin2, separating the 10 and 12 repeats.
A gene-based haplotype approach

Figure 5. A 2% agarose gel for separation of a typical SNP product after incubation with restriction enzyme. Figure 6 shows a schematic of a typical pyrosequencing pyrogram.

Table 1. Primers & conditions for PCR amplifications and pyrosequencing

<table>
<thead>
<tr>
<th>Marker</th>
<th>Position</th>
<th>Primer Sequence</th>
<th>Annealing Temp °C</th>
<th>Restriction Enzyme</th>
<th>Primer Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin Transporter Gene:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5HTTLPR</td>
<td>26958-26486</td>
<td>GGCGTTGCCGCTCTGAATGC&lt;br&gt;GAGGGACTGAGCTGGACAACCAC</td>
<td>60</td>
<td>-</td>
<td>26080-26099&lt;br&gt;26586-26608</td>
</tr>
<tr>
<td>STin2</td>
<td>41985-42146</td>
<td>TGGATTTCTTCTTCTCATGTATTG&lt;br&gt;TCATGTCTCTAGTCTTCAGCAGAGTG</td>
<td>60</td>
<td>-</td>
<td>41873-41897&lt;br&gt;42237-42261</td>
</tr>
<tr>
<td>rs140701</td>
<td>52061</td>
<td>GGGCACACAGTTTTCAAGATGGA&lt;br&gt;GCCAGGGATGCGAGGGAATTGAG</td>
<td>55</td>
<td>MaeIII</td>
<td>51919-51941&lt;br&gt;52331-52353</td>
</tr>
<tr>
<td>rs1042173</td>
<td>65582</td>
<td>ATATTTCCATGCTAGTGTG&lt;br&gt;TTCATTTTAGCTTATGGGACTCTTACATCCTT</td>
<td>55</td>
<td>XapI</td>
<td>65313-65333&lt;br&gt;65646-65669</td>
</tr>
<tr>
<td>Tryptophan Hydroxylase 1 Gene:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4537731</td>
<td>124889</td>
<td>TTTATGGCATTGAAGATAGGAC&lt;br&gt;TTTGGCTCTGGAGCCTAAAC</td>
<td>55</td>
<td>SauIIA</td>
<td>125089-125066&lt;br&gt;124772-124792</td>
</tr>
<tr>
<td>rs684302</td>
<td>116360</td>
<td>AGAGAGATGCGGACGAAAAACACTAC&lt;br&gt;CCAGTCTTTCAAAATCTGATAC</td>
<td>55</td>
<td>AluI</td>
<td>116248-116270&lt;br&gt;116459-116438</td>
</tr>
<tr>
<td>rs211105</td>
<td>111308</td>
<td>CAAGGCAAGATTTATATGAGTT&lt;br&gt;CCAGTCCCTCAAAATCTGATAC</td>
<td>55</td>
<td>CfrI</td>
<td>111050-111071&lt;br&gt;111481-111458</td>
</tr>
<tr>
<td>rs180053</td>
<td>103821</td>
<td>AATGGCATCTACCTATGGGTTTT&lt;br&gt;CTTATTTTCTCAGGGACCTCA</td>
<td>58</td>
<td>NheI</td>
<td>103993-103971&lt;br&gt;103655-103678</td>
</tr>
<tr>
<td>rs1799913</td>
<td>103260</td>
<td>ATGGGATTTCAGTTTTGATTG&lt;br&gt;GGCAATACTGACTGCAG&lt;br&gt;CAGGCTGACAAACTCGTACC</td>
<td>55</td>
<td>Pyrosequencing</td>
<td>103335-103316&lt;br&gt;103196-103213&lt;br&gt;103283-103264</td>
</tr>
<tr>
<td>rs7933505</td>
<td>101992</td>
<td>CCCCAGCGTTTTTCTGTTGTCCG&lt;br&gt;TCAGATCCACATCTGCGTGGAAC</td>
<td>55</td>
<td>BsmI</td>
<td>101746-101767&lt;br&gt;102194-102171</td>
</tr>
</tbody>
</table>

aPolymorphism ID number from the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP/).
bSNP and primer positions are shown based on NCBI clone AC104984 (5-HTT) and AC124058 (TPH1).
3.2.5 Pyrosequencing™

Pyrosequencing™ is sequencing by synthesis, a technique for accurate and quantitative analysis of DNA sequences. A sequencing primer is hybridized to a single stranded, PCR amplified DNA template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and a pyrase, and the substrates, adenosine 5’ phosphosulfate (APS) and luciferin. The first of four dNTP is added to the reaction. DNA polymerase catalyzes the incorporation of the dNTP into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi). ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5’ phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a pyrogram™ as presented in Figure 6. Each light signal is proportional to the number of nucleotides incorporated.

3.2.6 Pyrosequencing Conditions

SNP rs1799913 (Table 1) was analyzed by pyrosequencing, using a Pyrosequencer PSQ 96 and a PSQ 96 SNP Reagent Kit (Pyrosequencer, Uppsala, Sweden) according to the manufacturer’s instructions. PCR reactions were carried out in 50 µl volume containing 100 ng genomic DNA, PCR buffer (1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% v/v Tween 20), 200 µM dNTPs, 10 pmol of each primers (the reverse primer was biotinylated), and 1.5 units of Taq DNA polymerase (Roche Diagnostic GmbH, Mannheim, Germany) Thermal cycling was performed with an initial denaturation for 5 minutes at 96°C, followed by 50 cycles of denaturation for 30 seconds at 96°C, primer annealing for 30 seconds at 55°C, and synthesis for 30 seconds at 72°C. A final primer extension was conducted for 5 minutes at 72°C. The PCR products (140 bp) were run on 2% agarose gels (Roche Diagnostic GmbH, Mannheim, Germany) and visualized after an ethidium bromide staining. A total 45 µl of PCR product were used for pyrosequencing, and 15 pmol of the forward sequencing primer were applied to detect the polymorphisms.

3.2.7 DNA Sequencing

The purpose of sequencing is to determine the order of the nucleotides of a gene. For sequencing, PCR fragments or cloned genes can be used as templates. Similar to PCR there are three major steps in a sequencing reaction, denaturation, annealing and extension. The differences are however that in sequencing reactions, only one primer is used, so there is only one strand copied. The extension step is carried out at lower temperature in order to incorporate the labeled nucleotides. When a ddNTP is incorporated, the extension reaction stops because a ddNTP contains an H-atom on the 3rd carbon atom (dNTPs contain an OH-atom on that position). Since the ddNTPs are
fluorescently labeled, it is possible to detect the color of the last base of this fragment on an automated sequencer.

3.3 NON-EXPERIMENTAL ANALYSES

3.3.1 Statistical analysis

Genotype and allele frequencies, as well as Hardy-Weinberg equilibrium were calculated using Microsoft Excel macro PHARE version 2.1 (developed by David G. Cox, Lyon, France), which can be downloaded at http://bioinformatics.org/macroshack/programs/PHARE/description.html.

The Hardy-Weinberg model describes and predicts genotype and allele frequencies in a non-evolving population. The model has five basic assumptions: 1) the population is large (i.e., there is no genetic drift); 2) there is no gene flow between populations, from migration or transfer of gametes; 3) mutations are negligible; 4) individuals are mating randomly; and 5) natural selection is not operating on the population. Given these assumptions, a population’s genotype and allele frequencies will remain unchanged over successive generations, and the population is said to be in Hardy-Weinberg equilibrium. The Hardy-Weinberg model can also be applied to the genotype frequency of a single gene.

The Hardy-Weinberg model consists of two equations: one that calculates allele frequencies and one that calculates genotype frequencies. Because we are dealing with frequencies, both equations must add up to 1. The equation:

\[ p + q = 1 \]

describes allele frequencies for a gene with two alleles. In a diploid organism with alleles \(A\) and \(a\) at a given locus, there are three possible genotypes: \(AA\), \(Aa\), and \(aa\). If we use \(p\) to represent the frequency of \(A\) and \(q\) to represent the frequency of \(a\), we can write the genotype frequencies as \(p^2\) for \(AA\), \(q^2\) for \(aa\), and \(2pq\) for \(Aa\). The equation for genotype frequencies is:

\[ p^2 + 2pq + q^2 = 1 \]

For association analyses of individual genotypes and alleles, chi-square analyses on 2 x 2 or 2 x 3 contingency tables were carried out (http://www.georgetown.edu). The significance level for all statistical tests was 0.05. Chi-square tests the fit between a theoretical frequency distribution and a frequency distribution of observed data.

3.3.2 Haplotype analysis

For pairwise linkage disequilibrium (LD) and haplotype analyses the Arlequin program version 2.0 was used (Schneider S, Roessli D, and Excoffier L, 2000 Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva) which can
be downloaded at http://lgb.unige.ch/arlequin. Arlequin is an exploratory population genetics software able to handle large samples of molecular data (RFLPs, DNA sequences, microsatellites) and also data presented either in the form of genotype frequencies, or as haplotype frequencies. The program performs a total of 100,000 permutations in each analysis to estimate the expected frequencies of the haplotypes that could exist in the study population. The haplotypic frequencies are based on the genetic data of the input file.

D’values were used to illustrate the extent of LD, and the corresponding p-values are also shown. Bonferroni correction was used for multiple testing, using the total number of SNPs as correction factor.

### 3.3.3 Meta-analysis

A meta-analysis was performed of all published case-control studies analyzing the TPH-1 rs1800532 (A218C) polymorphism in the context of SCZ. Data were obtained by searching the National Library of Medicine’s database PubMed up to December 2005. The search terms “tryptophan hydroxylase”, “TPH”, and “SCZ” were used.

Financial support for this project was received from different sources; the Swedish Research Council (K2003-21X-05454-25A, Åsberg M and Leopardi R; K2004-21X-15078-01A, Jönsson EG), the Wallenberg foundation (Jönsson EG), the HUBIN project (Jönsson EG), and from the AFA insurance company (Åsberg M and Leopardi R).
4 RESULTS & DISCUSSION

When this study began, knowledge was spreading on SNPs as tools for characterization of gene variants. At about the same time, algorithms implemented by programs such as Arlequin made it possible to perform haplotype reconstruction work also for scientists not formally trained in population genetics. However, it was still unclear how to deal with dense SNP maps and especially with results from neighboring SNPs, often at significant LD. Knowledge about the haplotype block structure of the genome, which has simplified these investigations, developed only during the course of this study.

With SNP maps available almost for the whole genome, averaging 1 SNP/kb, formal haplotype reconstruction for an average human gene would have required genotyping 50-100 SNPs in an entire study population, typically several hundreds patients plus controls. Since SNP technology at that time was still at a pioneering stage, we considered this enterprise unrealistic, in terms of both time and finances.

A significant problem was to be solved, i.e. how to choose polymorphic loci in the absence of haplotype structure information for the genes this project wanted to focus on.

4.1 GENE VARIANTS OF TPH-1 IN ASSOCIATION TO MDD, SCZ & BPD

4.1.1 Choice of TPH-1 SNPs

The first part of this thesis focused on choosing SNPs that would allow us to construct risk haplotypes. We reasoned that any allele belonging to a common haplotype would be expected to be in homozygosity when all other alleles belonging to the block are. We therefore chose the two well-documented TPH-1 intron 7 SNPs (SNPs 4 and 5; Table 1). The entire MDD patient group was genotyped for these two SNPs. Subjects that were homozygous for both markers were sub-grouped into the four possible genotype combinations (CC/CC, CC/AA, AA/CC, AA/AA). From these four subgroups three subjects were chosen at random. If there were fewer than three subjects in any
subgroup, DNA samples from all subjects in that subgroup were used. For these subjects, DNA sequencing was carried out over a total of about 10 kb, including all exons, all intron regions within about 0.5 kb from the exon boundaries, and in some promoter regions. The rationale for choosing these gene regions was that they potentially could carry functional SNPs.

No novel polymorphism was identified (data not shown). For further analyses, we selected all SNPs from the sequenced regions where all subjects in any of the four subgroups were homozygous for at least one allele. Four SNPs were identified that met our criteria (indicated as #1, 2, 3, and 6 in Table 1). Thus, including the mentioned SNPs, totally six loci were employed for genotyping of controls and the three patient groups. The six SNPs cover 23 kb of the 29 kb gene length.

### 4.1.2 Genotyping

One goal of this project was to identify TPH-1 gene variants associated with MDD, SCZ and BPD. To this end, we started with genotyping the entire study populations. For each of the six SNPs, primers were designed and PCR reactions were carried out under conditions as previously described in Material & Method section. The table below represents the genotyping data for each marker, providing the single nucleotide polymorphism’s character, the restriction enzyme used for digestion of the PCR products, the size of the PCR products and their cleaved products separated on 2% agarose gel by electrophoresis.

#### Table 1. Genotyping details for the SNPs of TPH-1 gene

<table>
<thead>
<tr>
<th>Marker</th>
<th>Polym</th>
<th>Restriction enzyme</th>
<th>PCR product (size bp)</th>
<th>Cleaved product (size bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) rs4537731</td>
<td>A/G</td>
<td>Sau3A</td>
<td>318</td>
<td>318 201+117</td>
</tr>
<tr>
<td>2) rs684302</td>
<td>C/T</td>
<td>AluI</td>
<td>213</td>
<td>68+44+61+40 68+105+40</td>
</tr>
<tr>
<td>3) rs211105</td>
<td>G/T</td>
<td>Cfrl</td>
<td>432</td>
<td>258+174 432</td>
</tr>
<tr>
<td>4) rs1800532</td>
<td>A/C</td>
<td>Nhel</td>
<td>338</td>
<td>171+167 338</td>
</tr>
<tr>
<td>5) rs1799913</td>
<td>C/A</td>
<td>-</td>
<td>140</td>
<td>Pyrosequencing</td>
</tr>
<tr>
<td>6) rs7933505</td>
<td>A/G</td>
<td>Baul</td>
<td>449</td>
<td>244+205 449</td>
</tr>
</tbody>
</table>

Polym: Polymorphism; bp: base pair, AA: Homozygous for allele 1; aa: Homozygous for allele 2

### 4.1.3 Single locus analysis

All six SNPs were tested using a case-control design, searching for single locus associations with the disorders. All SNPs were at Hardy-Weinberg equilibrium in both the control and patient samples. Bonferroni method was applied to correct for multiple testing.
Several significant associations with MDD were observed. In the allelic association analysis (Table 2a), SNP 5 (rs1799913) A allele was found to associate with the MDD patient group (P=0.001), which was also reproduced in the genotypic association with the disease (P=0.003; Table 3a). Both these associations were preserved after correction for multiple testing (P=0.008 and P=0.021, respectively). This SNP, also known in the literature as SNP A779C, has been associated with CSF 5-HIAA concentrations, which suggests that TPH-1 genotypes may participate differentially in the regulation of serotonin turnover rate in the central nervous system [224]. Several reports have also shown an association with suicidal behavior [151, 238]. An association study with 58 volunteers revealed that the A779C TPH polymorphism significantly relates to aggressive hostility, with the highest aggression levels for the genotype AA and the lowest aggression levels for the genotype CC [250]. Other associations were observed which did not retain significance after Bonferroni correction. This will be discussed later.

Table 2a. Allelic association tests for MDD & SCZ

<table>
<thead>
<tr>
<th>SNP</th>
<th>Polym</th>
<th>Control</th>
<th>MDD</th>
<th>p</th>
<th>SCZ</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A/G</td>
<td>0.51(A)</td>
<td>0.51(A)</td>
<td>0.9735</td>
<td>0.64(A)</td>
<td>0.00003</td>
</tr>
<tr>
<td>2</td>
<td>C/T</td>
<td>0.57(C)</td>
<td>0.51(C)</td>
<td>0.0518</td>
<td>0.50(C)</td>
<td>0.054</td>
</tr>
<tr>
<td>3</td>
<td>G/T</td>
<td>0.75(T)</td>
<td>0.78(T)</td>
<td>0.3452</td>
<td>0.75(T)</td>
<td>0.837</td>
</tr>
<tr>
<td>4</td>
<td>A/C</td>
<td>0.40(A)</td>
<td>0.45(A)</td>
<td>0.0996</td>
<td>0.49(A)</td>
<td>0.008</td>
</tr>
<tr>
<td>5</td>
<td>C/A</td>
<td>0.40(A)</td>
<td>0.50(A)</td>
<td>0.0013</td>
<td>0.45(A)</td>
<td>0.118</td>
</tr>
<tr>
<td>6</td>
<td>A/G</td>
<td>0.40(A)</td>
<td>0.43(A)</td>
<td>0.4344</td>
<td>0.48(A)</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Table 2b. Allelic association tests for BPD

<table>
<thead>
<tr>
<th>SNP</th>
<th>Polym</th>
<th>Control</th>
<th>BPD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A/G</td>
<td>0.48(A)</td>
<td>0.45(A)</td>
<td>0.462</td>
</tr>
<tr>
<td>2</td>
<td>C/T</td>
<td>0.52(C)</td>
<td>0.63(C)</td>
<td>0.035</td>
</tr>
<tr>
<td>3</td>
<td>G/T</td>
<td>0.79(T)</td>
<td>0.73(T)</td>
<td>0.172</td>
</tr>
<tr>
<td>4</td>
<td>A/C</td>
<td>0.45(A)</td>
<td>0.39(A)</td>
<td>0.164</td>
</tr>
<tr>
<td>5</td>
<td>C/A</td>
<td>0.44(A)</td>
<td>0.36(A)</td>
<td>0.053</td>
</tr>
<tr>
<td>6</td>
<td>A/G</td>
<td>0.46(A)</td>
<td>0.40(A)</td>
<td>0.137</td>
</tr>
</tbody>
</table>

With respect to SCZ, on the level of single alleles we found significant associations for SNP 1A (rs4537731), SNP 4A (rs1800532) and SNP 6A (rs7933505) with the disorder.
(P=0.00003; P=0.0083 and P=0.0492, respectively; Table 2a). Of these, after Bonferroni correction the association between SNP 1A (rs4537731), SNP 4A (rs1800532) alleles and schizophrenic psychoses remained significant (P=0.0002 and P=0.0498, respectively). Regarding SNP 4A allele (also known as A218C), reports in the literature are conflicting as to its association with schizophrenia [233, 235, 236]. A218C variants may not serve as a major liability factor for the symptoms of major psychoses but a trend has been observed for males with the A genotype to have lower depressive symptoms compared with C subjects [236]. A two-fold risk increase has been observed for childhood-onset schizophrenia in individuals with the AA genotype compared to other genotype groups [235]. Although the A218C polymorphism is an intronic mutation unlikely to change the amino acid sequence of any exon directly, the A218C site is located in a potential GATA transcription factor binding site, and this may influence TPH-1 gene expression [153]. Alternatively, this polymorphism might be in LD with another mutation in the tryptophan hydroxylase coding sequence, in a regulatory region, or in a nearby gene.

Genotypic associations with SCZ were also found for SNPs 1 (rs4537731), 2 (rs684302), 4 (rs1800532) and 6 (rs7933505) (P=0.0001, P=0.0024, P=0.0148, and P=0.0338, respectively; Table 3a). After correction for multiple testing associations with SNP 1 (rs4537731) and 2 (rs684302) were maintained (P=0.0006 and P=0.0144, respectively). For these SNPs the significance level was high enough to entertain the possibility that a causal polymorphism may lay in their vicinity.

No association was observed with SNP 5 (A779C) although association between this SNP and violence among schizophrenic men has been reported [226]. Association for this SNP has also been found in Indian schizophrenics, although a family based association study was not able to replicate the findings [227].

Table 3a. Genotypic association tests for healthy controls, MDD & SCZ patients

<table>
<thead>
<tr>
<th>SNP</th>
<th>Control (n=253)</th>
<th>MDD (n=258)</th>
<th>SCZ (n=155)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA Aa Aa AA Aa aa</td>
<td>P&lt;sup&gt;b&lt;/sup&gt; AA Aa aa</td>
<td>P&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>A/G</td>
<td>24.9 48.2 26.9 31.1 35.5 33.3 0.019</td>
<td>39.4 49.7 11.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>C/T</td>
<td>36.8 40.3 22.9 35.5 30.3 34.2 0.012</td>
<td>21.9 56.1 21.9</td>
<td>0.002</td>
</tr>
<tr>
<td>G/T</td>
<td>5.5 37.9 56.5 4.8 34.2 61.0 0.612</td>
<td>5.8 36.1 58.1</td>
<td>0.933</td>
</tr>
<tr>
<td>A/C</td>
<td>15.0 49.8 35.2 23.2 43.9 32.9 0.068</td>
<td>20.6 57.4 21.9</td>
<td>0.015</td>
</tr>
<tr>
<td>C/A</td>
<td>34.0 52.2 13.8 24.6 51.3 24.1 0.003</td>
<td>26.5 56.1 17.4</td>
<td>0.241</td>
</tr>
<tr>
<td>A/G</td>
<td>15.4 50.6 34.0 21.9 42.5 35.5 0.106</td>
<td>17.4 60.6 21.9</td>
<td>0.034</td>
</tr>
</tbody>
</table>

<sup>a</sup>Allele 1/allele2; SNP numbering follows the physical location on the gene. Bold numerals highlight significant SNPs by a significant P-value. <sup>b</sup>P-values are provided by 2x2 contingency tables and are not corrected for multiple testing. MDD (Major Depression Disorder); SCZ (Schizophrenia)
In the case of BPD women (Table 2b & 3b) no genotypic associations were found with the disease. Allele association tests for BPD vs. controls showed significance for SNP 2C (rs684302) with \( P=0.035 \). However, this association did not maintain statistical significance after Bonferroni correction. The small sample size is a limiting factor to be considered. Other studies have associated SNP 5 (a.k.a. A779C) with features of BPD such as impulsive aggression \[^{130}\]. Also, the 779C allele has been associated to violent offenders with a history of suicide attempts \[^{153}\]. A meta-analysis of the relationship between the A218C (SNP 5) polymorphism and suicidal behavior indicated a significant association with a dose-dependent effect of the A allele on the risk for suicidal behavior \[^{156}\].

### Table 3b. Genotypic association tests for healthy female controls & BPD patients

<table>
<thead>
<tr>
<th>SNP( ^a )</th>
<th>Genotypic associations (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=98)</td>
<td>BPD (n=95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>Aa</td>
<td>aa</td>
<td>AA</td>
</tr>
<tr>
<td>1</td>
<td>A/G</td>
<td>27.6</td>
<td>41.8</td>
<td>30.6</td>
</tr>
<tr>
<td>2</td>
<td>C/T</td>
<td>33.7</td>
<td>37.8</td>
<td>28.6</td>
</tr>
<tr>
<td>3</td>
<td>G/T</td>
<td>3.1</td>
<td>35.7</td>
<td>61.2</td>
</tr>
<tr>
<td>4</td>
<td>A/C</td>
<td>29.6</td>
<td>51.0</td>
<td>19.4</td>
</tr>
<tr>
<td>5</td>
<td>C/A</td>
<td>29.6</td>
<td>52.0</td>
<td>18.4</td>
</tr>
<tr>
<td>6</td>
<td>A/G</td>
<td>28.6</td>
<td>50.0</td>
<td>21.4</td>
</tr>
</tbody>
</table>

\( ^a \)Allele 1/allele2; SNP numbering follows the physical location on the gene. \(^b\)P-values are provided by 2x2 contingency tables and are not corrected for multiple testing. BPD (Borderline Personality Disorder)

We also performed a meta-analysis (Table 4) of published case-control studies investigating the TPH-1 A218C allele in the context of schizophrenia, resulting in a significant association with the SNP4 (rs1800532) A allele (a.k.a. A218C; odds ratio \( 1.47; \) 95% confidence interval 1.10–1.95), for which we also obtained an association with the disorder \( P=0.008 \). When our results were added to the meta-analysis (Table 4) comprising 2097 subjects the significance was strengthened \( (\chi^2=18.01, P<0.0001; \text{OR 1.31, 95% C.I: 1.16 – 1.49}) \). Despite the limited sample number, the similarity in odds ratios in four out of five studies supports the validity of our results, and suggests that TPH-1 variants may contribute to susceptibility to schizophrenia.
### Table 4. TPH-1 rs1800532 (A218C) polymorphism and schizophrenia: Meta-analysis of published case-control studies

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects’ residence; ethnicity</th>
<th>Diagnostic system</th>
<th>Class</th>
<th>N</th>
<th>Gender (M%)</th>
<th>A allele freq (%)</th>
<th>A vs. C allele</th>
<th>O.R.</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paik et al. 2000</td>
<td>Seoul, South-Korea; Korean</td>
<td>DSM-III-R</td>
<td>P</td>
<td>217</td>
<td>51</td>
<td>53.2</td>
<td>1.03</td>
<td>0.79-1.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>236</td>
<td>53</td>
<td>52.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hong et al. 2001</td>
<td>Taipei, Taiwan Han Chinese</td>
<td>DSM-IV</td>
<td>P</td>
<td>196</td>
<td>46</td>
<td>52.3</td>
<td>1.42</td>
<td>1.09-1.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>251</td>
<td>49</td>
<td>43.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sekizawa et al. 2004</td>
<td>Hamamatsu, Japan; Japanese</td>
<td>DSM-IV</td>
<td>P</td>
<td>51</td>
<td>43</td>
<td>57.8</td>
<td>1.47</td>
<td>0.93-2.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>148</td>
<td>44</td>
<td>48.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Asian</td>
<td></td>
<td></td>
<td>P</td>
<td>464</td>
<td></td>
<td></td>
<td>1.24a</td>
<td>1.04-1.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>635</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serretti et al. 2001</td>
<td>Milan, Italy; Caucasian</td>
<td>DSM-IV</td>
<td>P</td>
<td>210</td>
<td>NR</td>
<td>45.2</td>
<td>1.36</td>
<td>1.07-1.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>380</td>
<td>50</td>
<td>37.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>P</td>
<td>674</td>
<td></td>
<td></td>
<td>1.29b</td>
<td>1.11-1.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>1015</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \chi^2=5.92, \text{ degree of freedom (df)=1, } P<0.02. \) Heterogeneity \( \chi^2=3.48, \text{ df}=2, \) not significant. \( ^b \chi^2=11.79, \text{ df}=1, P<0.001. \) Heterogeneity \( \chi^2=3.87, \text{ df}=3, \) not significant. NR, not reported; O.R, Odds ratios, C.I., confidence intervals; P, patients; C, controls; and fixed-effects meta-analysis \(^{[251]}\) were performed as described \(^{[252]}\).

#### 4.1.4 LD analysis

Data were calculated for all SNP pairs separately in cases and controls (see Tables 5a-5e), and indicated as Lewontin’s D’-values and corresponding P-values. Because of the strategy followed to select SNPs, based on our hypothesis we expected the SNPs to be at LD. Accordingly, each patient group shared almost a homogeneous linkage pattern compared to controls, with one exception observed in the the MDD group, where SNP 1 was found to be in strong LD only with SNP 3 (Table 5b). The results suggest that all the SNPs belong to the same haplotype block.

### Table 5a. D’ and P-values for controls

<table>
<thead>
<tr>
<th>SNPs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
</tr>
<tr>
<td>2</td>
<td>0.519</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
</tr>
<tr>
<td>3</td>
<td>0.782</td>
<td>0.720</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
</tr>
<tr>
<td>4</td>
<td>0.589</td>
<td>0.880</td>
<td>0.857</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
</tr>
<tr>
<td>5</td>
<td>0.540</td>
<td>0.804</td>
<td>0.747</td>
<td>0.837</td>
<td>(&lt;10^{-5})</td>
<td>(&lt;10^{-5})</td>
</tr>
<tr>
<td>6</td>
<td>0.551</td>
<td>0.865</td>
<td>0.687</td>
<td>0.948</td>
<td>0.892</td>
<td>(&lt;10^{-5})</td>
</tr>
</tbody>
</table>

Upper diagonal: P-values for pair wise LD; lower diagonal: D’-values for each SNP pair combination. SNP numbering follows the physical location on the gene. D’ values \(>0.5\) are shown in bold.
In the case of SCZ (Table 5c), SNP1 also differed between the controls and the patients. The displayed $D'$ values in controls (Table 5a) ranged between 0.50-0.75 while $D'$ values in patients ranged between 0.85-0.95, i.e. almost complete LD.

In the BPD analysis (Tables 5d and e) all SNPs were in high LD with each other in patients and controls with the exception of SNP1. In the control group SNP1 was in LD only with SNP3.

### Table 5b. $D'$ and P-values for MDD patients

<table>
<thead>
<tr>
<th>SNPs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.386</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.841</td>
<td>0.527</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.491</td>
<td>0.742</td>
<td>0.907</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.379</td>
<td>0.561</td>
<td>0.745</td>
<td>0.841</td>
<td></td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>6</td>
<td>0.470</td>
<td>0.704</td>
<td>0.733</td>
<td>0.814</td>
<td>0.806</td>
<td></td>
</tr>
</tbody>
</table>

Upper diagonal: P-values for pair wise LD; lower diagonal: $D'$-values for each SNP pair combination. SNP numbering follows the physical location on the gene. $D'$ values > 0.5 are shown in bold.

### Table 5c. $D'$ and P-values for SCZ patients

<table>
<thead>
<tr>
<th>SNPs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.922</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.589</td>
<td>0.851</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.897</td>
<td>0.924</td>
<td>0.844</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.905</td>
<td>0.616</td>
<td>0.954</td>
<td>0.930</td>
<td></td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>6</td>
<td>0.957</td>
<td>0.974</td>
<td>0.915</td>
<td>0.975</td>
<td>0.920</td>
<td></td>
</tr>
</tbody>
</table>

Upper diagonal: P-values for pair wise LD; lower diagonal: $D'$-values for each SNP pair combination. SNP numbering follows the physical location on the gene. $D'$ values > 0.5 are shown in bold.

### Table 5d. $D'$ and P-values for female controls

<table>
<thead>
<tr>
<th>SNPs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>2</td>
<td>0.467</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>3</td>
<td>0.771</td>
<td>0.785</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>0.0008</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.453</td>
<td>0.766</td>
<td>0.815</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.433</td>
<td>0.738</td>
<td>0.823</td>
<td>0.754</td>
<td></td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>6</td>
<td>0.452</td>
<td>0.824</td>
<td>0.664</td>
<td>0.866</td>
<td>0.816</td>
<td></td>
</tr>
</tbody>
</table>

Upper diagonal: P-values for pair wise LD; lower diagonal: $D'$-values for each SNP pair combination. SNP numbering follows the physical location on the gene. $D'$ values > 0.5 are shown in bold.
Table 5e. D' and P-values for BPD patients

Upper diagonal: P-values for pair wise LD; lower diagonal: D'-values for each SNP pair combination. SNP numbering follows the physical location on the gene. D' values > 0.5 are shown in bold.

<table>
<thead>
<tr>
<th>SNPs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10⁻⁵</td>
<td>&lt;10⁻⁵</td>
<td>&lt;10⁻⁵</td>
<td>&lt;10⁻⁵</td>
<td>&lt;10⁻⁵</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.606</td>
<td>&lt;10⁻⁵</td>
<td>&lt;10⁻⁵</td>
<td>&lt;10⁻⁵</td>
<td>&lt;10⁻⁵</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.779</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.636</td>
<td>0.700</td>
<td>1</td>
<td></td>
<td>&lt;10⁻⁵</td>
</tr>
<tr>
<td>5</td>
<td>0.628</td>
<td>0.686</td>
<td>1</td>
<td></td>
<td></td>
<td>&lt;10⁻⁵</td>
</tr>
<tr>
<td>6</td>
<td>0.563</td>
<td>0.646</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.1.5 Six-marker haplotype analysis

Single marker association studies are known to be prone to generate weak associations and/or inconsistent reproducibility [253, 254]. One reason is that parent-offspring transmission of genetic variants follows certain constraints, such that most individual alleles carry only limited information on gene variants [255]. Alleles are mostly organized in haplotype blocks, limited arrays of allelic combinations inherited with minimal recombination, and rather stably maintained in populations [256].

A haplotype analysis was carried out with the six SNPs across the TPH-1 gene (Tables 6a-6c). About 82% of the control subjects carried five common haplotypes, all with a frequency above 5% (Table 6a). As shown in Tables 6a, 6b, and 6c, all patients groups differed significantly in the compound common haplotype frequency.

Individual haplotype analysis provided further information. Several haplotypes displayed associations with the patient groups. In particular, haplotype GCTCCG frequency differed significantly from the control group in both MDD (Table 6a) and BPD patients (Table 6c). Also, the frequency of haplotype GCGCCG, which differs from this haplotype only in SNP3 (rs211105, G/T) varied significantly between controls and MDD patients, but the significance was lost after Bonferroni correction. Haplotype ACTCCG, which also differs from haplotype GCTCCG only by one SNP (rs4537731, A/G), showed no significant difference.

Table 6a. Estimated haplotype frequencies for control and MDD subjects

<table>
<thead>
<tr>
<th>Haplotypesa</th>
<th>Frequencies (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=253)</td>
<td>MDD (n=258)</td>
<td>χ²</td>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 ATTAAA</td>
<td>26.83</td>
<td>26.62</td>
<td>0.01</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 GCGCCG</td>
<td>18.56</td>
<td>12.71</td>
<td>6.19</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 GCTCCG</td>
<td>18.08</td>
<td>11.92</td>
<td>7.07</td>
<td>0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 ACTCCG</td>
<td>12.01</td>
<td>9.57</td>
<td>1.43</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 GTTAAA</td>
<td>6.56</td>
<td>7.56</td>
<td>0.32</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6c All rare&lt;3%</td>
<td>17.96</td>
<td>31.62</td>
<td>24.01</td>
<td>&lt;10⁻⁵</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aAlleles are ordered according to their physical location on the gene. bP-values are calculated for each haplotype versus all others by the χ² test and corrected by Bonferroni method. Significant P-values are shown in bold. cAll haplotypes with estimated frequencies <3% in either group. MDD (Major Depression Disorder); NS (not significant)
However, taken together, haplotypes GCGCCG, and GCTCCG, account for about 30% of the total study population, and their compound association with the control group was stronger than haplotype GCTCCG alone \((P=0.0003)\). This indicates that the shared five-SNP haplotype might have a protective role.

The other two common haplotypes in the MDD population, namely ATTAAA and GTTAAA, differing at only one SNP (rs4537731, A/G) and accounting totally for about 1/3 of the total study population, showed no association. Haplotype ATTAAA showed a significant association with both the SCZ and the BPD group (corrected \(P=0.00006\) and \(P<10^{-5}\), respectively). Further, haplotype GTTAAA frequency differed significantly between control and SCZ groups (corrected \(P=0.0004\)). The same haplotype was also significantly associated with BPD (corrected \(P<10^{-5}\)).

### Table 6b. Estimated haplotype frequencies for control and SCZ subjects

<table>
<thead>
<tr>
<th>Haplotypes (^a)</th>
<th>Control ((n=253))</th>
<th>SCZ ((n=155))</th>
<th>(\chi^2)</th>
<th>(p^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ATTAAA</td>
<td>26.83</td>
<td>41.98</td>
<td>19.83</td>
<td>0.00006</td>
</tr>
<tr>
<td>2 GCGCCG</td>
<td>18.56</td>
<td>21.18</td>
<td>0.89</td>
<td>NS</td>
</tr>
<tr>
<td>3 GCTCCG</td>
<td>18.08</td>
<td>11.71</td>
<td>5.94</td>
<td>NS</td>
</tr>
<tr>
<td>4 ACTCCG</td>
<td>12.01</td>
<td>13.45</td>
<td>0.38</td>
<td>NS</td>
</tr>
<tr>
<td>5 GTTAAA</td>
<td>6.56</td>
<td>0.76</td>
<td>16.17</td>
<td>0.0004</td>
</tr>
<tr>
<td>6 (^c) All rare&lt;3%</td>
<td>17.96</td>
<td>10.92</td>
<td>7.29</td>
<td>0.0414</td>
</tr>
</tbody>
</table>

\(^a\) Alleles are ordered according to their physical location on the gene. \(^b\)P-values are calculated for each haplotype versus all others by the \(\chi^2\) test and corrected by Bonferroni method. Significant P-values are shown in bold. \(^c\) All haplotypes with estimated frequencies <3% in either group.

SCZ (Schizophrenia); NS (not significant)

Haplotypes ATTAAA, GCGCCG, and GTGCCG showed an opposite pattern in BPD, where they were associated with the healthy control group. Haplotypes GCGCCG and GTGCCG differ only in their second SNP (rs 684302, C/T), and they both maintained significance after correction for multiple testing (corrected \(P<10^{-5}\), and \(P=0.0001\), respectively). It is noteworthy that haplotypes TTTAAA and CGCCG (configuration 2–3–4–5–6) were associated with disease depending on SNP 1 allelic phase, that is, the presence of SNP 1 alleles A or G seemed to determine whether the resulting six-loci haplotype was associated with risk or protection. However, SNP 1 alone did not display consistent individual associations, indicating that its role is probably context-specific. A striking observation was made for haplotype ACGCCG. While this haplotype was absent in the control group, and very rare in SCZ or MDD, it displayed almost a 25% frequency in the BPD group (corrected \(P<10^{-5}\)). Since this haplotype was very rare in MDD, this association is likely specific for BPD and/or suicidal behavior. Except for the first SNP (rs4537731, A/G) all alleles of this haplotype were opposite to those in haplotype ATTAAA, which was associated with the control group.
Table 6c. Estimated haplotype frequencies for control and BPD subjects

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Control (n=98)</th>
<th>BPD (n=95)</th>
<th>( \chi^2 )</th>
<th>( p^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ATTAAA</td>
<td>26.6</td>
<td>5.0</td>
<td>32.4</td>
<td>&lt;10^-5</td>
</tr>
<tr>
<td>2 GCGCCG</td>
<td>16.4</td>
<td>1.9</td>
<td>23.1</td>
<td>&lt;10^-5</td>
</tr>
<tr>
<td>3 GTTAAA</td>
<td>9.4</td>
<td>28.1</td>
<td>22.5</td>
<td>&lt;10^-5</td>
</tr>
<tr>
<td>4 GTGCCG</td>
<td>9.1</td>
<td>0.0</td>
<td>18.3</td>
<td>0.00001</td>
</tr>
<tr>
<td>5 GCTCCG</td>
<td>8.4</td>
<td>19.9</td>
<td>10.1</td>
<td>0.0084</td>
</tr>
<tr>
<td>6 ACTCCG</td>
<td>7.7</td>
<td>12.7</td>
<td>2.6</td>
<td>NS</td>
</tr>
<tr>
<td>7 ACGCCG</td>
<td>0.0</td>
<td>24.9</td>
<td>55.2</td>
<td>&lt;10^-5</td>
</tr>
<tr>
<td>8 All rare&lt;3%</td>
<td>22.4</td>
<td>7.4</td>
<td>17.2</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

\( ^a \) Alleles are ordered according to their physical location on the gene.  
\( ^b \) Bonferroni corrected significant P-values are shown in bold.  
\( ^c \) All haplotypes with estimated frequencies <3% in either group.  
SCZ (Schizophrenia); BPD (Borderline Personality Disorder); NS (not significant)

To avoid data fragmentation, all rare haplotypes below 3% were accumulated. This group of rare haplotypes was found to be strongly associated with MDD (31.6% vs. 18.0% in controls; Bonferroni corrected \( P=0.000006 \)). The data suggest a scenario whereby the most common TPH-1 haplotypes range from neutral to protective, while the less frequent haplotypes could contribute to genetic predisposition for MDD. Conceivably, given the diversity of these haplotypes, a causal polymorphism might be shared by a number of low-frequency haplotypes or several distinct causal variants might exist in this sample.

The pooled low-frequent haplotypes was instead found to associate with the control group in comparison to SCZ group. However, the association was marginal after Bonferroni correction (\( P=0.0414 \)). This compound group was also associated with the control group who compared to BPD (corrected \( P=0.0002 \)). Thus, it appears that the accumulated low-frequent haplotypes seem to carry a risk for MDD but are protective of SCZ and BPD. However, such data should be taken with caution, as frequency estimates of rare haplotypes are highly susceptible to error [257].

### 4.1.6 Three-locus haplotype “sliding window” analysis

The presence of a causal polymorphism within a gene region may be reflected in a particularly strong association with disease for that region. In order to define TPH-1 gene regions that may carry stronger associations, we analyzed haplotypes formed by 3-locus combinations with a “sliding window” approach, i.e. SNP combinations 1-2-3, 2-3-4, 3-4-5, and 4-5-6. Tables 7a-7c show the 3-locus haplotypes with significant associations after Bonferroni correction for each configuration.

The combination 4-5-6 provided the most informative data for MDD. This region carries the two SNPs, A779C (SNP4) and A218C (SNP5) which have been implicated in pathophysiology of mood disorders in several studies [236, 238]. Haplotype CCG, which was the overall most common 3-locus haplotype, displayed lower frequencies in
MDD patients than controls (corrected P=0.003). Haplotype CAG showed instead higher frequencies in MDD patients (corrected P=0.016). Haplotype AAG was also significantly associated with MDD (corrected P=0.002). Although the results for the latter two haplotypes are dubious because of their overall low frequency, since they differ by one SNP, their compound frequency reflects that of the 2-locus haplotype AG in configuration 5-6. This haplotype alone had a frequency of 11.0% in patients vs. 3.6% in controls, thereby associating strongly with MD (corrected P=0.000008).

Table 7a. Three-locus haplotype analysis for control and MDD subjects

<table>
<thead>
<tr>
<th>Frequencies (%)</th>
<th>Controls</th>
<th>MDD</th>
<th>χ²</th>
<th>P&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Configuration&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3-locus haplotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2-3</td>
<td>GTG 0.94</td>
<td>3.67</td>
<td>8.0</td>
<td>0.027</td>
</tr>
<tr>
<td>2-3-4</td>
<td>TGC 1.34</td>
<td>4.15</td>
<td>7.1</td>
<td>0.047</td>
</tr>
<tr>
<td>4-5-6</td>
<td>AAG 0.40</td>
<td>3.44</td>
<td>13.1</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>CAG 3.22</td>
<td>7.52</td>
<td>9.0</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>CCG 54.07</td>
<td>42.95</td>
<td>12.1</td>
<td>0.003</td>
</tr>
</tbody>
</table>

MDD (Major Depressive Disorder). The position of each SNP is according to their physical location on the gene. *Only haplotypes with significant associations are shown. **Totally 4 configurations are made by the 6 SNPs. Each configuration contains eight different combinations of 3-locus haplotypes (4×8=32). Numbers in each configuration indicate which SNPs are included. *P-values after Bonferroni correction.

Table 7b. Three-locus haplotype analysis control and SCZ subjects

<table>
<thead>
<tr>
<th>Frequencies (%)</th>
<th>Control</th>
<th>SCZ</th>
<th>χ²</th>
<th>P&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Configuration&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3-locus haplotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2-3</td>
<td>ATT 32.23</td>
<td>47.89</td>
<td>19.6</td>
<td>0.00006</td>
</tr>
<tr>
<td></td>
<td>GCT 20.22</td>
<td>12.56</td>
<td>7.7</td>
<td>0.0324</td>
</tr>
<tr>
<td></td>
<td>GTT 8.49</td>
<td>1.09</td>
<td>20.5</td>
<td>0.00003</td>
</tr>
<tr>
<td>2-3-4</td>
<td>TTA 36.23</td>
<td>47.02</td>
<td>9.5</td>
<td>0.0122</td>
</tr>
</tbody>
</table>

*Only haplotypes with significant associations are shown. **Totally 4 configurations are made by the 6 SNPs. Each configuration contains eight different combinations of 3-locus haplotypes (4x8=32). Numbers in each configuration indicate which SNPs are included. The position of each SNP is according to their physical location on the gene. *P-values after Bonferroni correction. SCZ (Schizophrenia).

Most of the significant associations regarding SCZ mapped instead in the SNP combination 1-2-3, encompassing the gene region comprised between the promoter and intron 3 (Table 7b). In this SNP combination haplotypes ATT and GTT differed significantly in frequency between patients and controls (P=0.00006 and P=0.00003, respectively). The association of this region is in agreement with the allelic and genotypic associations of SNP 1 and 2 with the disease reported earlier. It is noteworthy that the TPH-1 promoter region, into which SNP1 but not SNP 2 maps, has
been reported to be characterized by very few haplotypes, with SNPs almost invariably in complete LD resulting in two major haplotype blocks \[^{[258]}\]. This implicates that SNP 1 alleles might be considered representative of the entire promoter region sequence (approx. 2 kb), suggesting that risk or protection is dependent on specific sequence combinations between promoter and gene transcript. Such different combinations may be reflected in the different LD estimates observed between SNP 1 and other SNPs when comparing controls vs. patients. The TPH-1 region spanning over SNPs 1 and 2 might potentially be affected even by minor mutations, as it contains promoter sequences, transcription and translation start, as well as intron 1 and 2 splicing sites. This region is therefore a candidate for the location of a possible haplotype-linked functional polymorphism.

### Table 7c. Three-locus haplotype analysis control and BPD subjects\(^a\)

<table>
<thead>
<tr>
<th>Configurations(^b)</th>
<th>3-locus haplotypes</th>
<th>Control</th>
<th>BPD</th>
<th>(\chi^2)</th>
<th>(P)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2-3</td>
<td>ACG</td>
<td>0.49</td>
<td>23.6</td>
<td>49.1</td>
<td>(&lt;10^{-5})</td>
</tr>
<tr>
<td></td>
<td>ATT</td>
<td>33.8</td>
<td>5.8</td>
<td>47.9</td>
<td>(&lt;10^{-5})</td>
</tr>
<tr>
<td></td>
<td>GCG</td>
<td>18.8</td>
<td>1.8</td>
<td>30.4</td>
<td>(&lt;10^{-5})</td>
</tr>
<tr>
<td></td>
<td>GCT</td>
<td>11.5</td>
<td>23.4</td>
<td>10.4</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>GTG</td>
<td>9.1</td>
<td>1.0</td>
<td>13.8</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>GTT</td>
<td>12.1</td>
<td>29.7</td>
<td>17.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>2-3-4</td>
<td>CTC</td>
<td>19.9</td>
<td>34.5</td>
<td>10.6</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>TGC</td>
<td>10.1</td>
<td>1.0</td>
<td>15.9</td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
<td>TTC</td>
<td>6.8</td>
<td>1.0</td>
<td>8.6</td>
<td>0.020</td>
</tr>
<tr>
<td>3-4-5</td>
<td>TCA</td>
<td>4.8</td>
<td>0.0</td>
<td>10.5</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>TCC</td>
<td>21.7</td>
<td>35.5</td>
<td>8.9</td>
<td>0.017</td>
</tr>
<tr>
<td>4-5-6</td>
<td>CAG</td>
<td>4.1</td>
<td>0.0</td>
<td>8.3</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>CCG</td>
<td>46.4</td>
<td>61.0</td>
<td>8.4</td>
<td>0.022</td>
</tr>
</tbody>
</table>

\(^{a}\)Only haplotypes with significant associations are shown. \(^{b}\)Totally 4 configurations are made by the 6 SNPs. Each configuration contains eight different combinations of 3-locus haplotypes (4x8=32). Numbers in each configuration indicate which SNPs are included. The position of each SNP is according to their physical location on the gene. \(^{c}\)P-values after Bonferroni correction. BPD (Borderline Personality Disorder)

The same gene region accounted for most of the significant associations seen with BPD (Table 7c). Haplotype combinations ACG, GCT and GTT in this configuration were observed to associate with the BPD patient group (corrected \(P<10^{-5}\), \(P=0.007\), and \(P=0.0001\), respectively), while haplotypes ATT, GCG and GTG were associated with the control group (corrected \(P<10^{-5}\), \(P<10^{-5}\), and \(P=0.0012\), respectively). Configuration 2-3-4 carried also 3 significant combinations, namely haplotype CTC.
was associated with BPD (corrected $P=0.007$), while haplotypes TGC and TTC were found associated with healthy controls (corrected $P=0.0006$ and $P=0.020$, respectively). Configurations 3-4-5 and 4-5-6 each provided 2 significant haplotype combinations. In particular, haplotype TCC (configurations 3-4-5) was among the most frequent haplotypes (35.5% in BPD patients vs. 21.7% in controls, corrected $P=0.017$). The most common three-locus haplotype among those tested was CCG (configuration 4-5-6), with frequencies of 61% in BPD patients vs. 46% in controls (corrected $P=0.022$). Interestingly, as observed in SCZ the region containing SNPs 4 and 5, which have been often reported in the literature associated with suicide and violent behavior, showed the least significant associations [259].

4.2 GENE VARIANTS OF 5-HTT IN ASSOCIATION TO MDD AND SCZ

4.2.1 Choice of 5-HTT SNPs

Another goal of this project was to identify 5-HTT gene variants associated with MDD and SCZ. This part of the study was initiated after the data reported so far had been obtained. Our hypothesis was that the screening strategy we devised was potentially applicable to other genes as well. Thus, we employed the same strategy to screen for polymorphic loci within 5-HTT. We chose the two well-studied 5-HTT polymorphisms, 5-HTTLPR and STin2, reported in the literature to be associated with a number of psychiatric disorders. The entire MDD patient group was genotyped for these two loci. Subjects that were homozygous for both markers were sub-grouped into the four possible genotype combinations (LL/10.10, LL/12.12, ss/10.10, ss/12.12). From these four subgroups three subjects were randomly chosen. If there were fewer than three subjects in any subgroup, DNA samples from all subjects in that subgroup were used. DNA sequencing was carried out in these samples over all exons and intron regions within about 0.5 kb from the exon boundaries. No novel polymorphism was identified. For further analyses, we selected those SNPs that were homozygous for at least one allele in all subjects in any of the four subgroups. Two SNPs were identified that met our criteria, thus, totally 4 loci were included (Table 10).

4.2.2 Genotyping

Genotyping was carried out as described in the Material & Method section. The table below presents the genotyping data for each marker, providing the single nucleotide polymorphisms character, the size of the PCR products, and their cleaved products separated on 2% agarose gel by electrophoresis.
Table 10. Genotyping details for the SNPs of 5-HTT gene

<table>
<thead>
<tr>
<th>Marker</th>
<th>Polym</th>
<th>Restriction enzyme</th>
<th>PCR product (size bp)</th>
<th>Cleaved product (size bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AA</td>
</tr>
<tr>
<td>1) 5HTTLPR*</td>
<td>L/s</td>
<td>-</td>
<td>528/484</td>
<td>-</td>
</tr>
<tr>
<td>2) STin2*</td>
<td>9/10/12</td>
<td>-</td>
<td>345/360/390</td>
<td>-</td>
</tr>
<tr>
<td>3) rs140701</td>
<td>A/G</td>
<td>MaeIII</td>
<td>435</td>
<td>172+263</td>
</tr>
<tr>
<td>4) rs1042173</td>
<td>G/T</td>
<td>Xap1</td>
<td>357</td>
<td>269+88</td>
</tr>
</tbody>
</table>

No incubation with restriction enzyme; Polym: Polymorphism; bp: base pair; s: 5-HTTLPR short allele; L: 5-HTTLPR long allele; AA: Homozygous for allele 1; aa: Homozygous for allele 2

4.2.3 Single locus analysis

The study population was not tested for any other 5-HTT polymorphisms. Genotypic distributions for all four loci were in Hardy-Weinberg equilibrium among all cases and control subjects. STin2.9 allele frequency was very low in all groups, namely 1.6% in controls, 2% in MDD patients and 0.8% in SCZ patients. When performing allele and genotype tests, STin2.9 allele frequency was accumulated with STin2.10 as one unit termed short alleles (S). This was done to allow reliable chi-square calculations, not applicable in the presence of empty or almost empty cells [260].

Table 11. Genotypic association tests for healthy controls, MDD & SCZ patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control (n=246)</th>
<th>MDD (n=194)</th>
<th>SCZ (n=155)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>Aa</td>
<td>aa</td>
</tr>
<tr>
<td>L/s</td>
<td>30.5</td>
<td>51.2</td>
<td>18.3</td>
</tr>
<tr>
<td>S/12</td>
<td>11.8</td>
<td>50.8</td>
<td>37.4</td>
</tr>
<tr>
<td>A/G</td>
<td>20.3</td>
<td>30.9</td>
<td>48.8</td>
</tr>
<tr>
<td>G/T</td>
<td>24.0</td>
<td>52.4</td>
<td>23.6</td>
</tr>
</tbody>
</table>

SNP numbering follows the physical location on the gene. P-values are provided by 2x2 contingency tables and are not corrected for multiple testing. Bold numerals highlight significant SNPs by a significant P-value. Genotype frequencies are shown in percentage. MDD (Major Depression Disorder); SCZ (Schizophrenia); #1 L (5-HTTLPR long allele); s (5-HTTLPR short allele); #2 S (STin2.9 allele + STin2.10 allele); 12 (STin2.12 allele)

No single locus association with MDD passed Bonferroni correction. 5-HTT has been considered a promising candidate for genetic vulnerability to depression, and both 5-HTTLPR and STin2 have been intensively investigated for association with MDD [71]. Thus, it is interesting that even using a haploype-based approach we failed to find any
association with depression. However, the literature data on 5-HTT association with MDD are not consistent, and although several studies have shown correlations of the S variant with depression-related trait scores, based on meta-analysis this polymorphism has not been significantly associated with MDD \[^{71}\].

Association with SCZ was shown for SNP rs140701, occurring in higher heterozygote and lower G homozygote frequencies among SCZ patients (corrected \(P=0.0036\)). When comparing alleles, there was marginally significant evidence for higher A allele frequencies in SCZ patients than controls (corrected \(P=0.052\)). SNP rs140701 (position 52061 according to NCBI clone AC104984) is located about 90 nt from the intron 8/exon 8 junction (position 52151), halfway between the branch site (position 52031) and the splice acceptor site (position 52149), a region that could be potentially affected even by a single point mutation.

### Table 12. Allelic association tests

<table>
<thead>
<tr>
<th>SNP</th>
<th>Marker(^a)</th>
<th>Allele frequencies</th>
<th>Allele frequencies</th>
<th>Allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MDD (P^b)</td>
<td>SCZ (P^b)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>L/s</td>
<td>0.56 (L)</td>
<td>0.57 (L)</td>
<td>0.743</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.59 (L)</td>
<td></td>
<td>0.318</td>
</tr>
<tr>
<td>2</td>
<td>S/12</td>
<td>0.63 (12)</td>
<td>0.59 (12)</td>
<td>0.240</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.60 (12)</td>
<td></td>
<td>0.426</td>
</tr>
<tr>
<td>3</td>
<td>A/G</td>
<td>0.36 (G)</td>
<td>0.41 (G)</td>
<td>0.150</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45 (A)</td>
<td></td>
<td>0.013</td>
</tr>
<tr>
<td>4</td>
<td>G/T</td>
<td>0.50 (T)</td>
<td>0.57 (T)</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50 (T)</td>
<td></td>
<td>0.885</td>
</tr>
</tbody>
</table>

\(^a\)Allele 1/allele2. Alleles are ordered according to their physical location on the gene.

\(^b\)P-values are not corrected for multiple testing.

MDD (Major Depression Disorder); SCZ (Schizophrenia);
#1 L (5-HTTLPR long allele); s (5-HTTLPR short allele);
#2 S (STin2.9 allele + STin2.10 allele); 12 (STin2.12 allele)

#### 4.2.4 LD analysis

LD data indicated as Lewontin’s \(D\)'-values and corresponding \(P\)-values were calculated for all SNP pairs in control MDD and SCZ groups. Significant \(D\)'-values (>0.5) were observed between loci STin2, SNP rs140701 and SNP rs1042173 in the population studying MDD, indicating that these loci are likely to belong to the same haplotype block. 5-HTTLPR on the other hand did not seem to belong to the block, as the \(D\)' value between 5-HTTLPR and STin2 was below 0.5 in both MDD patient and control groups, and further decayed with distance from SNP rs140701 and SNP rs1042173 (Tables 13 and 14). Also in the SCZ group, except for 5-HTTLPR all loci shared an almost homogeneous linkage pattern in both cases and controls (Table 15). The LD patterns between the 5-HTTLPR and STin2 polymorphisms have been studied in several populations, and shown to be variable \[^{202}\] ranging from moderate in Europeans to very strong in Native Americans, while it is completely absent in the Chinese population. A recent meta-analysis indicate that the two loci belong to separate haplotype blocks \[^{200}\]. Our results are in agreement with the latter studies.
Table 13. D' and P-values for healthy controls

<table>
<thead>
<tr>
<th>SNPs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.373</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.252</td>
<td>0.874</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.191</td>
<td>0.523</td>
<td>0.739</td>
<td></td>
</tr>
</tbody>
</table>

Table 14. D' and P-values for MDD patients

<table>
<thead>
<tr>
<th>SNPs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10^{-5}</td>
<td>0.0009</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.462</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.229</td>
<td>0.746</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.158</td>
<td>0.572</td>
<td>0.741</td>
<td></td>
</tr>
</tbody>
</table>

Table 15. D' and P-values for SCZ patients

<table>
<thead>
<tr>
<th>SNPs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.521</td>
<td>&lt;10^{-5}</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.452</td>
<td>0.823</td>
<td>&lt;10^{-5}</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.408</td>
<td>0.791</td>
<td>0.891</td>
<td></td>
</tr>
</tbody>
</table>

Upper diagonal: P-values for pair wise LD; lower diagonal: D'-values for each pair. D' values > 0.5 are shown in bold. SNP numbering follows the physical location on the gene.

4.2.5 Haplotype analysis

Haplotype analyses revealed the existence of six common haplotypes, all with a frequency above 5% in all groups. In the case of MDD, none of the haplotypes were found to associate with patient or control group. A discussed above for single locus analysis, 5-HTT association with MDD is not confirmed. In contrast, there is convincing evidence for a 5-HTT association with anxiety-related traits [206]. With this in mind, and to circumvent potential phenotype stratification problems, MDD patients with co-morbidity for other Axis I or II disorders were not included in these studies. Since co-morbidity between MDD and anxiety disorders is commonly estimated to about 50% [74] studies showing 5-HTT association with MDD could, at least in part, reflect a phenotype stratification due to co-morbidity with anxiety or other disorders. A recent large German study supported the involvement of 5-HTTLPR in the etiology of MDD, but also emphasized that size and homogeneity are key elements for detecting small genetic effects [211]. In our case, we found marginal associations for SNP rs140701 (P= 0.048) and for allele T of SNP rs1042173 (P= 0.034), but both associations failed to retain significance after correction for multiple testing. However, our sample size was not large, and small genetic effects are hardly expected to be captured. Our studies will need to be replicated with similar population and study design.
In the case of SCZ one of the common haplotypes, L.10.G.T showed a significant association with the SCZ group (corrected P=0.035). Haplotype L.10.G.G, differing from L.10.G.T by only one allele in the fourth position was carried more by controls (6% in controls vs. 3% in patients). However, this association did not reach the significance level possibly due to limited sample size (corrected P=0.20). Since both haplotypes carry the SNP rs140701 G allele, the risk associated with this SNP conceivably depends on other polymorphism configurations, such that only certain allele combinations contribute to genetic liability.

To avoid further data fragmentation, all rare haplotypes below 3% in all groups were accumulated. Rare haplotypes were carried by approximately 11% of controls vs. 8% of SCZ patients and 13% of MDD patients, with no significant distribution differences among groups.

### Table 16a. Estimated haplotype frequencies for control and MDD subjects

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Control</th>
<th>MDD</th>
<th>χ²</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 s. 12. A. G</td>
<td>22.63</td>
<td>18.08</td>
<td>2.512</td>
<td>NS</td>
</tr>
<tr>
<td>2 L. 10. G. T</td>
<td>19.97</td>
<td>24.37</td>
<td>2.213</td>
<td>NS</td>
</tr>
<tr>
<td>3 L. 12. A. G</td>
<td>15.77</td>
<td>14.55</td>
<td>0.192</td>
<td>NS</td>
</tr>
<tr>
<td>4 L. 12. G. T</td>
<td>9.39</td>
<td>8.29</td>
<td>0.227</td>
<td>NS</td>
</tr>
<tr>
<td>5 s. 10. G. T</td>
<td>8.28</td>
<td>7.52</td>
<td>0.184</td>
<td>NS</td>
</tr>
<tr>
<td>6 s. 12. G. T</td>
<td>6.53</td>
<td>9.88</td>
<td>2.799</td>
<td>NS</td>
</tr>
<tr>
<td>7 L. 10. G. G</td>
<td>6.09</td>
<td>3.98</td>
<td>1.838</td>
<td>NS</td>
</tr>
<tr>
<td>8 All rare &lt; 3%</td>
<td>11.3</td>
<td>13.33</td>
<td>0.763</td>
<td>NS</td>
</tr>
</tbody>
</table>

### Table 16b. Estimated haplotype frequencies for control and SCZ subjects

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Control</th>
<th>SCZ</th>
<th>χ²</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 s. 12. A. G</td>
<td>22.63</td>
<td>25.75</td>
<td>0.983</td>
<td>NS</td>
</tr>
<tr>
<td>2 L. 10. G. T</td>
<td>19.97</td>
<td>28.15</td>
<td>6.848</td>
<td><strong>0.0088</strong></td>
</tr>
<tr>
<td>3 L. 12. A. G</td>
<td>15.77</td>
<td>15.35</td>
<td>0.016</td>
<td>NS</td>
</tr>
<tr>
<td>4 L. 12. G. T</td>
<td>9.39</td>
<td>7.75</td>
<td>0.497</td>
<td>NS</td>
</tr>
<tr>
<td>5 s. 10. G. T</td>
<td>8.28</td>
<td>5.84</td>
<td>1.563</td>
<td>NS</td>
</tr>
<tr>
<td>6 s. 12. G. T</td>
<td>6.53</td>
<td>5.84</td>
<td>0.168</td>
<td>NS</td>
</tr>
<tr>
<td>7 L. 10. G. G</td>
<td>6.09</td>
<td>3.96</td>
<td>3.810</td>
<td>NS</td>
</tr>
<tr>
<td>8 All rare &lt; 3%</td>
<td>11.33</td>
<td>8.37</td>
<td>1.674</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Alleles are ordered according to their physical location on the gene. P-values are calculated for each haplotype versus all others by the χ² test and corrected by Bonferroni method. Significant P-values are shown in bold. All haplotypes with estimated frequencies <3% in both groups. MDD (Major Depressive Disorder) SCZ (Schizophrenia); NS (not significant)**
In order to define 5-HTT gene regions that may carry stronger associations, we analyzed haplotypes formed by two-locus combinations with the “sliding window” approach described earlier. SNP configurations were 1-2, 1-3, 1-4, 2-3, 2-4 and 3-4 (data not shown). Only one haplotype (10-A, configuration 2-3) was observed to associate with MDD group ($\chi^2=6.55$; df=1; $P=0.01$, data not shown). However, this association did not pass correction for multiple testing. No other significant associations were found for this or for SCZ group.

Taken together, our results indicate that 5-HTT is not involved in the pathogenesis of MDD per se but rather in other co-morbid components, especially anxiety disorders, that are often incorporated in MDD populations. Also, in agreement with previous genetic studies on 5-HTT \cite{200}, our results suggest that the 5-HTT gene region potentially liable for genetic risk for SCZ could be within the haplotype block containing STin2 but not 5-HTTLPR. Although perhaps less expected than association with MDD, the association of 5-HTT with schizophrenia is well documented (see Introduction), with data pointing at an involvement of STin2 but not 5-HTTLPR \cite{200}. Accordingly, our data show no association with 5-HTTLPR, and through the association of SNP rs140701 they point at an involvement of the STin2-containing haplotype block. Our results thus strengthen the possibility that the 5-HTT gene region potentially liable for genetic risk for schizophrenia might lie within the haplotype block containing STin2 but not 5-HTTLPR. As noted by Fan and Sklar in their meta-analysis, given the focus on the 5-HTT promoter polymorphism it is unlikely that a systematic search for other functional polymorphisms has been carried out. Thus, it appears that 5-HTT is a gene with a potential liability to schizophrenia, although the context-specific role of its molecular variants remains to be elucidated.

\subsection*{4.2.6 Impaired decision making as a candidate endophenotype of BPD \textbf{(Paper VI)}}

When we found the highly significant association between TPH-1 and a population of suicidal BPD women reported in Paper II, other members of our group failed to obtain similar results with a group of suicidal patients with mixed gender and diagnoses (De Franciscis et al., unpublished data). The results were somewhat unexpected, as the hypothesis of a biological association between BPD and the serotonin system was based mainly on suicidal behavior, common in BPD patients. It should be noted, however, that the BPD group had been selected for severe suicidality, and the subsequent suicide mortality in this group has been much higher than in our comparison group of suicide attempters. Thus, we cannot know whether the gene variant was primarily associated with BPD or with severe suicidality. The results led us to hypothesize that a disturbance in impulse control might associate with TPH-1. We therefore conducted a study, in collaboration with Arne Öhman’s group at Karolinska Institute to test for association between the specific TPH-1 gene variant previously found associated with BPD women and impulsiveness in decision-making. Impulsiveness was measured using the Iowa Gambling Test (IGT), originally devised to score cognitive deficits following brain damage (see Paper VI). The TPH-1 gene variant ACGCCCG (see previously shown Table 6c) was significantly more common
among low IGT performers (Figure 7b). We therefore propose that impaired decision-making is a potential endophenotype of borderline personality disorder.

Figure 7a. Net scores (number of choices from good decks minus choices from bad decks at the Iowa Gambling Task (IGT) showed a linear improvement over five blocks of 20 cards each (a total number of 100 cards), $F(1,76)=49.58$, $P<0.001$. This improvement was stronger for controls compared to BPD patients, $F(1,76)=3.01$, $P<0.04$ (one-tailed), and the groups differed significantly across the last three blocks, $t(76)=2.05$, $P<0.04$ (two-tailed), but not the first two blocks, $t(76)<1$.

Figure 7b. The TPH-1 haplotype was carried by 35% of the BPD patients with low IGT performance versus 12% of the BPD patients with higher net scores ($\chi^2[1, N=42] =5.4$, $P<0.02$).
The objective of these studies was the identification of risk gene variants within a classical case-control study design. Approaches to construct haplotypes carrying risk for disease are generally complex, and when this study began information available on haplotype blocks was limited, and extensive genotyping and/or sequencing would have been required in all subjects within a study population, a prohibitive effort for most research groups. To make this project realistic, we introduced a hypothesis-based screening strategy that would reduce complexity. Using this approach, only a small number of samples were sequenced in limited regions, and the comparatively low number of markers found necessary to carry out haplotype analyses made cost and time acceptable even to small research groups. Overall, the study design allowed the reconstruction of informative haplotype markers using a simple strategy. Several significant associations were observed between the chosen serotonergic genes and different psychiatric disorders.

Analyses done with individual polymorphisms mostly yielded in weak associations at both allelic and genotypic levels. This was to be expected, as single-locus association studies are prone to generate weak associations, as well as inconsistent reproducibility across replica studies. Parent-offspring transmission of genetic variants follows certain constraints that could partly explain the poor reproducibility of the association studies outcome. Sample size is also factor to consider which may lead to false negative results, as common variants with small effects are difficult to detect.

The results obtained from 5-HTT association studies are worth some additional considerations. After a large number of genetic association studies done on this gene, the conclusions obtained from meta-analysis (see Introduction, section 1.8.1) diverge from the “popular consensus” notion that the gene is associated with depression. Also, association with SCZ, not widely acknowledged in the field, according to meta-analysis is reliable, although related to STin2 not to the more popular 5-HTTLPR (see Introduction, section 1.8.1). Interestingly, our data on 5-HTT are fully in agreement with meta-analysis conclusions, suggesting that the approach is both sensitive and specific.
In the course of gene-base haplotype association analyses, several haplotypes were identified that may carry risk for a disease, but also some with an opposite pattern, potentially “protective”. Such knowledge may contribute to future genetic assessments, such as gene functional studies, through which one might gain more insight into the possible role a gene may have in “normal” human physiology versus pathophysiology.

Differences were sometimes observed in LD values between groups, although mostly not significant. These may be related to population stratification. For instance, as noted in the Materials & Methods, our BPD patients also had one to several diagnoses on Axis I. Alternatively, as further discussed below, in certain cases there might be other reasons more pertinent to disease pathogenesis.

Although we tried to match our controls, a number of hidden phenotypes are likely to exist because of limited knowledge in biological psychiatry. Another major limitation in our studies was sample size, as well as lack of replica groups. Therefore, as noticeable in the abstracts of individual papers, it was carefully avoided to draw strong conclusions for associations with P-values just below the significance level, even though Bonferroni correction was used. This correction for multiple testing is very conservative, particularly in these studies, where measurements were not independent since most SNPs were in LD. This was done with the awareness that association studies are influenced by several factors, with phenotypic stratification probably being the most common in the case of complex psychiatric disorders. The results of this thesis work should therefore be interpreted with caution until replicated.
SUMMARY IN PERSIAN

بیماری های روایی، بیماری های مورونی بهبودی و هستند که از نتیجه وراثت مشتق شده است. در این مقاله، به احتمال‌هایی مربوط به عوامل مختلف و احتمالاتی مربوط به بیماری های دانشگاهی می‌پردازیم.

در این اثر، در اصل به احتمالاتی مربوط به بیماری های دانشگاهی مربوط به عوامل مختلف و احتمالاتی مربوط به بیماری های دانشگاهی می‌پردازیم.

در این اثر، در اصل به احتمالاتی مربوط به بیماری های دانشگاهی مربوط به عوامل مختلف و احتمالاتی مربوط به بیماری های دانشگاهی می‌پردازیم.

در این اثر، در اصل به احتمالاتی مربوط به بیماری های دانشگاهی مربوط به عوامل مختلف و احتمالاتی مربوط به بیماری های دانشگاهی می‌پردازیم.

در این اثر، در اصل به احتمالاتی مربوط به بیماری های دانشگاهی مربوط به عوامل مختلف و احتمالاتی مربوط به بیماری های دانشگاهی می‌پردازیم.
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Ghazal Zaboli
Stockholm, 2006-05-10
8 REFERENCES


A gene-based haplotype approach


A gene-based haplotype approach


